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ABSTRACTS

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OL1

Sustainable API Process Applications Through Biocatalysis

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OL2

Biocatalytic Systems for the Biomanufacturing of Metabolites and Metabolite-like Compounds

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Small molecular weight components from biobased resources have accompanied and supported the quality of life throughout human history in many highly relevant areas, such as nutrition, diagnostics and therapy of diseases, dyes, cosmetics, and well-being. The great scientific progress and the revitalized interest in metabolism and the small molecular weight components of biological cells has also brought increasing attention to the rising gap between the metabolites identified and the metabolites which have been manufactured as pure compounds [1]. As metabolites are not only needed as standards, enzyme substrates, inhibitors, or for the discovery of novel biological functions, but also as important final products at large scale, the design of efficient and straightforward biomanufacturing routes to desired metabolites is not only of fundamental but also of much practical interest [2,3]. This interest applies also to metabolite-like compounds, which are needed as stable isotope-labelled metabolites, damaged metabolites or synthetic intermediates [2].

Biocatalytic systems have become key enabling tools in sustainable chemistry and asymmetric synthesis and have also been applied for the biomanufacturing of an increasing number of metabolites and metabolite-like compounds [4,5]. The design and selection of resource-efficient transformation routes based on retrosynthetic analysis in the chemical and biochemical domain, as well as the development of biocatalytic reaction systems and reaction conditions are thereby essential [6]. Examples of rapid prototyping [2] and bottleneck identification, which support the design of resource-efficient processes, are shown in applications of highly selective biocatalytic one-step systems for defunctionalization reactions of biobased starting materials [7], for phosphorylations with molecular economy [8,9], and for building molecular complexity from simple metabolites by Michael addition reactions [10]. Analytical tools and methods with high information content are thereby instrumental for the rapid development of biocatalytic processes, from one-step to multi-step reactions and biocatalytic total synthesis [11]. Biocatalytic reaction engineering and product recovery aspects are illustrated by the examples of the two lactaldehyde enantiomers and all four limonene-1,2-epoxide stereoisomers. Biocatalytic functions remain a key frontier for biomanufacturing and connect back to the future of metabolism [12].

References

[1] R. Wohlgemuth, *Biotechnol. J.* 2018, *13(6)*, 1700620.

[2] R. Wohlgemuth, *Metabolites* 2023, *13(10)*, 1097.

[3] R. Wohlgemuth, J. Littlechild, *Front. Bioeng. Biotechnol*. 2022, *10*, 958606.

[4] A.R. Alcántara, P. Dominguez de Maria, J.A. Littlechild, M. Schürmann, R.A. Sheldon, R. Wohlgemuth, *ChemSusChem* 2022, *15(9)*, e202102709.

[5] R. Wohlgemuth, in: *Biocatalysis in Asymmetric Synthesis* (G. De Gonzalo, A.R. Alcántara, Eds.), 2024, 431-463, Academic Press.

[6] R. Wohlgemuth, *React. Chem. Eng.* 2023, *8(9)*, 2109-2118.

[7] R. Wohlgemuth, *ChemSusChem*, 2022, *15(9)*, e202200402.

[8] R. Wohlgemuth, *Catalysts* 2022, *12(11)*, 1436.

[9] R. Wohlgemuth, *Int. J. Mol. Sci.* 2023, *24(4)*, 3150.

[10] B. Schoenenberger, A. Wszolek, R. Meier, H. Brundiek, M. Obkircher, R. Wohlgemuth, in: *Applied Biocatalysis* (J. Whittall, P.W. Sutton, Eds.), 2021, 204–210, John Wiley & Sons.

[11] R. Wohlgemuth, *New Biotechnol*. 2021, *60*, 113-123.

[12] R. Wohlgemuth, *Life* 2024, *14(3)*, 364.

OL3

CFD-model based small scale bioreactor design for large-scale bioprocess development

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Although shaken bioreactors are the most commonly used vessels for cultivating microorganisms, particularly for strain screening and early process development, their design has practically not changed since their inception. This is especially noteworthy given that Erlenmeyer flasks and microtiter plates were not even originally designed for microbial cultivation. Intense characterization during the last decades led not only to a much more reliable applicability for bioprocess development with shaken bioreactors, but it also revealed deficiencies compared to large scale bioreactors, e.g. significantly lower maximum oxygen transfer capacities.

The presentation will illustrate how hydrodynamic models can be used to design and characterize novel types of shaken bioreactors with the purpose of mimicking relevant large-scale bioprocess conditions already in small-scale. The concept will be outlined with the help of an example of a new type of shaken bioreactor that overcomes a relevant problem in the scale-down of many industrial bioprocesses.

IL1-1

Development and Implementation of AI-tools for accelerated in silico screenings and biocatalytic process development

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IL1-2

Protein design 2.0

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IL1-3

Fast Yet Accurate Computational Enzyme Design

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Enzymes are essential for supporting life by accelerating chemical reactions in a biologically compatible timescale. These remarkable catalysts possess unique features like high specificity and selectivity, and they function under mild biological conditions. These extraordinary characteristics make the design of enzymes for industrially relevant targets highly appealing.

Enzymes exist as an ensemble of conformational states, and the populations of these states can be altered through substrate binding, allosteric interactions, and even by introducing mutations into their sequence. These conformational states can be altered through mutations, which facilitates the evolution of enzymes towards acquiring novel activities.[1] Interestingly, many laboratory-evolved enzymes exhibit a common pattern—a significant impact on the catalytic activity is often observed due to remote mutations located distal from the catalytic center.[2] Similar to allosterically regulated enzymes, distal mutations play a role in regulating enzyme activity by stabilizing pre-existing conformational states that are crucial for catalysis.

In this talk, the rational approaches we have developed for enzyme design along the years will be discussed. These approaches rely on inter-residue correlations derived from microsecond time-scale Molecular Dynamics (MD) simulations, enhanced sampling techniques, and more recently, the incorporation of AlphaFold2 predictions.[1-4] Over the years, our research on various enzyme systems has provided compelling evidence that the current challenge of predicting distal active sites to enhance functionality in computational enzyme design can ultimately be addressed.[3]

References:

[1] Maria-Solano, M. A.; Serrano-Hervás, E.; Romero-Rivera, A.; Iglesias-Fernández, J.; Osuna, S. Role of conformational dynamics for the evolution of novel enzyme function, *Chem. Commun.* **2018**, 54, 6622-6634.

[2] Osuna, S. The challenge of predicting distal active site mutations in computational enzyme design, *WIREs Comput Mol Sci*. **2020**, e1502.

[3] Casadevall, G.; Duran, C.; Osuna, S. AlphaFold2 and Deep Learning for Elucidating Enzyme Conformational Flexibility and Its Application for Design, *JACS Au* **2023**, 3, 1554.

[4] Casadevall, G.; Duran, C.; Estévez-Gay, M.; Osuna, S. Estimating conformational heterogeneity of tryptophan synthase with a template-based AlphaFold2 approach, *Prot. Sci.* ***2022****, 31, e4426.*

L1-1

Computational mathematic model for the immobilization of cells on charged solid surfaces by electrostatic interactions

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Non-covalent immobilization techniques, such as ionic adsorption on ionic exchanger supports, may be a good option because immobilization is very simple and produces very little work and time consumption, and the supports may be reused after desorption and, in this way, reduce the final price and generate less residues. Modulation or Rational Design of Immobilized Derivatives (RDID) is a strategy that combines mathematical and bioinformatics tools for designing optimal immobilization processes. In this work, there are described new mathematical algorithms, belonging to RDID strategy, to optimize cells immobilization via electrostatic interactions on ionic exchangers. The support maximum loading capacity on the immobilization of *Scenedesmus obliquus* cells, *Aspergillus niger* spores, *Escherichia coli* BL21, *Micrococcus luteus* ATCC 10240, *Pseudomonas aureginosa* ATCC 27853, *Bacillus* sp. RC9 and RC15 strains (originally isolated from coffe rhizosphere), *Bacillus wiedmannii* TAN-125, TAN-113 and TAN-311 strains (originally isolated from rhizosphere of the hydrophyte plant *Typha dominguensis*), *Pichia pastoris* KM71H, *Saccharomyces cerevisiae* ATCC 9763, *Vibrio harveyi* CBM-784, CBM-976 and CBM-992 strains (originally isolated from Cuban coastal waters) were estimated. At the same time, the immobilized derivatives were predicted by RDID and predictions were highly accurate when comparing with experimental results.

L1-2

Computer-Aided Enzyme Discovery and Engineering for Industrial Biocatalysis

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Question

Enzyme engineering through directed evolution offers the potential to optimize virtually any enzyme, transforming it into a potent industrial catalyst suitable for practical applications under industrial process conditions. However, the directed evolution process faces challenges due to time constraints and cost limitations. Traditional enzyme discovery from (meta)genomic diversity often falls short in delivering high-performance catalysts ready for industrial use. Additionally, experimental enzyme variant screening under process conditions [1] is restricted by the limited exploration of protein sequence space.

Methods

To address these limitations, Enzymaster has developed the BioEngine®, a proprietary directed evolution platform. This platform integrates computational tools, including the BioNavigator®, to enhance enzyme identification and engineering utilizing physics-based and parameterized semiempirical QM/MM methods for protein variant prediction.[2] To explore a significantly larger sequence space in shorter time and include experimental data from previous rounds of evolution we additionally employ machine learning and artificial intelligence. Our EnzyMaster Machine Learning (EM2L)-platform combines activity, 3D structure, and sequence information for predictive in silico screening.

Results:

In this presentation, we showcase how Enzymaster utilizes its digital toolbox to accelerate the BioEngine® through computer-aided enzyme engineering on different industrial enzymes.[3,4,5] Our approach combines in silico pre-screening, high-throughput screening and next-generation sequencing to generate fast and reliable data.

Conclusion:

Enzymaster"s ultimate goal is to provide practical solutions, bridging the gap from innovative ideas to tangible products in the emerging data-driven bioeconomy. By integrating 3D-protein modeling, AI and machine learning, we achieve virtual enzyme identification and in silico enzyme variant screening for fast and efficient industrial biocatalyst development.

[1] M.M.C.H. van Schie et al. Applied biocatalysis beyond just buffers – from aqueous to unconventional media. Options and guidelines. Green Chem., 2021, 23, 3191-3206. DOI: 10.1039/D1GC00561H

[2] A computational methodology for designing artificial enzyme variants with activity on non-natural substrates WO2022233232A1

[3] Artificial ketoreductase variants and design methodology thereof. WO2022233233A1

[4] T. Sehl et al.; Enzymatic (2R,4R)-Pentanediol Synthesis – "Putting a Bottle on the Table". Cem.Ing.Tech., 2023, 95, 4, 557-564

[5] X. Yang et al. A Self-regulated Process from Bench to Pilot Production of Non-natural Heterocycles Using Halohydrin Dehalogenases. Online 2024, DOI: 10.1002/cctc.202400095

L1-3

Helix Engineering: Combining the Power of 3DM with AI to Disrupt Protein Engineering

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The high dimensionality and practically infinite size of the sequence space requires effective techniques to explore, navigate and improve proteins. Machine learning methods have enabled the in silico screening of variants with vastly improved speed, but the current techniques are underwhelming in their accuracy and mainly resort to recombining variants present in the training data. This means that current techniques have a hard time finding novel promising mutations outside their training set.

With its 3DM technology[1] Bio-Prodict has long been at the forefront of providing protein engineering solutions. Recently we generated over 35.000 3DM databases that each contain large amounts of highly integrated protein related data for all protein superfamilies. Using this massive amount of information, we were able to develop Helix[2], a best-in-class AI pathogenicity predictor[3,4]. Currently we are applying this signature innovation of Bio-Prodict to solve protein engineering problems.

We have automated conventional 3DM-based search strategies so that the program can now smartly pre-select positions to mutate initially. This step is designed to replace the need for randomization of complete proteins often used by large pharmaceutical and biotech companies to find promising starting positions. It also removes the need for laborious bioinformatics analysis of the target protein to select positions manually.

For next rounds of evolution, we have developed a deep learning based ensemble architecture. Using multiple deep mutational scanning datasets we showed that this pipeline outperforms legacy machine learning methods[5] on average by 71.5% when mutations were selected randomly and with 115% when the 3DM-based initial selection step was used. Furthermore, we have shown that even as few as 50 initial mutations are needed to train a target specific AI network that already yields competitive hit rates.

In conclusion, we present an integrated methodology that combines the powerful 3DM technology with multiple state-of-the-art AI techniques to smartly optimize proteins. This can drastically decrease the number of rounds and samples required, thereby lowering costs and labtime. We expect that soon, once we have fully utilized all data inside 3DM to our AI methods, the Helix Engineering platform will become even more accurate, just like we did for Helix Pathogenicity, making Helix Engineering the best-in-class tool to solve protein engineering problems.

[1] R. Kuipers et al, 3DM: systematic analysis of heterogeneous superfamily data to discover protein functionalities. Proteins, 78(9):2101–2113, July 2010.
[2] B. Vroling, S Heijl, White paper: The Helix Pathogenicity Prediction Platform, arXiv:2104.01033 [q-bio.GN], 2021
[3] L. Dorling et al, Breast cancer risks associated with missense variants in breast cancer susceptibility genes, https://doi.org/10.1101/2021.09.02.21262369, 2021
[4] R. Boonen, Functional analysis identifies damaging CHEK2 missense variants associated with increased cancer risk, Cancer Research, December 13, 2021
[5] Y. Xu et al., Deep Dive into Machine Learning Models for Protein Engineering, J. Chem. Inf. Model., vol. 60, no. 6, pp. 2773–2790, June, 2020

L1-4

Regression Models as Cornerstone of AI-guided Protein Design

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Protein engineering plays a central role in developing biocatalysts for biotechnology, biomedicine, and life science. Over recent years it has evolved significantly with the integration of machine learning (ML) techniques. Our study focuses on the application of ML algorithms in enhancing biocatalyst functionalities, including enzyme stability, function, and solubility. We have pioneered the use of ML algorithms as effective tools in protein engineering, specifically targeting biocatalysts.

Our methodology involves a two-step ML model application. Initially, our models proficiently predict protein sequence-to-function mappings. The approach does not require but can integrate detailed mechanistic or structural data. The application has proven particularly effective in low data regimes, even when only a few dozen functionally assayed sequence variants are available. Subsequently, we employ these predictions in a Bayesian optimization framework to guide the selection of candidates for experimental validation. This process allows simultaneous optimization of multiple parameters, such as stability, catalytic speed, and substrate specificity.

A notable achievement of our research is the superior performance of our prediction algorithms. They consistently outperform current state-of-the-art methods, including a recent algorithm developed by Novartis, across various datasets and benchmarks. The practical applicability of our algorithms was further validated through successful protein engineering campaigns, enhancing the functionality of complex enzymes like carboxylases, hydrogenases, and phosphohydrolases.

Our findings underscore the potential of ML methods in expediting directed evolution and rational design of proteins. By harnessing the power of existing sequence variant data, these methods effectively predict and select sequences with enhanced properties. During the talk, we will delve into these advancements in detail, highlighting practical applications, limitations and their significant impact on the future of protein engineering.

Fig. 1

Fig. 2


exazyme

L1-5

The EnzymeML framework: improving efficiency and quality of biocatalytic science

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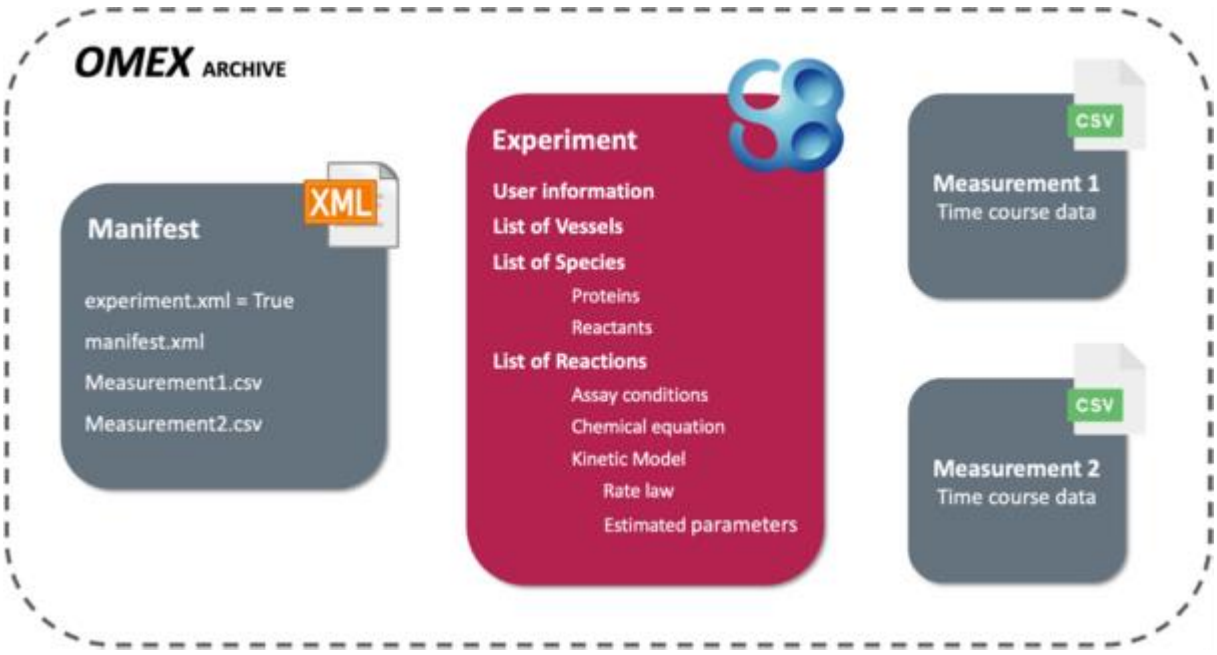
Biocatalysis is entering a promising era as a data-driven science. High-throughput experimentation generates a rapidly increasing stream of biocatalytic data, which is the raw material for mechanistic and data-driven modeling to design improved biocatalysts and bioprocesses. However, data management has become a bottleneck to progress in biocatalysis. In order to take full advantage of rapid progress in experimental and computational technologies, biocatalytic data should be findable, accessible, interoperable, and reusable (FAIR).
The EnzymeML framework (https://github.com/EnzymeML) provides reusable and extensible tools and a standardized data exchange format for FAIR and scalable data management in biocatalysis.1 To enable storage, retrieval, and exchange of enzymatic data, the XML–based markup language EnzymeML has been developed.2 An EnzymeML document contains information about reaction conditions and the measured time course of substrate or product concentrations (Fig. 1). Kinetic modelling is performed by uploading EnzymeML documents to the modelling platforms COPASI or PySCeS or by using the JAX platform (Fig. 2). The rate equation and the estimated kinetic parameters are then added to the EnzymeML document. The EnzymeML document containing the experimental and the modelling results is then uploaded to a Dataverse installation or to the reaction kinetics database SABIO-RK. The workflow of a project is encoded as Jupyter Notebook, which can be re-used, modified, or extended The feasibility and usefulness of the EnzymeML toolbox was demonstrated in six scenarios, where data and metadata of different enzymatic reactions are collected, analysed, and uploaded to public data repositories for future re-use3.
FAIRification of data and software and the digitalization of biocatalysis improve the efficiency of research by automation and guarantee the quality of biocatalytic science by reproducibility4. Most of all, they foster reasoning and creating hypotheses by enabling the reanalysis of previously published data, and thus promote disruptive research and innovation.

1. Range, J. et al. EnzymeML—a data exchange format for biocatalysis and enzymology. FEBS J. 289, 5864–5874 (2022).
2. Pleiss, J. Standardized Data, Scalable Documentation, Sustainable Storage – EnzymeML As A Basis For FAIR Data Management In Biocatalysis. ChemCatChem 13, 3909–3913 (2021).
3. Lauterbach, S. et al. EnzymeML: seamless data flow and modeling of enzymatic data. Nat. Methods 20, 400–402 (2023).
4. Pleiss, J. FAIR data and software: improving efficiency and quality of biocatalytic science. ACS Catalysis (in press)

Figure captions
Fig. 1 An EnzymeML document is a ZIP container in OMEX format and contains the experiment file (SBML) and the measurement files (CSV).
Fig. 2 An EnzymeML document serves as a seamless communication channel between experimental, modelling, and publication platforms.

Fig. 1


OMEX ARCHIVE
Manifest
XML
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manifest.xml
Measurement1.csv
Measurement2.csv
Experiment
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List of Vessels
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Proteins
Reactants
List of Reactions
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Kinetic Model
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Estimated parameters
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Measurement 1
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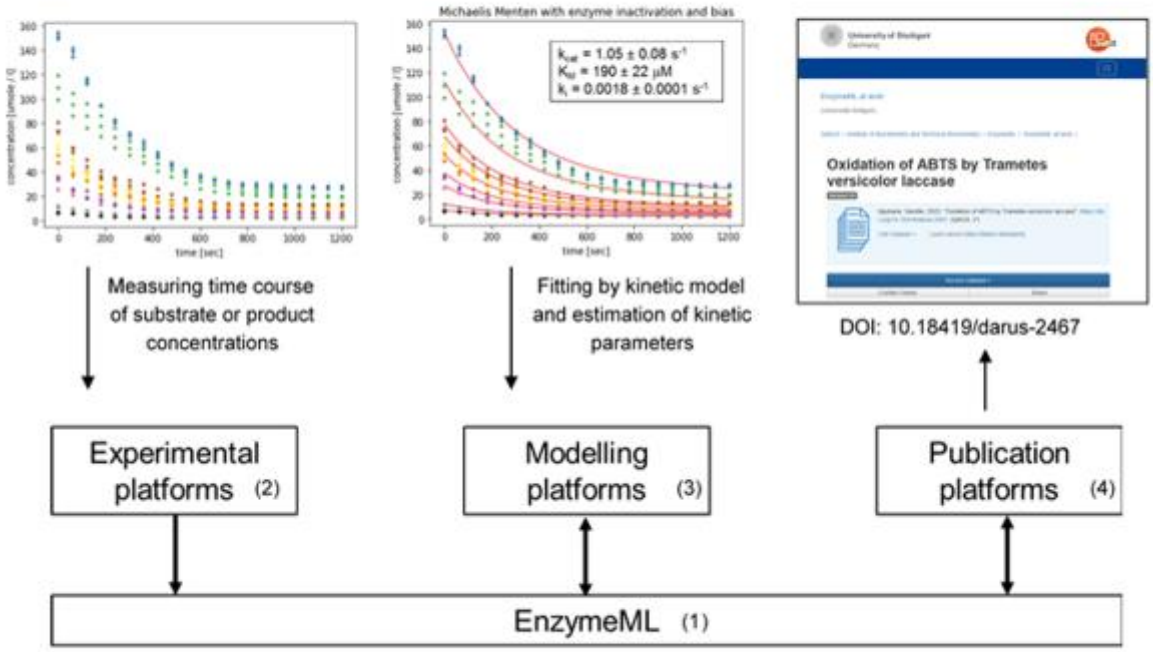
Measurement 2
Time course data

Fig. 2


Michaelis Menten with enzyme inactivation and bias
Oxidation of ABTS by Trametes versicolor laccase
Measuring time course of substrate or product concentrations
Fitting by kinetic model and estimation of kinetic parameters
DOI: 10.18419/darus-2467
Experimental platforms (2)
Modelling platforms (3)
Publication platforms (4)
EnzymeML (1)

L1-6

Computational Pipeline for Structure-based Prediction of Temperature-induced Unfolding of Proteins

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Question: Protein stability is a highly desirable property in biocatalysis. Next to the natural gene pool also diverse novel engineering tools are at hand to create diversified sequences that in the ideal case encode for enzymes with the same fold and function. The question we address is: Can we extract structural attributes from models of proteins with a known melting point to train algorisms to predict the melting point of other similar proteins? (1)

Methods: A set of phenolic acid decarboxylases was generated via ancestral sequence reconstruction and provided by collaboration partners. The melting points of the respective enzymes were determined via circular dichroism spectroscopy. Structure determination was done via X-ray crystallography. Various structural attributes were calculated from alpha fold models. Extracted were salt bridge networks, hydrogen bond networks, hydrophobic clusters and surface properties using a computational pipeline (code available at https://github.com/ugSUBMARINE/structural-properties). After applying statistics to the respective attributes they were used in different combinations to train regression models like k-nearest neighbors" regression or linear regression.

Results: The pipeline generates two outputs; First of all scripts that allow the visualization of the localization of the respective attributes in the protein structures. All decarboxylases share a similar hydrophobic core with salt bridge networks that a distributed in the outer layers of the proteins. Secondly the quantification of the respective attributes allows the training of regression models. When asked to recall the three proteins with the highest melting temperatures, the k-nearest neighbors model can successfully recall the three proteins with the highest melting temperatures out of the six proteins in a test data set.

Figure 1: Overview of the model training and prediction workflow: b) protein unfolding temperatures: Experimental and predicted values of the three best performing models and the SCooP server. LR - linear regression, KNN - k-nearest neighbors", RF - random forest.

Conclusion: Structural attributes can be extracted from alpha fold models of proteins with known melting points to train models to predict the melting points of unknown similar proteins. We have demonstrated the feasibility of our approach on decarboxylases but also tested it successfully on other publicly available data sets.

References:

1: Myrtollari *et al*. *ACS Sustainable Chem. Eng.* 2024, 12, 9, 3575–3584 https://doi.org/10.1021/acssuschemeng.3c06513

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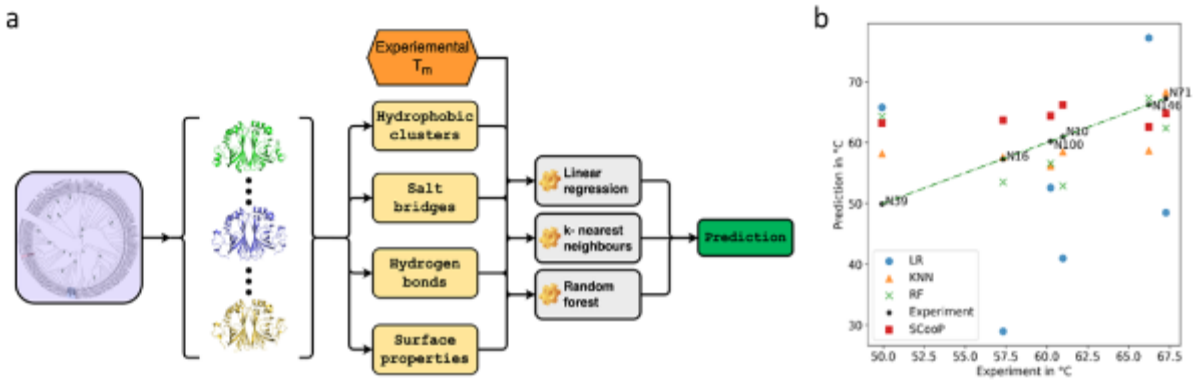
Fig. 1



P1-1

Enhancing Low-N Enzyme Engineering through Zero-Shot Predictor Integration

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Enzymes present a promising avenue for environmentally sustainable chemical processes, yet their adaptation for industrial contexts often necessitates substantial engineering efforts (1). To address this challenge, leveraging the capabilities of Machine Learning (ML) offers a viable approach, wherein predictive models can be developed based on empirical enzyme data. However, the efficacy of such models hinges on the availability of extensive mutational datasets, which can be hindered by the limited throughput of experimental assays which is common for biocatalysis (2). An alternative strategy involves the utilization of zero-shot (ZS) predictors, which are unsupervised models pre-trained on vast protein datasets, thereby encapsulating a profound understanding of underlying molecular principles (3). These ZS predictors obviate the need for experimental characterization by providing predictive estimates for all conceivable variants. Nonetheless, the profusion of pre-trained models presents a conundrum in selecting the most suitable ZS predictor (4).

In this investigation, we examine the correlation between a selection of pre-trained models and in-house experimental data in the low-n regime. Following the identification of the ZS model exhibiting the highest correlation, we construct supervised ML models incorporating the ZS predictions as features. Subsequently, each variant within the mutant library undergoes evaluation through both the optimized ZS model and the supervised ML model. The resultant predictions inform a subsequent round of experimental characterization, thereby facilitating informed decision-making regarding enzyme engineering strategies.

References:

1. Reetz, M. T., Qu, G. & Sun, Z. Engineered enzymes for the synthesis of pharmaceuticals and other high-value products. *Nature Synthesis 2024 3:1* **3**, 19–32 (2024).
2. Biswas, S., Khimulya, G., Alley, E. C., Esvelt, K. M. & Church, G. M. Low-N protein engineering with data-efficient deep learning. *Nature Methods 2021 18:4* **18**, 389–396 (2021).
3. Wittmann, B. J., Yue, Y. & Arnold, F. H. Informed training set design enables efficient machine learning-assisted directed protein evolution. *Cell Syst* **12**, 1026-1045.e7 (2021).
4. Notin, P. *et al.* ProteinGym: Large-Scale Benchmarks for Protein Fitness Prediction and Design. Preprint at (2023).

P1-2

Single-step biosynthesis of a novel LSD1 inhibitor using an IRED engineered rapidly using machine learning and laboratory automation

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Rapidly identifying sites within an enzyme's complex structure that can be mutated to improve biochemical characteristics is of high value for the adoption of biocatalytic routes. Standard approaches to directed evolution require the construction and iterative screening of tens of thousands of variants, making the process highly laborious and time consuming. Machine learning can be used to identify beneficial mutations and guide experiments but requires large experimental datasets to train effectively. We have used artificial intelligence combined with automated laboratory techniques to significantly reduce the time taken to pick out beneficial mutations in an engineered imine reductase (IRED), with no prior knowledge of the enzyme structure or experimental data sets.

We identified IMP023, which can synthesize a promising anti-tumour drug through the chiral resolution of the racemic substrate by reductive amination (albeit with low yield), by screening a panel of IREDs. By modelling IMP023 in silico, and training a machine-learning model, we selected a handful of mutations that had a high probability of improving reaction yield. These selections were used to rapidly construct four small genetic libraries using laboratory automation, alongside a small library focussed on the enzyme's active site, which were subsequently screened for improved activity and combined in multiple rounds of engineering based on activity gains.

After just three rounds of engineering, an enzyme was identified that produced the target compound at a conversion efficiency of 90%, whilst retaining its high chiral selectivity (a yield suitable to progress this variant into product development). This result is an example of how we are using machine learning to accelerate the enzyme engineering process, allowing the discovery of optimised enzyme variants more quickly by reducing the experimental screening burden and number of iterations required to obtain desired results.

P1-3

Using artificial intelligence for the identification of enzymes with desired properties

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In order to replace conventional fossil-based processes that are harmful to the climate, there is an increasing interest to develop innovative reaction pipelines using enzymes as biocatalysts for the creation of value chains. Enzymes operate under mild reaction conditions (in comparison to chemical catalysts), are biodegradable and enantio- / stereoselective. However, the identification of enzymes with predetermined properties for technical applications under harsh conditions (e.g. organic solvents, inhibiting molecules, etc.) is still limited.

To circumvent this limitation, algorithms can be developed based on public databases to predict the occurrence of a target enzyme class with the desired properties, for example thermostability of enzymes. [1+2] We are currently expanding the scope of properties, which can be predicted. Applying such algorithms to DNA datasets, acquired from environmental sampling of diverse habitats, resulted in the identification of candidate organisms possessing a potential enzyme with the desired properties. In order to efficiently grow these organisms, different media are currently being evaluated regarding the preferred media for achieving high optical densities and rapid growth. Furthermore, cell fractions of the microorganisms are being incubated together with different artificial electron acceptors / donors and the enzymatic activity was monitored using a chromatographic analysis. Initial experiments provide the first evidence of enzyme activity with the desired properties, confirming the AI's prediction. Such functional predictions and their verification might be transferable to other enzyme classes and will improve future enzyme discovery.

[1] Li, G., Rabe, K. S., Nielsen, J., Engqvist, M. K. M. (2019) Machine Learning Applied to Predicting Microorganism Growth Temperatures and Enzyme Catalytic Optima. ACS Synthetic Biology 8, 1411-1420; 10.1021/acssynbio.9b00099

[2] Peng, M., Mittmann, E., Wenger, L., Hubbuch, J., Engqvist, M. K. M., Niemeyer, C. M., Rabe, K. S. (2019) 3D-Printed Phenacrylate Decarboxylase Flow Reactors for the Chemoenzymatic Synthesis of 4-Hydroxystilbene. Chemistry – A European Journal 25, 15998-16001; https://doi.org/10.1002/chem.201904206

P1-4

STRENDA Biocatalysis Guidelines

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Introduction:

Research and the analysis of experiments are crucial for obtaining new insights and driving innovation.[1] Therefore, a detailed description and documentation of these processes are essential across all research domains. Also in biocatalysis, there is need to improve the reproducibility of experiments and quality of research data reporting. The interdisciplinary nature of biocatalysis with experts of various fields leads to challenges in standardized data acquisition and documentation. The inconsistent recording of metadata causes irreproducible experiments and results in literature.[2] As a consequence, extensive review and unnecessarily repeated experiments harm scientific progress.

Objective:

In order to improve the transparency, reproducibility and quality of reporting of biocatalytic experiments a collaborative, extensible and flexible metadata catalog is under development. The collaboration of an interdisciplinary and international team seems to be optimal to meet the requirements. The already established STRENDA guidelines for enzymological data[3] were used as a basis and extended with expert knowledge for the requirements of biocatalysis. The aim was to create a structured framework to comprehensively describe complex biocatalytic experiments and to offer this to the biocatalysis community in a freely accessible, extensible version.

Results:

The STRENDA Biocatalysis Guidelines provide a structured metadata catalog enhancing transparency and reproducibility of biocatalytic experiments by identifying and defining essential metadata. The catalog is accessible online in an interactive format via GitHub [https://w3id.org/strenda/biocatalysis-guidelines] and encourages the community to use it, suggest changes and discuss catalog-related issues. This iterative process, which is kept transparent by automatic versioning, ensures ongoing improvement and updating of the guidelines [**Figure 1**]. Its modular design enables easy, structured entry of metadata and a user-friendly interface which is flexible and adaptable for extensions and enables seamless integration into Python code, allowing the catalog to be used in modeling and software development. This also promotes collaboration and communication between experimenters, engineers and software developers.

Figure 1: Overall structure and motivation of the STRENDA Biocatalysis Guidelines project.

Conclusion:

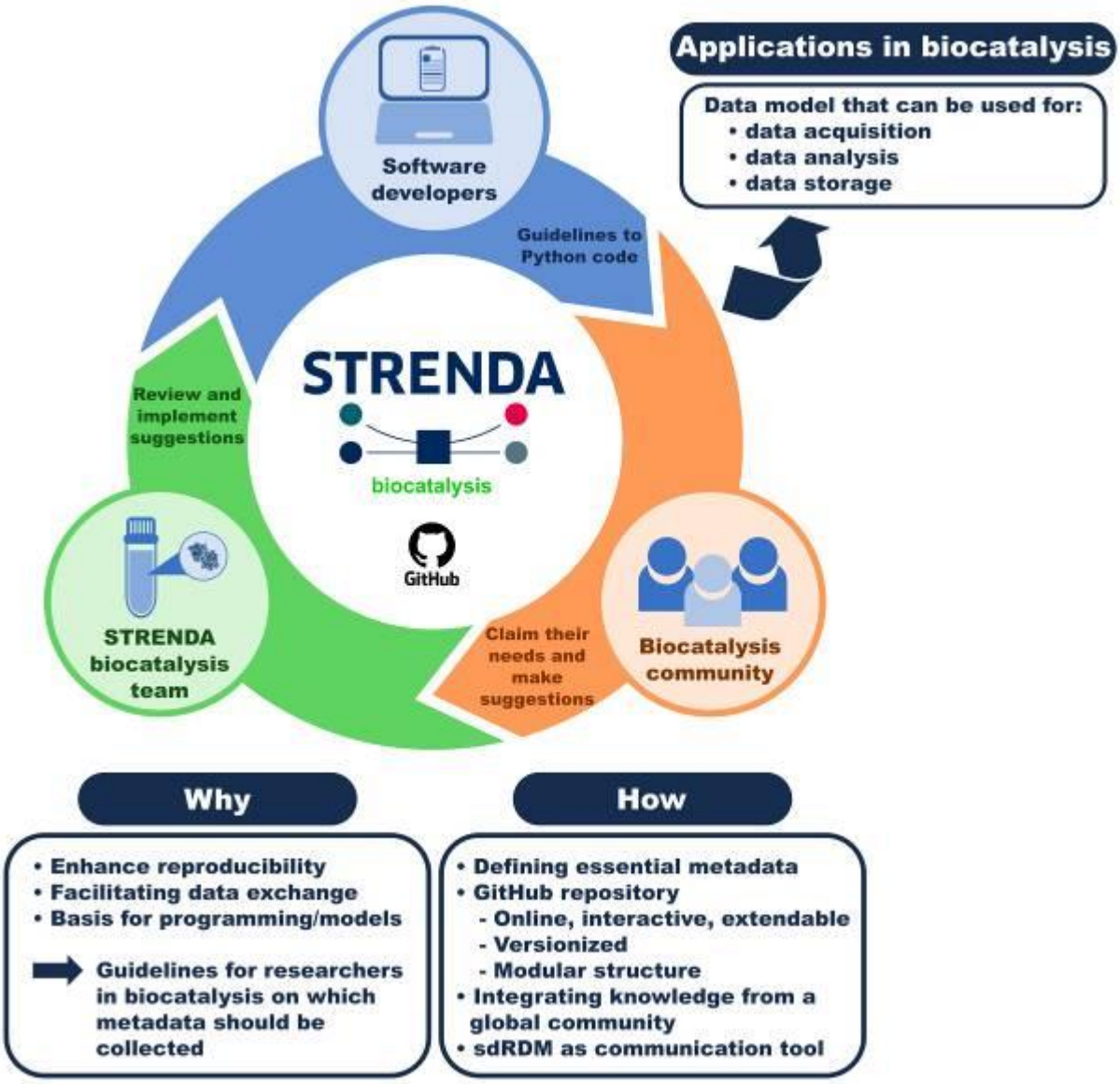
The STRENDA Biocatalysis Guidelines define essential metadata and therefore represent a significant step forward in improving the quality in the reporting of biocatalytic experiments. As a platform for the biocatalysis community, it can be used as a data model in data acquisition, data analysis and data storage.

[1] H. Kitano, *Science* **2002**, *295*, 1662-1664.

[2] a) C. Kettner, M. G. Hicks, *Current Enzyme Inhibition* **2005**, *1*, 171-181; b) P. Halling *et al.*, *Biophysical Chemistry* **2018**, *242*, 22-27.

[3] K. F. Tipton *et al.*, *Perspectives in Science* **2014**, *1*, 131-137.

Fig. 1



IL2-1

The golden age of peroxygenase engineering

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The selective insertion of oxygen into non-activated organic molecules has to date been considered of utmost importance to synthesize existing and next generation industrial chemicals or pharmaceuticals. In this respect, the minimal requirements and high activity of fungal unspecific peroxygenases (UPOs) situate them as the jewel in the crown of C-H oxyfunctionalization biocatalysts. Although their limited availability and development has hindered their incorporation into industry, the conjunction of directed evolution and computational design is approaching UPOs to practical applications. This lecture will address the most recent advances in UPO engineering, while discussing the future prospects in this fast-moving field of research.

Monterrey, D.T., Menés-Rubio, A., Keser, M., Gonzalez-Perez, D. and Alcalde, M. (2023). Unspecific peroxygenases: The pot of gold at the end of the oxyfunctionalization rainbow?. *Current Opinion in Green and Sustainable Chemistry* 41: 100786.

Beltran-Nogal, A., Sanchez-Moreno, I., Mendez-Sanchez, D., Gomez de Santos, P., Hollmann, F. and Alcalde, M. (2022). Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases. *Current Opinion in Structural Biology* 73: 102342.

Hobisch, M., Holtmann, D., Gomez de Santos, P., Alcalde, M., Hollmann, F. and Kara, S. (2021). Recent developments in the use of peroxygenases – exploring their high potential in selective oxyfunctionalisations. *Biotechnology Advances* 51: 107615.

IL2-2

A Journey towards Sustainable Catalysis – Examples of Biocatalysis in Development at Novartis Pharma

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Biocatalysis is a powerful and versatile tool for the synthesis of complex molecules in the pharmaceutical industry, gaining momentum as an enabling technology. It offers several advantages over conventional chemical methods, such as high selectivity, mild reaction conditions, reduced waste and environmental sustainability. By implementing the CodeEvolver technology from Codexis in our development projects, we advanced from pure enzyme users to experienced enzyme designers. Examples of the implementation of biocatalysis at Novartis Pharma from various projects will be presented. Our journey towards new and sustainable catalysis, enzyme engineering and our efforts to change the culture in favor of an early implementation of biocatalysis during development will be discussed.

References

G. Mann, F. V. Stanger, A Bio-logical Approach to Catalysis in the Pharmaceutical Industry, *Chimia*, **2020**, 74, 407.

G. Mann, F. V. Stanger, T. Peschke, K. Schroer, N. Q. Nguyen Trung, N. Burrer, F. Grassinger, L. Piccioni, O. Erdogan, J. Heimburger, H. Koehl, H. Ertle, R. Denay, P. Dreier, T. Schlama, Biocatalytic assembly of chemically modified oligonucleotides, *Tetrahedron Letters*, **2022**, 93, 153696.

E. Siirola, F. Eggimann, C. Moore, K. Schroer, A. Vargas, T. Peschke, T. Schlama, R. Snajdrova, Evolution of Biocatalysis at Novartis over the last 40 Years, *Chimia*, **2023**, 77, 376.

IL2-3

Exploring IREDs with Catalophore-AI: Shifting Frontiers for Broad-Scope Reductive Aminations

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Chiral amines are a crucial class of compounds in the pharmaceutical industry. Asymmetric reductive amination is a highly efficient method for synthesizing chiral amines. The recently widely used biocatalysts for asymmetric reductive amination are NADPH-dependent imine reductases (IREDs) or reductive aminases (RedAms). These enzymes catalyze a variety of transformations, such as the asymmetric reduction of imines as well as direct asymmetric reductive amination.

We have harnessed the power of our Catalophore™ technology to search and identify novel IREDs for the synthesis of a wide range of chiral amines. Through meticulous analysis of the entire IRED/RedAm sequence space and active-site catalophores, we have unearthed novel NADPH-dependent imine reductases (IREDs) that demonstrate high substrate versatility and selectivity for various carbonyl and amine substrate combinations.

Our in-depth research has not only led to the identification of highly efficient IREDs for direct synthesis of a wide range of amines and carbonyls through reductive amination, but it has also showcased the potential of combining catalophores with machine learning, deep learning and AI to virtually predict amination activities and improve IREDs for specific target reactions.The use of large, high-quality experimental data sets of diverse aminations has benefited the machine learning, deep learning and AI approach. The practical applications of our *in-silico* approach have been successfully demonstrated through its use in (semi)-preparative amine synthesis.

Our collaborative work has provided a comprehensive understanding of the reductive amination performance of the IRED family of enzymes, leading to the identification of catalysts that catalyze reductive aminations with high activity and remarkable selectivity. This cooperative and cross-disciplinary approach will undoubtedly have a significant impact on the synthetic capabilities of the pharmaceutical industry.

IL2-4

Synthesis of Pharmaceutical Amides Using Amide Bond Synthetases

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Introduction: The synthesis of amide bonds is one of the most important reactions in medicinal chemistry, accounting for some 16% of all reactions performed.[1] However, the requirement for activated starting materials and coupling reagents in standard amide couplings has stimulated research into biocatalysis as a more sustainable alternative.[2]

Objectives: We have focused on a class of Amide Bond Synthetases, derived from bacterial secondary metabolism, in which an amine and a carboxylic acid can be coupled *via* an adenylate intermediate within one enzyme active site. Specifically, we have employed enzymes related to McbA from the marinacarboline biosynthetic pathway of *Marinactinospora thermotolerans*[3] for the ATP-dependent ligation of a range of amine and carboxylic acid substrates, with the aim of synthesising amide pharmaceuticals.

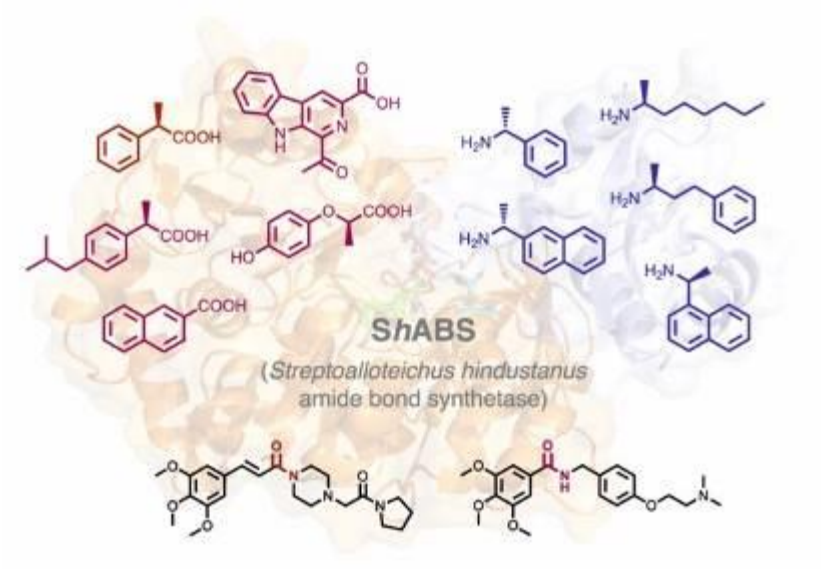
Results: We have used a mixture of organic synthesis, molecular biology, structural biology and enzyme engineering to enable the use of ABSs including McbA and *Sh*ABS from *Streptoalloteichus hindustanus*, for a wide range of amide bond synthesis reactions (**Figure 1**), including asymmetric transformations for the synthesis of chiral amides. We have observed complementarity between the activity of McbA homologs, and also a much wider range of substrate specificity for the carboxylic acid and amine substrates than had been envisaged previously.[4-6]

Figure 1. Formation of Pharmaceutical Amides by the Amide Bond Synthetase *Sh*ABS.

Conclusion: ABSs have proved to be an interesting new addition to the biocatalytic toolbox for amide bond formation, with advantages that are complementary to the available enzymatic reactions for these reactions.

References: Dunetz, *Org. Proc. Res. Devel.*, **2016**, *20*, 140; [2] Petchey, *Adv. Synth. Catal.*, **2019**, *361*, 3895; [3] Ji, *Tetrahedron Lett.*, **2014**, *55*, 4901; [4] Petchey, *Angew. Chem. Int. Ed.*, **2018**, *57,* 11584; [5] Petchey, *ACS Catal.*, **2020**, *10*, 4659; [6] Tang, *ACS Catal.*, **2024**, *14*, 1021.

Fig. 1



L2-1

Bioinformatic-assisted identification and engineering of promiscuous amidases for multi-purpose applications

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The overall production of plastics has been ever-increasing since the introduction of synthetic polymers in the early 1900s. Their resistance to degradation and the simple upscaling for mass production have made plastics indispensable materials in basically every aspect of modern life and technology. However, these features have culminated in today"s global ecological crisis due to the accumulation of plastic waste and insufficient recycling plans. Hence, effective and sustainable recycling strategies, ideally, leading to a circular plastic economy, are urgently needed. In this regard, the biodegradation of synthetic polymers by microorganisms or isolated enzymes and the reuse of monomers are fields of vivid research. While the biocatalysis-based hydrolysis of polyethylene terephthalate (PET) has been established, other abundantly used plastics such as polyurethanes (PUs) and polyamides (PAs) largely resist biodegradation.

In this work, the bioinformatic-assisted mining of public databases yielded a new panel of amidases with promiscuous activity against low molecular weight carbamates as well as structurally related compounds including amides and (thio)esters. Following the structrual elucidation of a metagenomic urethanase, which belongs to the amidase superfamily, the evolvability of various promiscuous amidases was demonstrated successfully and yielded enzyme variants with enhanced hydrolysis profiles. Together, these findings not only expand the number of available amidases/urethanases and resolved - to date - the first structure of a metagenome-derived urethanase; the broad hydrolysis profile of newly identified wildtype enzymes and derived variants suggests versatile applications for the bio-based recycling of plastic waste, the removal of commonly used protecting groups in organic and medicinal chemistry like the Cbz group, or the (bio)remediation of pesticides and other contaminants.

L2-2

Spiroluchuene A Synthase: A Cyclase from *Aspergillus luchuensis* Forming a Spirotetracyclic Diterpene

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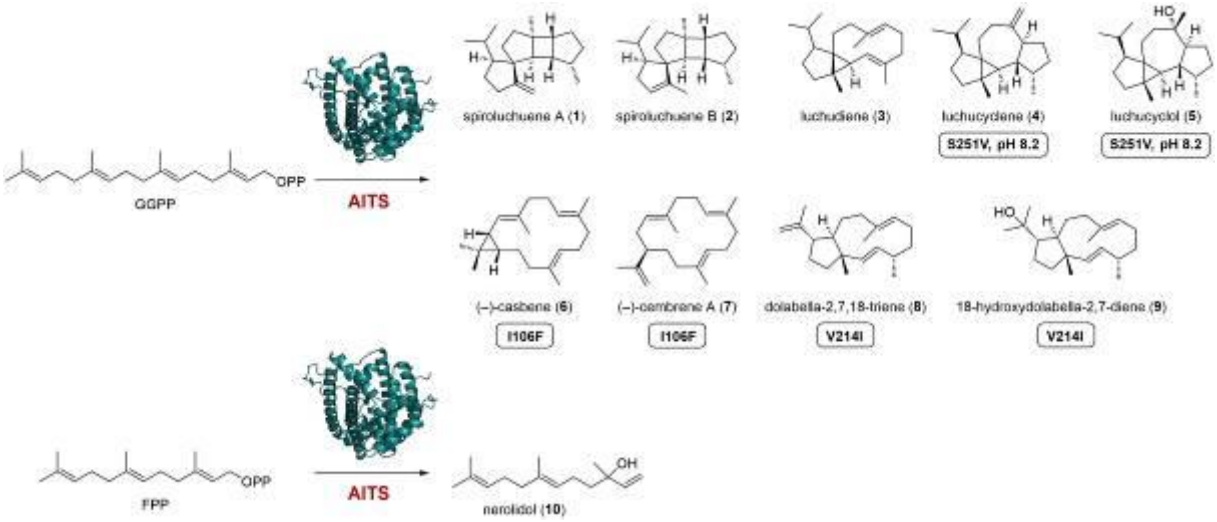
The diterpene synthase AlTS was identified from *Aspergillus luchuensis*. AlTS catalyses the formation of the diterpene hydrocarbon spirochuluene A that exhibits a novel skeleton characterised by a spirocyclic ring system. The cyclisation mechanism towards this compound was elucidated through isotopic labelling experiments in conjunction with DFT calculations and metadynamic simulations. Through analysis of model built by AlphaFold, several mutants were constructed to obtain new diterpenoids. The biosynthetic intermediate luchudiene, besides the derivative spiroluchuene B, was captured from an enzyme variant obtained through site-directed mutagenesis. With its 10-membered ring luchudiene is structurally related to germacrenes and can undergo a Cope rearrangement to luchuelemene. Under a lower temperature (120 °C) in the presence of oxygen (air), the oxidation product luchucyclol B epoxide is obtained. Its formation can be rationalised through epoxidation of luchudiene at the C2=C3 double bond. Epoxide opening may induce a ring closure to luchucyclol B that may again be epoxidised to form luchucyclol B epoxide. This hypothesis is supported by the isolation of luchucyclol B from a thermal reaction at 60 °C in the presence of air.

Fig. 1



L2-3

Enzymatic late-stage functionalization – Creating compound diversity

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Introduction:

Selective C-H functionalization under mild conditions suitable for late-stage functionalization (LSF) is one of the key challenges medicinal chemists are currently facing. The need for speed in drug development is pressuring our small molecule timeline and novel solutions are required to accelerate lead identification.[1]The application of enzymes offers an obvious solution to the problem due to the bening conditions used for enzymatic reactions. In the past years the advances in high-throughput screening (HTS) have helped to broaden our understanding for substrate promiscuity of the tested enzymes. Together with state of the art in silico tools rapid enzyme optimisation for industrial purposes was achieved.[2] However, the application of enzymes for late-stage functionalisation of compound libraries for rapid drug molecule diversification in the early phase of drug discovery remains underexplored. The implementation and interconversion of functional groups of interest is diffcult as only few revlevant enzyme libraries for selective hydroxylation, halogenation or alkylation are currently available.[3] Data on enzyme promiscuity for a diverse set of molecules is rarely available and thus HTS is required to firstly identify active enzymes and secondly profile enzyme promiscuity as well as selectivity.[4]

Objective:

To adress the latter challenges we are building our own enzyme libraries that will systematically be analysed for their apllicability in drug molecule diversification. The data generated by HTS is used as training set for machine learning and combined with in silico analysis for active site interaction mapping. Data guided rational protein engineering is used to optimize and tailor candidate enzymes to match compound scaffolds and thus decrease screening efforts and experiments required.

Results:

Current efforts are focussed on the application of methyltransferase for site-selective methylation of drug molcules. We were able to demonstrate enzyme dependent scaffold promiscuity upon building block profiling.The data was used to train a machine learning model and scaffold promiscuity was shown to be translatable ressulting in a highly efficient methyltransferase for piperazine containing drug molecule alkylation.

Conclusion:

Based on the promising results similar wofklows will be implemented for biocatalytic halogenation and hydroxylation to broaden our understanding for biocatalytic LSF and apply discovery biocatalysis for compound library diversification to enable rapid structure-activity realtionship (SAR) as well as structure property relationship (SPR) exploration and thus accelerate drug discovery to match the increasing demand for novel drugs.

References

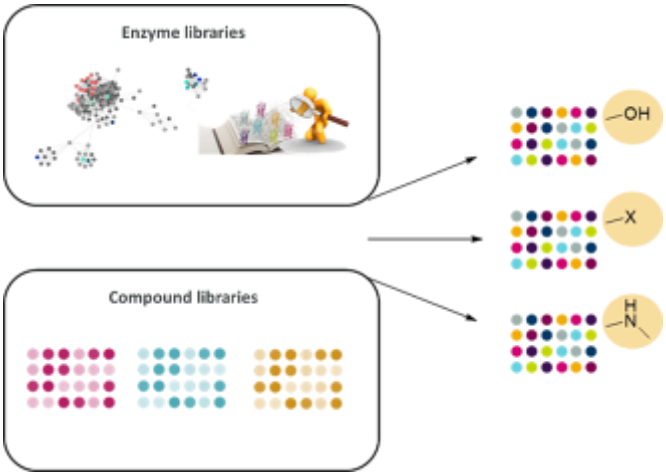
[1] M.D. Truppo *et al*. *ACS Med. Chem. Lett.* **2017***,* 8(5), 476-480.

[2] R. Buller *et al*. *Science* **2023**, 382(6673).

[3] E. Romero *et al*. *Angew. Chem. Int. Ed.* **2021**, 60(31), 16824-16855.

[4] M. Braun *et al*. *ACS catalysis* **2023**, 13(21), 14454-14469.

Fig. 1


Enzyme libraries
Compound libraries
OH
X
H
N

L2-5

How do metalloproteins manipulate their cofactor's reactivity to determine the metal preference of their catalysis?

N. Kwiatos[1], M. Esmaeeli[1], R. Mazgaj[1], S. Ciofi Bafoni[2], M. Dadlez[1], T. Kehl-Fie[3], K. Waldron[1]
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1. Introduction

Superoxide dismutases (SODs) are crucial enzymes for most organisms as they detoxify superoxide, a reactive oxygen radical harmful to cells. *Staphylococcus aureus* possesses two SODs, one manganese dependent and another one cambialistic, i.e. active with either iron or manganese. These two SODs are closely related, likely derived from a recent gene duplication event followed by the divergence of the new SOD to gain cambialistic function, and therefore show extensive sequence and structural homology. This has enabled us to make important fundamental discoveries about SOD function by leveraging them as a model system.

Evolutionary studies of the superoxide dismutase family (SodFM) show that phylogenetics does not correlate with metal specificity. We identified isozymes with multiple metal preferences in all five subfamilies (SodFM1-SodFM5). Hence, it is unclear what makes one enzyme active with one metal but inactive with the other, and still others cambialistic.

2. Objectives

Our current work aims to:

1. a) Discover molecular mechanisms that determine metal specificity of *aureus* SODs
2. b) Identify evolutionary processes that have manipulated their metal specificity
3. c) Determine how these enzymes work in atomic resolution, including protein dynamics

3. Results

Building on previous work that combined bioinformatic and biochemical approaches to study SODs, here we applied protein engineering and biochemistry with biophysical methods, such as NMR and HDX-MS, to study structure-function relationships in SodFM proteins. We investigated an atlas of amino acids localised within the metal ion's outer coordination sphere and studied their influence on metal specificity. Furthermore, unpublished data on the internal dynamics of these enzymes, obtained using magnetic resonance and mass spectroscopy approaches, will be described.

4. Conclusion

The SODs are a unique model system in which the molecular properties of proteins that can carefully control and manipulate the reactivity of bound metal cofactors can be studied. Furthermore, they have proven to be a useful system in which to study how the preference of a metalloenzyme for a specific metal cofactor can be altered and optimized by evolutionary processes to enable organisms to adapt to changes in environmental metal availabilities. Our studies shed light on how these ubiquitous enzymes' molecular dynamics and catalytic metal preferences are controlled chemically and biophysically, and how these properties evolve. Such studies apply to understanding the function and evolution of diverse natural metalloproteins and have potential application in the design of bespoke artificial metalloenzymes to perform novel chemical reactions of biotechnological value.

L2-6

Predictive biotechnology: Revealing sequence-function relationships of 4-phenol oxidases

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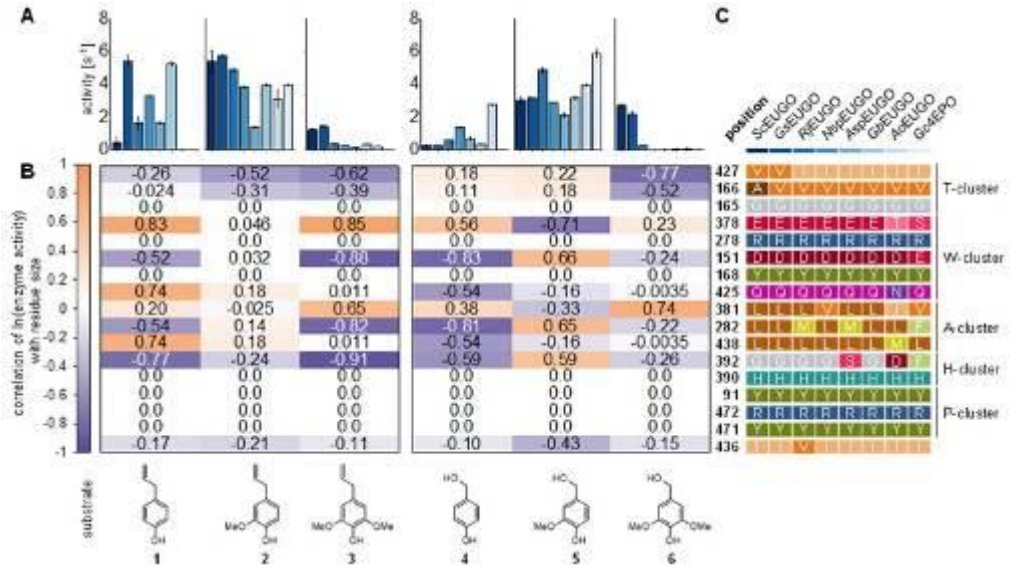
Enzymes are Natures" catalysts and have a great impact on (organic)synthesis either in their natural role or by being applied in technical processes. Certainly, the knowledge about enzymes and their functionality is steadily increasing. In addition, frequently novel enzymes and reactions are described. Herein, we present a streamlined approach of enzyme mining to rationally select enzymes with proposed functionalities from the ever-increasing amount of available sequence data. Hence, we want to predict enzyme functionality and applicability based on the amino acid sequence: a work related to predictive biotechnology.

In a case study on 4-phenol oxidoreductases, to be precise on the clade of the eugenol oxidases of the VAO/PCMH flavoprotein family, eight enzymes were selected from about 300 sequences on basis of the properties of first shell residues of the catalytic center. Thus, a broad sequence space was covered on base of a first bioinformatic screening approach including known enzyme studies. Thereafter, selected candidates were produced and fully characterized. Correlations between important residues identified and enzyme activity yielded robust sequence-function relations, which were exploited by site-saturation mutagenesis. Application of a novel oxidase screening resulted in 16 active enzyme variants which were up to 90-times more active than respective wildtype enzymes. The results were supported by kinetic experiments and structural models. The newly introduced amino acids confirmed the correlation studies (Fig. 1) which overall highlights the successful logic of the presented approach.

Fig. 1. Analysis of the catalytic pocket on the acceptance of substrates with varying *o*-methoxylation. **A:** Activity on the indicated substrates by the color-coded oxidases of this study. **B:** Heatmaps for the correlation of the logarithmic enzyme activity with the change in the size of each individual residue among the selected oxidases. A negative correlation of the amino acid size and the activity means that for enzymes containing larger residues, lower activities were observed and *vice versa*. **C:** Natural diversity of residues at the indicated position of each oxidase is shown. They are color-coded according to A and the residues are grouped according to functional clusters in the catalytic center of *Rj*EUGO.

Fig. 1



L2-7

Biocatalytic Ether Lipid Synthesis by an Ultrastable Archaeal Glycerolprenylase

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[2]Helmholtz Centre for Infection Research, Braunschweig, Germany
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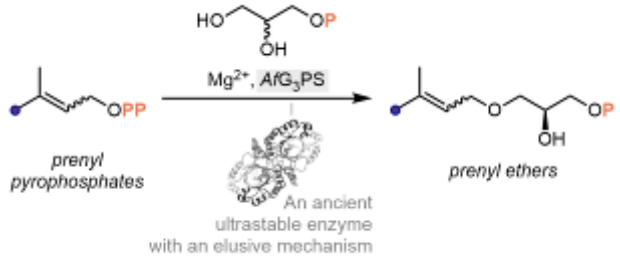
Although ethers are common in secondary natural products, they are an underrepresented functional group in primary metabolism. As such, there are comparably few enzymes capable of constructing ether bonds in a general fashion. However, such enzymes are highly sought after for synthetic applications as they typically operate with higher regioselectivity and under milder conditions than traditional organochemical approaches. To expand the repertoire of well characterized ether synthases, we recently reported on a promiscuous archaeal prenyltransferase from the scarcely researched family of geranylgeranylglyceryl phosphate synthases (GGGPSs or G3PSs)[1] and described methods to facilitate the analytical entry into research with the enzymes.[2] We found that the ultrastable *Archaeoglobus fulgidus* G3PS makes various (*E*)- and (*Z*)-configured prenyl glycerol ethers from the corresponding pyrophosphates, while exerting perfect control over the configuration at the glycerol unit. Remarkably, this enzyme orchestrates these transformations in a tremendously solvent-exposed active site and without a classical binding site for catalytically essential Lewis acids such as Mg2+. Based on experimental and computational data, we proposed a mechanism for this enzyme which involves an intermediary prenyl carbocation equivalent. As such, our work provides the fundamental understanding and methods to introduce G3PSs into the biocatalytic alkylation toolbox.

References

[1] F. Kaspar, L. Eilert, S. Staar, S.W. Oung, M. Wolter, C.S.G. Ganskow, S. Kemper, P. Klahn, C.R. Jacob, W. Blankenfeldt, A. Schallmey, *ChemRxiv,* **2024**, preprint, https://doi.org/10.26434/chemrxiv-2024-2lmv8-v2.

[2] L. Eilert, A. Schallmey, F. Kaspar, *Anal. Chem.,* **2022**, *94*, 3432–3435, https://doi.org/10.1021/acs.analchem.1c05356.

Fig. 1



L2-8

Engineering Unspecific Peroxygenase from *Agrocybe aegerita* Towards Efficient Aliphatic Substrate Oxyfunctionalization

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Alkanes are a cheap raw material originating from raw natural gas. They are primarily burned to generate heat or to power engines instead of using them in synthesis due to their chemically inert nature. Considering the demand for sustainable production routes, new ways of alkane valorization must be explored. Chemical hydroxylation requires high pressures and temperatures and reaches poor selectivities. Comparatively, enzyme catalyzed oxyfunctionalization of inert C-H bonds is regarded as "dream reaction" due to high selectivity and mild reaction conditions with low catalyst loading. Among the oxyfunctionalizing enzymes, the hydrogen peroxide dependent unspecific peroxygenases (UPOs) are especially attractive due to their robustness, high activity and independency from molecular oxygen and expensive cofactors, making them a good candidate for alkane valorization.

The short family of UPOs typically features aliphatic amino acids lining the access channel to their active site and show a preference to aliphatic substrates while the long family of UPOs often features aromatic amino acids in their access channel, corresponding to a preference for aromatic substrates. The long-family UPO from *Agrocybe aegerita* (especially its variant PaDaI) is a well-established model UPO and can be produced to high titers by the heterologous production yeast *Komagataella phaffii* (*Pichia pastoris*). We individually exchanged each of *Aae*UPOs 4 phenylalanines in its access channel and 3 in its active site with aliphatic ones (Ala, Gly, Ile, Leu, Val) to investigate the enzymes performance to hydroxylate alkanes to their corresponding alcohols and combine the process-related advantages of *Aae*UPO with the high aliphatic substrate affinity of short-family UPOs.

The 35 enzyme variants with single amino acid exchanges were expressed with a yeast surface display system to allow for rapid purification of multiple variants. They were investigated for alkane hydroxylation, determining kM values, regioselectivity and alcohol production rates. 15 variants featured amino acid exchanges directly in the enzymes active site and were additionally characterized for chiral selectivity of alkane hydroxylation. Docking studies were conducted for the active site variations of the enzyme to gain insight into the rationale of the amino acid exchanges during catalysis.

Exchanging the 3 active site phenylalanines lead to changes in regio- and stereoselectivity but also influences catalytic efficiency through steric effects, strongly dependent on the used substrate. Exchanging the 4 phenylalanines in the upper access channel expectedly had little effect on the selectivity of the reaction but could improve affinity towards certain substrates. Exchanges of phenylalanine to leucine and isoleucine were especially beneficial for hydroxylation activity. This work advances UPO-based alkane hydroxylation towards industrial feasibility.

P2-1

Protein engineering of formolases for the bioconversion of C1 compound formaldehyde

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C1 molecules represent attractive long-term feedstocks which can potentially sustain production of fuels and fine chemicals at hundreds of millions of tons per year. Among the C1 molecules, **formaldehyde is a particularly versatile and reactive compound which offers great promise for the production of value-added chemicals**. **Formolases are new enzymes catalyzing the building of three-carbon compound (1,3-dihydroxyacetone) and two-carbon compound (glycolaldehyde) using formaldehyde as the sole substrate.** Formolases, obtained through protein engineering, have been used in many artificial routes of C1 compounds conversione. However, their application is still limited by its low resistance towards formaldehyde *in vitro* and low activity for formaldehyde *in vivo*.

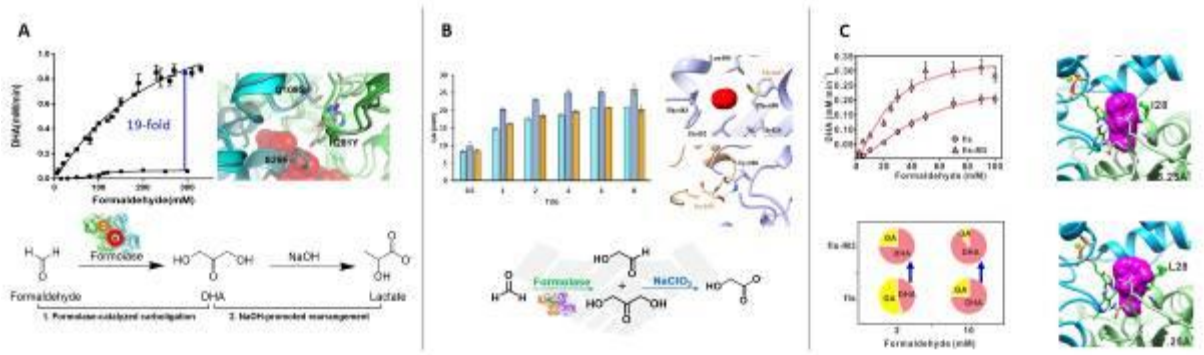
To tackle these problems, we engineered formolase$_{BFD}$ and formolase$_{BAL}$ through iterative rounds of directed evolution and elucidated the molecular insights of the property improvement through molecular dynamic simulations. We then designed chemoenzymatic conversion routes for the formaldehyde utilization. The engineered formolase$_{BFD}$ variant M6 exhibits substantially improved activity by 19-fold, improved formaldehyde resistance (up to 500 mM) and altered product selectivity from two-carbon glycolaldehyde (GA) to three-carbon dihydroxyacetone (DHA) as the main product. We designed a two-step chemoenzymatic route to convert formaldehyde into lactic acid using the engineered formolase and NaOH as catalysts, with 100% atom economy and 82.9% overall yield under near ambient condition[1]. Furthermore, we enhanced the formaldehyde resistance of formolase to 1M formaldehyde. With the obtained formolaseBFD variant M4V2 and the green oxidant sodium chlorite, we developed a two-step chemoenzymatic route to convert formaldehyde into glycolic acid[2]. In oder to increase the substrate affinity, we engineered formolase$_{BAL}$ to enable efficient conversion of formaldehyde at millimolar concentration using directed evolution. The formolaseBAL variant M3 allowed the successful starch synthesis from carbon dioxide[3]. Crystallization and MD simulations elucidated that the stabilization of the dimeric structure of formolase and enhancing the stability of the reaction intermediates are essential for the activity and formaldehyde resistance of formolases.

***Figure 1**. A. Engineered formolaseBFD M6 with significantly improved activity and a chemoenzymatic conversion of formaldehyde into lactic acid. B. Engineered formolaseBFD M4V2 with significantly improved foraldehyde resistance and a chemoenzymatic conversion of formaldehyde into lactic acid. C. Engineered formolaseBAL M3 exhibiting improved activity at millimolar concentration of formaldehyde.*

References

[1]. **Zhu L**. et al. Totally atom-economical synthesis of lactic acid from formaldehyde: combined bio-carboligation and chemo-rearrangement without the isolation of intermediate. **Green Chem**, 2020, 22(20), 6809-6814. **[2]**. **Zhu L**. et al. One-pot chemoenzymatic synthesis of glycolic acid from formaldehyde. **Green Chem**,2022, 24(13), 5064-5069. **[3]**. **Zhu L**. et al. Cell-free chemoenzymatic starch synthesis from carbon dioxide. **Science**, 2021, 373(6562), 1523-1527.

Fig. 1



P2-2

Engineering of D-Lactate Dehydrogenase Towards Acceptance of Nicotinamide Biomimetics

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Oxidoreductases are a widely applied enzyme family which is dependent on redox cofactors such as nicotinamide adenine dinucleotide (NADH).[1] There is a growing interest in employing these NADH-dependent oxidoreductases into microbial glycolytic pathways for fermentative production of chemicals. Changing their cofactor dependence would allow separation of growth and productive fermentation.[2] This channelling of the electron flux eliminates the need for extensive strain engineering.[3] An example for such a nicotinamide biomimetic is nicotinamide mononucleotide (NMNH) which is an intermediate in the nicotinamide biosynthesis. Thus, it offers the advantage of being available in bacterial cells, albeit in low concentration.[3]

To this end, our objective is to engineer the NADH-dependent D-lactate dehydrogenase from *E. coli* (EcLDH) towards increased NMNH affinity. NMNH is deficient of the adenosine monophosphate (AMP) moiety, which functions as a recognition handle for the cofactor. Structurally modifying the highly conserved Rossmann fold motif is challenging. We built a model structure of EcLDH with bound cofactor (NADH, NMNH) and identified interactions in the cofactor binding pocket. A rationally designed variant library was constructed with two focuses: a) breaking interactions with the AMP moiety to repel NADH and b) forming interactions with the monophosphate moiety to increase NMNH affinity.

We set up a screening platform featuring enzyme purification, photometric activity determination and hit validation by monitoring the D-lactate production by high-performance liquid chromatography with photodiode array detection. The wild-type enzyme showed very low affinity towards NMNH with the specific activity being >105 fold lower compared to the native cofactor. Mutations increasing the NMNH affinity while decreasing the NADH affinity are being identified and recombined.

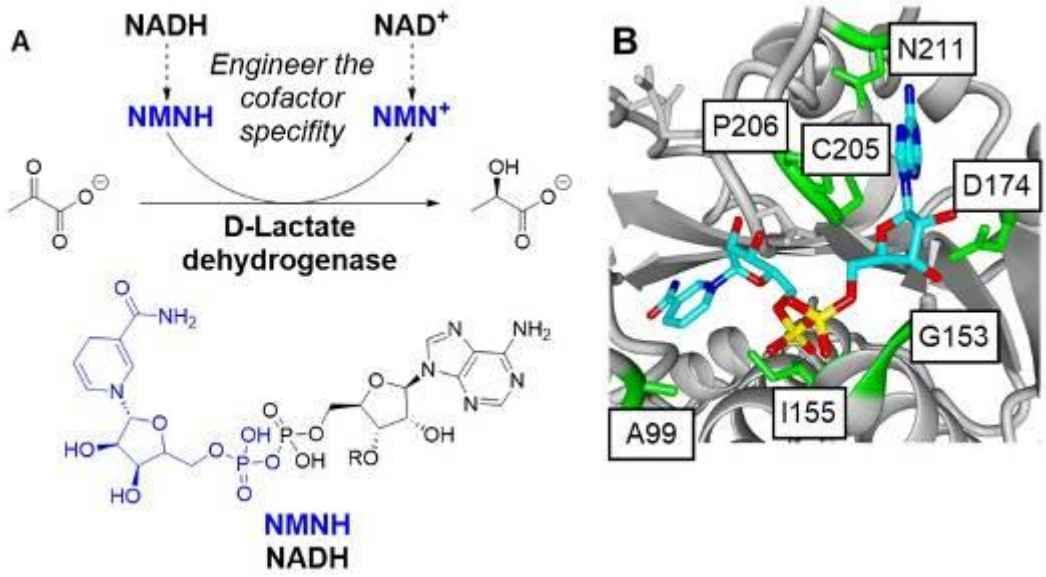
Figure 1. A: EcLDH-catalyzed reduction of pyruvate to D-lactate employing NMNH as cofactor biomimetic, B: Insight into the cofactor binding pocket of EcLDH with bound NADH and target residues (green).

[1] Y. Cárdenas-Moreno, J. González-Bacerio, H. García Arellano, A. Del Monte-Martínez, *Biotechnol. Appl. Biochem.* **2023**, *70*, 2108-2135.

[2] R. A. Weusthuis, P. Folch, A. Pozo-Rodríguez, C. E. Paul, *iScience* **2020**, *23*, 101471.

[3] E. King et al., *Nat. Commun.* **2022**, *13*, 7282.

Fig. 1



P2-3

A robust growth-based selection platform for (continuous) enzyme engineering

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Introduction

Directed evolution has revolutionized the application of enzymes in industrial settings by allowing users to tailor the properties and activities of biocatalysts to their needs. However, classic directed evolution is notoriously labor- and time-intensive, as it manually stages mutation, screening/selection, and amplification cycles. For example, routinely-used multi-well screens that assess biocatalysts one-by-one are laborious and slow, in contrast to growth-based selections which enable the rapid and straightforward identification of highly efficient biocatalysts on a population level. Unfortunately, selection-based improvement of synthetically useful biocatalysts is challenging, as such biocatalysts typically do not confer a growth advantage to producing organisms.

Objectives

To tackle this challenge, we first set out to establish an in vivo selection platform to reliably link diverse enzymatic activities to the replicative fitness of *Escherichia coli.* Then, we aimed to interface it with gene-directed hypermutation strategies to elicit improved biocatalysts with minimal researcher intervention (=continuous evolution).

Results

In this presentation, I will showcase the robustness and scalability of a growth-based selection platform that is based on the complementation of *E. coli* addicted to noncanonical amino acids (ncAAs) (Figure 1).[1,2] In this system, host survival under selective carbenicillin pressure depends on the activity of an enzyme to convert a suitable ncAA precursor, thus enabling the selection of highly active biocatalysts by continuous growth-dilution cycles (Figure 2). Specifically, I will discuss how we applied our strategy to elicit a diverse panel of efficient biocatalysts from a large and diverse library containing up to a million variants. Lastly, I will also showcase our efforts to drastically increase the mutation rate of genes of interest in vivo, in order to develop a versatile, scalable, and low-tech continuous evolution platform, which can engineer efficient biocatalysts autonomously.

Conclusion

Overall, I will highlight the advantages of applying growth-based selections in the engineering of synthetically useful biocatalysts, and how our fast and user-friendly platform enables an exhaustive sampling of the available sequence space, which thus far presents a persistent bottleneck in most directed evolution campaigns.

[1] Rubini, R.; Jansen, S. C.; Beekhuis, H.; Rozeboom, H. J.; Mayer, C. Selecting Better Biocatalysts by Complementing Recoded Bacteria. *Angew. Chem., Int. Ed.* **2023**, *62*, No. e202213942.

[2] Jansen, S.C.; Mayer, C. A Robust Growth-Based Selection Platform to Evolve an Enzyme via Dependency on Noncanonical Tyrosine Analogues. *JACS Au* **2024**. DOI: 10.1021/jacsau.4c00070.

Fig. 1

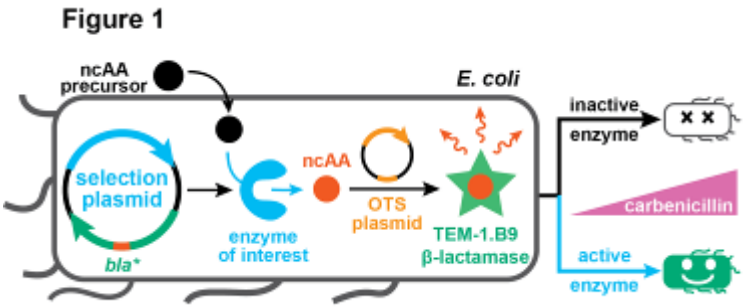

Fig. 2

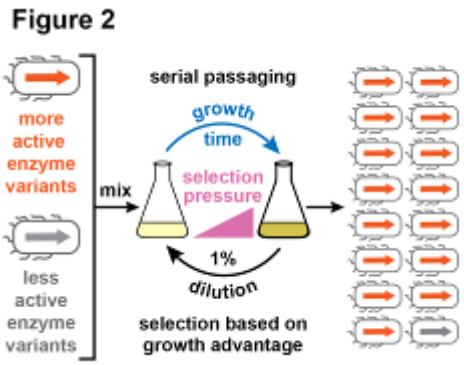
Figure 2
more active enzyme variants
less active enzyme variants
mix
serial passaging
growth
time
selection pressure
1%
dilution
selection based on growth advantage

P2-4

Elucidating the function of enzyme oligomerization in the fluorinase

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[4]Aarhus University, Department of Chemistry, Aarhus, Denmark

Organofluorides are critical to society, making up over 50% of agrochemicals (1) and 20% of pharmaceuticals (2). Currently, all industrially produced organofluorides are chemically synthesized using harsh, complex reaction schemes. Producing these organofluorides enzymatically would provide a route for the green transition of the organofluoride industry, but the lack of enzymes capable of performing these reactions precludes such a transition. The fluorinase remains the only enzyme known to catalyze direct, stable C-F bond formation in nature, but it has an incredibly slow catalytic rate and a very narrow substrate range (3). Engineering efforts to improve the fluorinase have largely failed, likely due to the lack of understanding of the factors that govern its catalysis. We believe that the elucidation of the structure-function relationship will provide insights into new routes for fluorinase engineering. Its unique hexameric structure is a dimer of trimers, with a trimeric subunit structure homologous to the functional unit of the related chlorinase (4). Although it is established that active site formation requires trimerization, the functional consequence of further oligomerization is largely unknown. Recent data from our lab demonstrate that a trimeric fluorinase mutant retains the wildtype catalytic rate, but has a greatly increased Michaelis-Menten constant, revealing that oligomerization is not required for activity but provides a significant benefit to enzymatic function (5). Using small-angle x-ray scattering (SAXS) and cryogenic electron microscopy (cryoEM), we have collected data which reveal a previously undescribed "open" hexameric conformation (Fig. 1). This conformation has only two contact points instead of the three in seen *in crystallo* (Fig. 2). These data, together with the kinetics of the trimeric fluorinase, suggest that fluorinase hexamerization and its dynamics could play a critical role in substrate shuttling that is essential for efficient catalysis. To further understand the role of hexamerization and its dynamics in fluorinase function, we are currently performing mutational analysis on the hexamer interface. Our approaches include analyzing the oligomeric state and activity of cross-linked fluorinase variants which are forced to remain in a closed conformation and fluorinase variants with mutations at the hexamer interface. We hope that the understanding of the essentiality of the hexamer conformational dynamics will provide a new avenue for fluorinase engineering and lead to the development of faster and more promiscuous fluorinases.

Figure 1. *Ab initio* model from cryoEM data.

Figure 2. Crystal structure of the fluorinase from *Streptomyces cattleya (PDB ID 2C2W).*

References

1. L. Martinelli, P. I. Nikel, *Microb. Biotechnol.* 2019

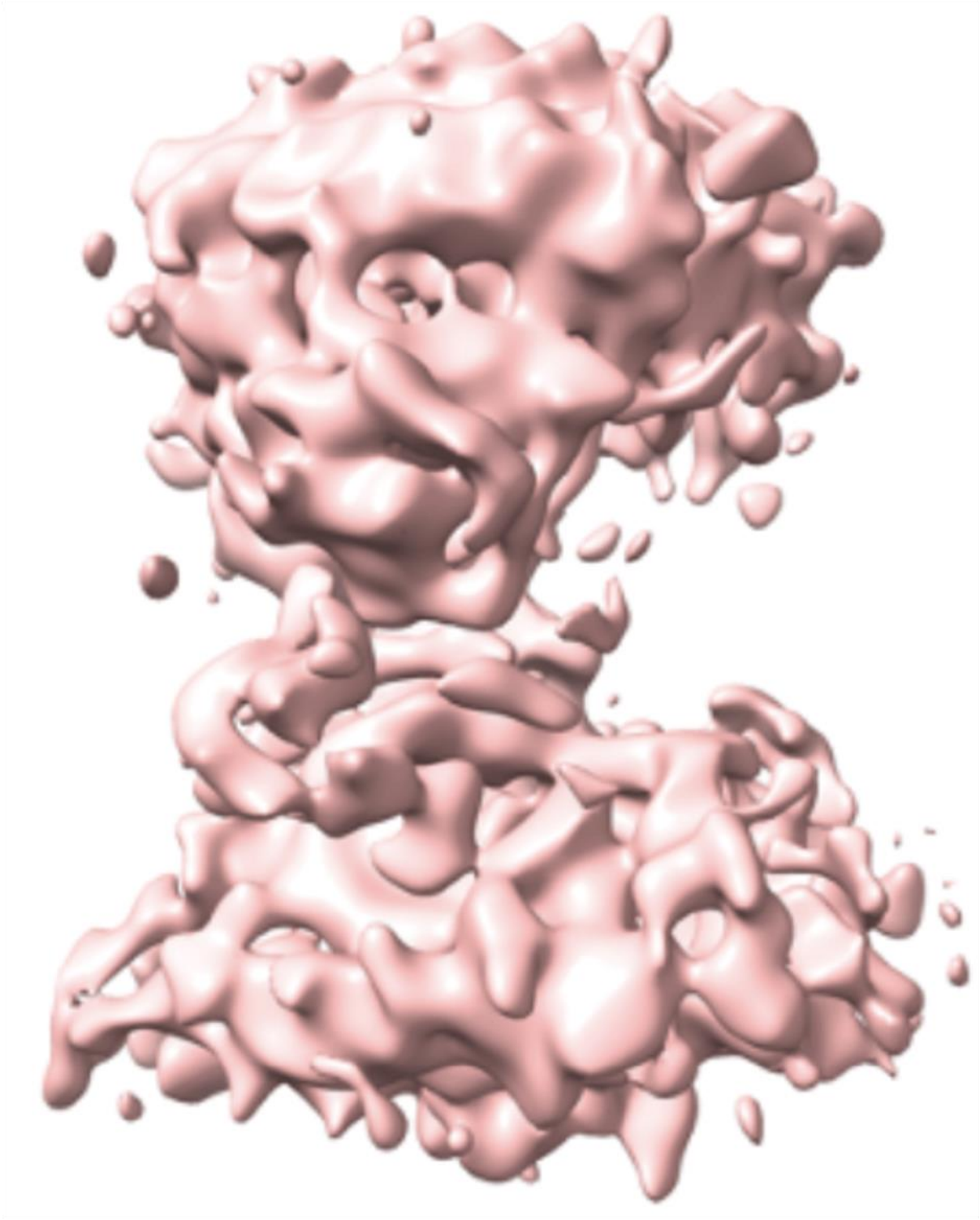
2. G. Chandra et al. *Chem. Pap.* 2023

3. C. Cadicamo et al. *Chem. Bio. Chem.* 2004

4. H. Deng et al. *Angew. Chem. Int. Ed.* 2006

5. T. Kittilä et al. *Microb. Biotechnol.* 2022

Fig. 1



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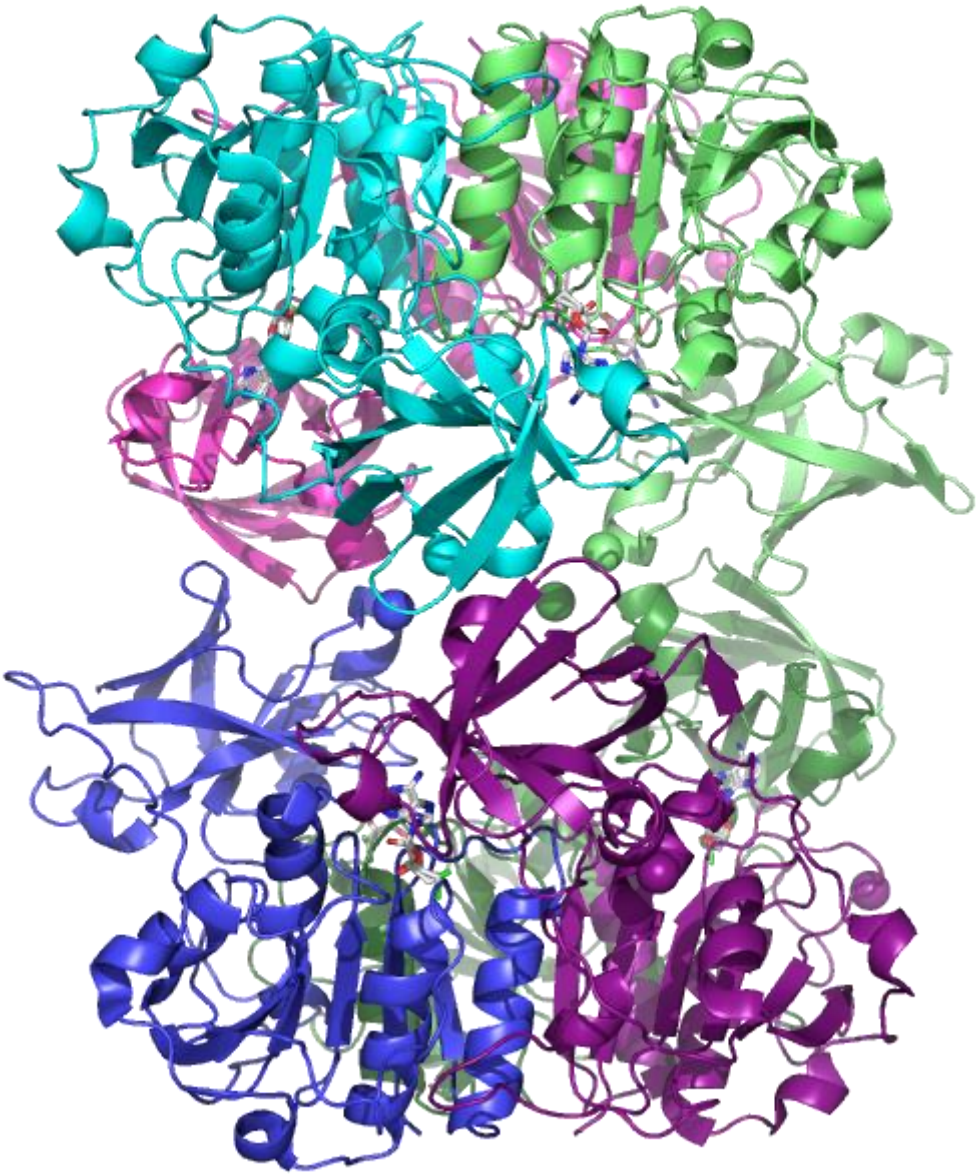

P2-5

Groundbreaking overall solution of mutation library generation and ultra-high throughput screening enpowering directed enzyme evolution application

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Introduction:

The advancement of synthetic biology has brought about new development in whole cell bio-catalysis, making it possible to produce products using cell factories. The ideal artificial biological pathway is unobstructed, efficient, and directional. However, the lack of key enzymes, imbalanced co factors, and low enzyme catalytic activity, lead to blockages, inefficiencies, and randomness. Rational design based on structural aspects is a powerful means to obtain specific enzymes. This method requires detailed knowledge of the structure and function, which has a certain technical threshold. The directed enzyme evolution is similar to the conceptual idea of Darwin's theory of evolution, which can improve the characteristics of enzymes with almost no structural understanding.

Goal:

During the directed enzyme evolution, a highly diverse enzyme library is constructed, followed by multiple rounds of screening to obtain enzyme molecules with specific performance. However, the diversity of enzyme library places higher demands on screening throughput, as the spatial structure of enzymes is more arranged than the total number of atoms in the universe. Traditional screening techniques are no longer sufficient at this level of quantity and has become a bottleneck in the process of enzyme directed evolution. Therefore, it is necessary to develop new technologies to improve screening throughput and promote further development of enzyme engineering.

Result:

Our team has developed a comprehensive solution for library generation and ultra-high throughput cell analysis and sorting platform. The developed atmospheric room temperature plasma has the characteristics of high mutation rate, safe and efficiency, which can be used to generate the large mutation library. Compared to the traditional orifice plate screening system, our team's single-cell culture and sorting system can increase the screening rate by 104-5 times and reduce the reagent consumption to 1/106-7. Thanks to its ultra-high throughput advantage, we have achieved significant breakthroughs in cell screening, enzyme directed evolution, key expression gene finding, and rare microbial resources exploration. On this basis, the microbial microdroplet culture system can carry out miniaturized, automated, and intelligent high-throughput microbial cultivation work. Through the addition of multiple gradient chemical factors and automated strain subculture, enzyme capable of working under specific environments can be further obtained.

Conclusion:

Nowadays, research on the relationship between protein structure and function is still in its early stages. The flux issue in the construction and screening of enzyme library has become a limited factor for the development of enzyme engineering. The use of plasma mutagenesis and high-throughput screening integrated solutions will help accelerate the evolution of enzymes and promote the development of future enzyme engineering research and large-scale manufacturing.

P2-6

From sequence to industrial application: How specialty enzymes are designed

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"Specialty enzymes" are developed to fulfil the needs of a particular application. Capability of mining the enormous diversity of enzymes provided by nature, offers a wealth of fascinating opportunities for these applications.

The sequence space of public digital sequence databases can be enlarged by the proprietary and highly diverse metagenome library MetXtra™. This rapidly expanding database derived from over 60 different metagenomes consists of > 99.8% novel sequences compared to public domain. To gain even deeper access to the natural sequence space, BRAIN Biotech continues to apply next generation sequencing technologies to digitalize metagenomes for sequence- and structure-driven enzyme discovery.

In case the discovered enzyme does not ideally match the desired application, enzyme engineering can enable targeted optimization of properties. Whether it is stability, substrate specificity or solvent resistance, rational design provides opportunities for enzyme improvements. Moreover, current development of machine learning tools can enable faster and easier adaptation of enzymes.

Methods of enzyme discovery and engineering are equally valuable for providing specialty enzymes. Using these approaches individually, simultaneously, or consecutively allows BRAIN Biotech to adapt the process of developing next generation enzymes on a case-by-case basis.

By adopting techniques of enzyme discovery and engineering, we can generate new specialty enzymes for a more sustainable circular economy. A prime example is the development of thermostable enzymes for functionalization of fatty acids. BRAIN Biotech identified new thermostable enzymes implementing multiple sequence alignment, sequence similarity network and various machine learning tools. Simultaneously, an enzyme candidate underwent series of engineering rounds where structure prediction, docking analysis and molecular dynamic calculations were of use. Each step of the development provided novel insights into the enzyme thermostability and brought the enzymes closer to industrial application.

P2-7

Application of rational enzyme engineering in a new route to Etonogestrel and Levonorgestrel: Carbonyl reductase bioreduction of ethyl secodione

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Women in developing countries still face enormous challenges when accessing reproductive health care. Access to voluntary family planning empowers women allowing them to complete their education and join the paid workforce. This effectively helps to end poverty, hunger and promotes good health for all. According to the United Nations (UN) organization, in 2022, an estimated 257 million women still lacked access to safe and effective family planning methods globally. One of the main barriers is the associated cost of modern contraceptive methods. Funded by the Bill & Melinda Gates Foundation, Almac Group worked on the development of a novel biocatalytic route to Etonogestrel and Levonorgestrel, two modern contraceptive APIs, with the goal of substantially decreasing the cost of production and so enabling their use in developing nations. This present work combined the selection and engineering of a carbonyl reductase (CRED) enzyme from Almac"s selectAZymeTM panel with process development to enable efficient and economically viable bioreduction of ethyl secodione to (13R,17S)-secol, the key chirality introducing intermediate en route to Etonogestrel and Levonorgestrel API. CRED library screening returned a good hit with an Almac CRED from Bacillus weidmannii, which allowed for highly stereoselective bioreduction at low enzyme loading of less than 1% w/w under screening assay conditions. However, the only co-solvent tolerated was DMSO up to ~30% v/v, and it was impossible to achieve reaction completion with any enzyme loading at substrate titres of 20 g/L and above, due to the insolubility of the secodione. This triggered a rapid enzyme engineering program fully based on computational mutant selection. A small panel of 93 CRED mutants were rationally designed to increase the catalytic activity as well as thermal and solvent stability. The best mutant Mutant-75 enabled a reaction at 45 °C to go to completion at 90 g/L substrate titre in a buffer/DMSO/Heptane reaction medium fed over 6h with substrate DMSO stock solution, with a low enzyme loading of 3.5% w/w wrt substrate. In screening assay conditions, Mutant-75 also showed a 2.2-fold activity increase. The paper shows which computations and rational decisions enabled this outcome.

P2-8

Unspecific Peroxygenases UPOs: From Expression to Selective Transformations

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Fungal unspecific peroxygenases (UPOs) can transfer peroxide-borne oxygen to a vast diversity of substrates, including aromatic and sp3-carbons. They are independent of external electron sources and additional reductase units and have demonstrated outstanding activities.[1] These highly glycosylated proteins currently have **two significant challenges** for their broader applicability: **1) The heterologous expression** in a fast-growing host permitting directed evolution endeavors and **2) the lack of chemo- and regioselectivity** for specific substrates.

We aimed to address these challenges by developing several techniques. To enable the heterologous expression of UPOs in *S. cerevisiae* and *P. pastoris*, a modular system consisting of i) the signal peptide, ii) the UPO gene, and iii) a *C*-terminal tag for split-GFP detection was established. With a Golden Gate protocol, these three biobricks can be rapidly shuffled and enable the expression of eleven UPOs.[2]

The *S. cerevisiae* system was utilized for the directed evolution of *Mth*UPO from a thermophilic fungus.

We were able to demonstrate the protein engineering on UPOs toward chemoselective conversions[3], light-controlled conversions,[4] and were able to obtain enantioselective conversions of terpenoids using a combination of computational and lab screening approaches.[5] Recently, in collaboration with Sarel Fleishman's group, we achieved the expression of non-fungal UPOs by synergistically employing AlphaFold2, PROSS, and Signal-Peptide Shuffling techniques.[6]

My talk will be about the whole process, from developing the UPO expression system to protein engineering culminating in preparative scale conversions.

References

[1] J. Münch, P. Püllmann, W. Zhang, M. J. Weissenborn, *ACS Catal.* **2021**, *11*, 9168-9203.

[2] P. Püllmann, A. Knorrscheidt, J. Münch, P. R. Palme, W. Hoehenwarter, S. Marillonnet, M. Alcalde, B. Westermann, M. J. Weissenborn, *Commun. Biol.* **2021**, *4*, 562.

[3] A. Knorrscheidt, J. Soler, N. Hünecke, P. Püllmann, M. Garcia-Borràs, M. J. Weissenborn, *ACS Catal.* **2021**, *11*, 7327-7338.

[4] P. Püllmann, D. Homann, T. A. Karl, B. König, M. J. Weissenborn, *Angew. Chem. Int. Ed.* **2023**, *62*.

[5] J. Münch, J. Soler, N. Hünecke, D. Homann, M. Garcia-Borràs, M. J. Weissenborn, *ACS Catal.* **2023**, *13*, 8963-8972.

[6] J. Münch, N. Dietz, S. Barber-Zucker, F. Seifert, S. Matschi, P. Püllmann, S. J. Fleishman, M. J. Weissenborn, *ACS Catal.* **2024**, 4738-4748.

P2-9

Linkers in Action: Exploring Fusion Enzymes for Oxyfunctionlization in Biphasic System through Experiments and Simulations

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Baeyer Villiger Monooxygenases (BVMOs) exhibit immense potential for the selective oxidation of a wide range of ketones using solely oxygen to synthesize diverse esters.[1] Despite the importance, their technical application remains challenging due to the reliance on oxygen and nicotinamide cofactor, inherent instability, and susceptibility to substrate/product inhibition.[2] Using non-conventional media offers an alternative, yet they lack sufficient water for enzyme activation and cofactor regeneration, leading to decreased stability. Fusion approaches aim to streamline cofactor regeneration by minimizing diffusion distances between active sites and hence are promisingly applicable for low-water conditions.[3,4] However, trial-and-error linker design and time-intensive fusion enzyme construction hinder their development, and often do not guarantee success.

Herein, we present the research on 12 novel fusions of cyclohexanone monooxygenase (CHMO) and alcohol dehydrogenase (ADH) with multiple linkers of varying lengths and flexibilities in both orientations for oxyfunctionalization cascades in aqueous-organic biphasic systems (**Fig. 1**). Our focus is on understanding the effects of linkers on the structural and catalytic properties of fusion enzymes. This study pioneers the utilization of structural prediction and molecular dynamics simulations to gain insights into fusion enzymes, encompassing linker conformation and flexibility, as well as the distance between active sites of the two enzymes. The integration of experimental and computational analyses has provided an in-depth understanding of the relationship between linkers and fusion enzymes, offering valuable foresight for the rational design of future fusion enzymes.

Fig. 1. *Design of fused alcohol dehydrogenase (ADH) and cyclohexanone monooxygenase (CHMO) for oxyfunctionlization of cyclohexanol to ε-caprolactone.*

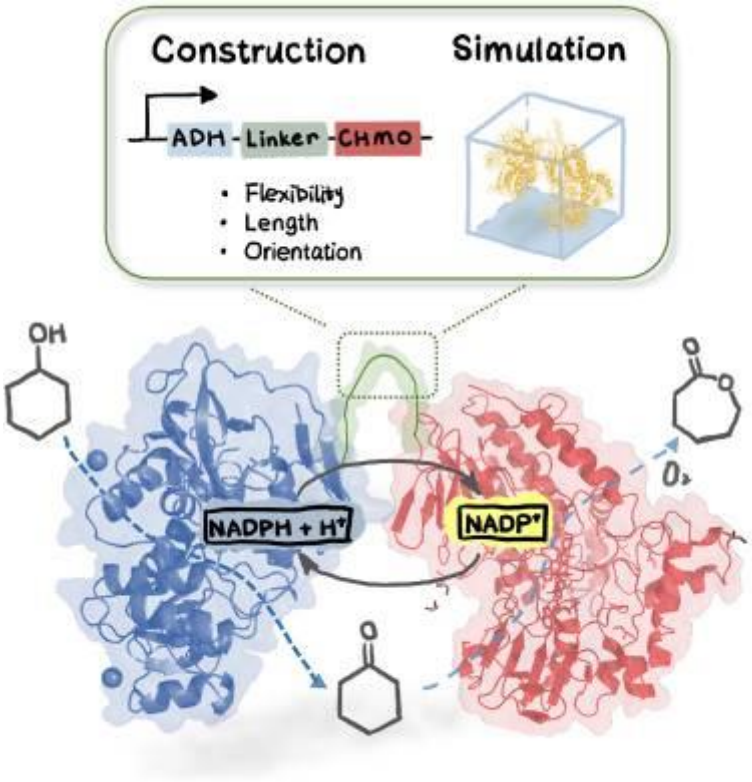
[1] G. de Gonzalo, M. D. Mihovilovic, M. W. Fraaije, *ChemBioChem* **2010**, *11*, 2208–2231.

[2] L. Huang, P. Domínguez De María, S. Kara, *Chemistry Oggi-Chemistry Today* **2018**, *36*, 48–56.

[3] Y. Ma, N. Zhang, G. Vernet, S. Kara, *Frontiers in Bioengineering and Biotechnology* **2022**, *10*, 944226–944237.

[4] G. Vernet, Y. Ma, N. Zhang, S. Kara, *ChemBioChem* **2023**, *24*, e202200794.

Fig. 1


Construction
Simulation
ADH - Linker - CHMO
• Flexibility
• Length
• Orientation
OH
NADPH + H+
NADP+
O2

P2-10

Engineering of an organic solvent tolerant esterase based on computational predictions

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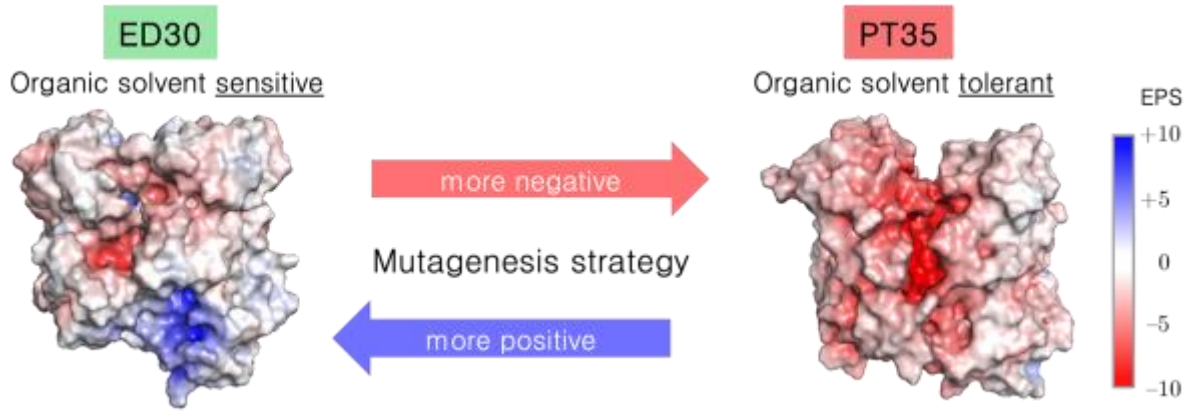
Biocatalytic reactions in synthetic chemistry often require the presence of organic solvents to enhance substrate solubility or steer the reaction equilibrium towards synthesis. Given that enzymes have evolved in aqueous environments, the discovery of rare organic solvent-tolerant enzymes in nature is immensely valuable for elucidating the molecular mechanisms underlying their resistance. The esterase PT35 from Pseudomonas aestusnigri, which was found in crude oil contaminated sand samples, exhibit extraordinary activity and stability (Tm 49°C; t1/2 35 h) in the presence of 50% acetonitrile.[1] Molecular dynamics (MD) simulations comparing PT35 with the organic solvent-sensitive structural homolog ED30 revealed a more pronounced hydration shell around PT35 due to its distinctive negative surface charge. We developed a mutagenesis strategy involving both enzymes, PT35 and ED30, that aimed to decrease or strengthen the hydration shell surrounding the enzyme by modifying its surface charge. Consequently, we generated PT35 variants with reduced negative surface charge and ED30 variants with enhanced negative surface charge. We successfully produced 10 variants for each enzyme, with 7 PT35 variants exhibiting decreased tolerance (e.g. Δt1/2 -37.2 h) and 8 ED30 variants displaying increased tolerance (e.g. Δt1/2 +25.6 h) compared to the wild type. Our findings underscore the potential of engineering an enzyme's surface charge as a means to boost its tolerance to organic solvents. Currently, our focus is directed towards conducting inactivation assays with an ED30 variant harboring 8 mutations that are strategically distributed across its surface to strengthen the hydration shell surrounding the entire enzyme.

[1] A. Bollinger, R. Molitor, S. Thies, R. Koch, C. Coscolín, M. Ferrer, and K.-E. Jaeger. 2020. Organic-solvent-tolerant carboxylic ester hydrolases for organic synthesis. *Applied and environmental microbiology*, 86(9).

Fig. 1



P2-11

Discovery of novel 'split' transketolases for application in biocatalysis

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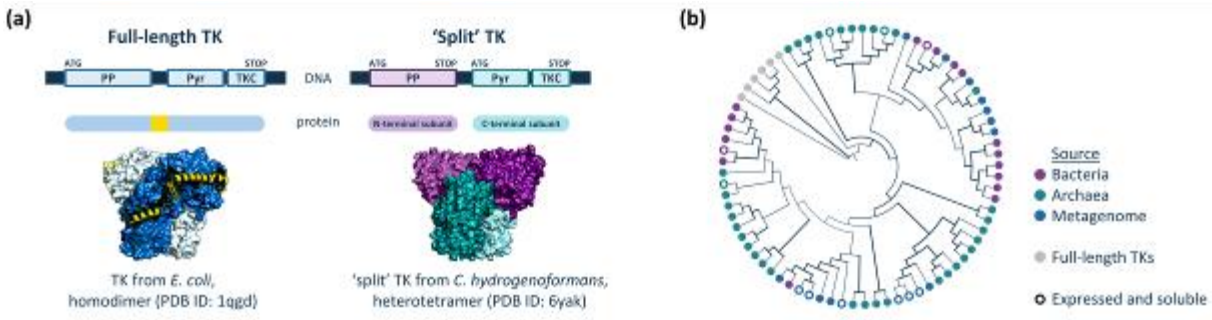
Transketolases (TKs) are thiamine diphosphate dependent enzymes, able to catalyse the transfer of two-carbon units in a stereoselective fashion. They are therefore considered to be biocatalysts with huge potential and applicability in the field of industrial biocatalysis, for the establishment of more sustainable processes[1]. Most well-known and characterised TKs are encoded as proteins of around 650 amino acids[2]. However, a second type of TK has been identified in many Archaea and Bacteria, which instead consists of two proteins of around 300 amino acids. To date, there is only a single example of these so-called "split" TKs that has undergone biochemical characterization and whose crystallographic structure has been unveiled[3]. Some examples of split TKs are present in literature, including a homologue of split TKs identified as part of a pathway responsible for the production of a phosphonate with herbicidal properties[4]. Exploring the unique features and differences of split TKs may help in assessing their potential use in biocatalysis and for uncovering new reactivities. Additionally, it could provide valuable information on how their structure relates to their function, especially when compared to full-length TKs.
The project aims to use genome and metagenome mining to broaden the panel of available split TKs, to investigate their phylogenetic spread across domains, and to explore their relatedness to full-length TKs. A selection of these enzymes will be produced and characterised, particularly for substrate scope and thermostability.
In this study, four in-house metagenomes, alongside various (meta)genomic online databases were queried for potential split TKs. This search resulted in the retrieval of over 70 enzymes, which were then assembled into a phylogenetic tree to examine their distribution across different species, as well as their relationship with full-length TKs. Out of the full array of putative split TKs, 27 were cloned into a plasmid with a synthetic two-gene operon, from which 11 were successfully produced in soluble form in *E. coli*. Following confirmation of their enzymatic activity, these enzymes were investigated for various properties, including thermostability and substrate scope.
This study successfully identified and produced a diverse group of split TKs from both genomic and metagenomic sources. Some of these enzymes showed activity on various unnatural substrates, which are promising features for further employment in biocatalysis. Moreover, one enzyme displayed enhanced thermostability, a highly desirable characteristic for industrial application.

Figure 1. (a) Comparison of subunits organization of full-length versus split TKs. (b) Phylogenetic tree of identified split TKs.

[1]Hailes, H.C. *et al. Curr. Org. Chem.* 14, 1883-1893 (2010)
[2]Abdoul-Zabar, J. *et al. Adv. Synth. Catal.* 355, 116-128 (2013)
[3]James, P. *et al. Front. Microbiol.* 11, 592353 (2020)
[4]Zhu, Y. *et al. J. Am. Chem. Soc.* 144, 16715-16719 (2022)

Fig. 1



P2-12

Droplet-based microfluidics for efficient high-throughput screening in biotechnology applications

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Introduction

High-throughput screening (HTS) platforms are used in drug discovery, enzyme engineering, disease diagnosis, and biotechnology for the rapid and simultaneous assessment of numerous samples or compounds to discover their desired activity and isolate potential candidates from large pools of variants like in mutant, environmental, or metagenomic libraries[1,2]. However, modern screening platforms based on robotic liquid handling are very labor, cost, and equipment-intensive as they require extensive set-up, maintenance processes, large number of consumables and reagents[3,4]. In addition, these platforms offer limited throughput compared to the size of mutant libraries, making the screening process time-consuming and less efficient.

Objective

We aim to establish a picolitre droplet-based HTS platform capable of screening millions of enzyme variants/reactions and testing it for improved biocatalysts from mutant libraries. We want to demonstrate the enhanced screening efficacy of the droplet platform through the evaluation of industrially significant protease enzymes from bacterial mutant libraries, serving as a benchmark test case.

Results

A microfluidic screening platform was established by integrating (1) a parallelized cultivation strategy for millions of droplets[5], (2) a fluorescent-based enzymatic assay, (3) an optical fiber-based detection technique, and (4) a droplet recovery platform for single droplet dispensing enabling single-droplet-to-single-colony relation. With a mixed population of control strains for protease activity, we achieved a 1000-fold enrichment of active cells. Mutant libraries were generated by UV-mutagenesis of a Bacillus strain expressing a protease enzyme or by error-prone PCR targeting the subtilisin gene. A rapid screening was performed on the established platform resulting in active variants with ~ 250 % higher activity.

Conclusion

Droplet microfluidic platform enables robust and efficient screening of improved enzymatic variants. Miniaturization and parallelization allow the screening of thousands of variants in a single experimental run with lower costs and enhanced sensitivity. This droplet HTS platform aims to transform screening methodologies across diverse applications like directed evolution, metagenomics, and drug discovery. We envision a broader use of droplet platforms paving the way for transformative breakthroughs in biocatalysts, biofuel production, and various other biotechnological applications.

References

1. Kulesa *et al.*, 2018, *PNAS*
2. Mahler *et al.*, 2021, *Elife*
3. Stucki *et al.*, 2021, *Angew. Chemie – Int. Ed.*
4. Gantz *et al.*, 2022, *Chem. Rev.*
5. Mahler *et al.*, 2015, *RSC Adv.*

P2-13

Epistasis & Context Dependency in Enzyme Evolution of Borneol-type Dehydrogenases

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The exceptional stereoselectivity of enzymes is a key feature for industrial applications, enabling the design of promising biocatalyst-based synthesis routes for enantiopure compounds. While directed evolution and rational design are powerful tools for the artificial improvement of stereoselectivity [1], it remains unclear how Nature evolved stereoselectivity within enzyme classes. As such, enzymes within the family of plant borneol-type dehydrogenases (BDHs) display striking differences in enantioselectivity towards borneol enantiomers despite close relations [2,3]. Mimicking Nature, we investigated active site and peripheral mutations' impact on BDH selectivity, to rationalize natural enzyme evolution. In this context, ancestral sequence reconstruction (ASR) acts as a guide to construct a plausible evolutionary pathway of BDH towards the enantioselective *Sr*BDH1 [2]. Common ancestors of selective and non-selective dehydrogenases were inferred and key mutations contributing to their stepwise diversification were identified (Figure 1). Ancestral enzymes were recombinantly produced, biochemically characterized, and their structures predicted. Consecutively, guided by *de-novo* protein structure prediction, selected residues were substituted to elucidate the structural basis thereof. This work highlights the importance of context-dependent evolutionary changes in enzymes and exemplifies how the application of computationally guided enzyme engineering tools leads to a deeper understanding of natural evolutionary principles in less investigated enzyme classes.

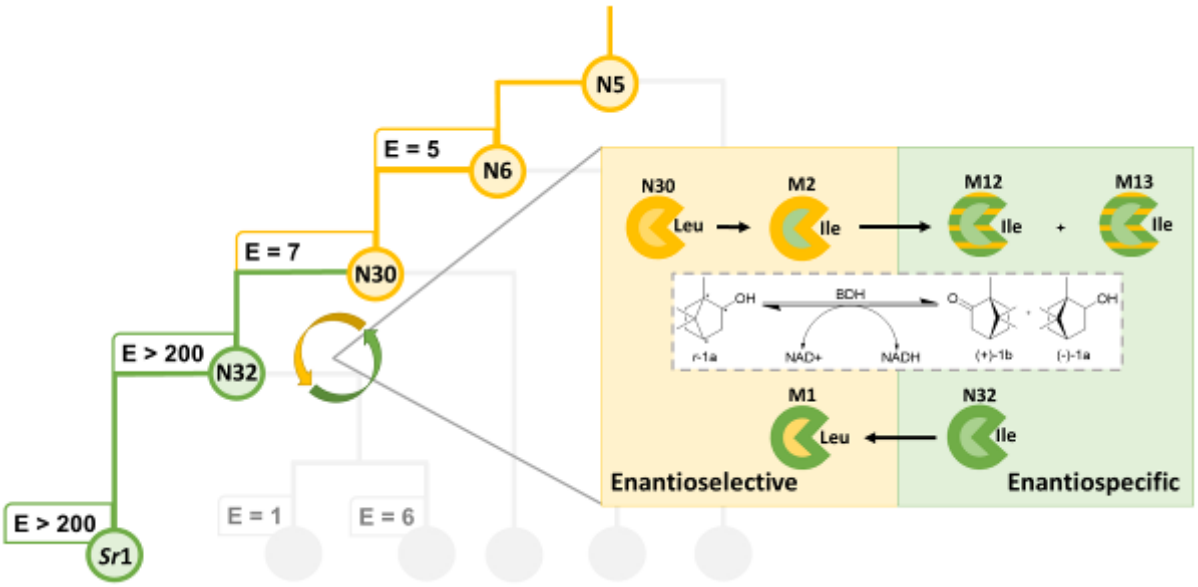
Figure 1. Ancestral sequence reconstruction of BDH enzymes as a guide to identifying peripheral and active site mutations leading to changes in enantioselectivity and -specificity.

[1] Bornscheuer, U. *et al.* Engineering the third wave of biocatalysis. *Nature* **485**, 185–194 (2012). https://doi.org/10.1038/nature11117

[2] Chánique, A.M. *et al.* A Structural View on the Stereospecificity of Plant Borneol-Type Dehydrogenases. *ChemCatChem* **Vol. 13, Issue 9**, 2262-2277 (2021). https://doi.org/10.1002/cctc.202100110

[3] Croteau, R. *et al.* Biosynthesis of monoterpenes: Partial purification and characterization of a bicyclic monoterpenol dehydrogenase from sage (Salvia officinalis). *Archives of Biochemistry and Biophysics* **Vol. 188, Issue 1**, 182-193 (1978). https://doi.org/10.1016/0003-9861(78)90371-5

Fig. 1



P2-14

Structure and identification of catalytic residues of D-Threonine aldolase from the green alga *Chlamydomonas reinhardtii*

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1. Introduction

D-Threonine aldolase (DTA, EC 4.1.2.42) catalyzes the reversible aldol reaction of glycine with aldehydes to yield the D-forms of β-hydroxy-α-amino acids such as D-threonine and D-*allo*-threonine. DTAs possess potential as a powerful tool in synthetic organic chemistry (1,2) due to their high enantioselectivity at the α-carbon of the substrate and broad selectivity at the β-carbon, enabling the enzyme to generate various types of D-amino acids. Several DTA products are available as chiral building blocks, which include intermediates for antibiotics and Parkinsonism drugs (3-5). DTAs belong to the fold-type III group of the pyridoxal 5'-phosphate (PLP) dependent enzyme family. We had produced and investigated the crystal structure of DTA from *Chlamydomonas reinhardtii* at 1.85 Å resolution (6). The present study was aimed at clarifying the structures of enzyme-substrate complexes and the catalytic residues of the enzyme reaction.

2. Experimental

We have succeeded in crystallizing the complexes of the enzyme, glycine, and aldehydes and analyzing their structures. The active site structure shows that the amino group of glycine forms a Schiff base (external aldimine) with PLP, confirming that glycine is incorporated into the active site as the primary substrate. It was predicted that the catalytic residue that withdraws the proton of β-OH is Tyr210 or His216. His216 is farther away from the substrate than Tyr210, suggesting that it must be mediated by a water molecule to act as a catalytic residue (Fig. 1). Mutant enzymes, Y210F and H216A were prepared, and their activities were examined. The specific activity of H216A remained 17% of that of the wild-type enzyme. The activity of Y210F was measured and showed no activity in the case of using partially purified enzyme after ammonium sulfate fractionation.

3. Conclusion

The specific activity of H216A, which was expected to be a catalytic residue in other studies (e.g. 7), remained 17% of that of the wild type, suggesting that H216 was not the essential catalytic residue. Y210F exhibited no activity, however, since this result was obtained with a partially purified enzyme, it is necessary to further purify the enzyme and measure the activity.

(1) Dückers et al., Appl. Microbiol. Biotechnol. 88, 409–424 (2010)

(2) Kataoka et al., Appl. Microbiol. Biotechnol. 100, 5747–5757 (2016)

(3) Liu et al., Appl. Microbiol. Biotechnol. 51, 586–591 (1999)

(4) Liu et al., Appl. Microbiol. Biotechnol. 54, 44–51 (2000)

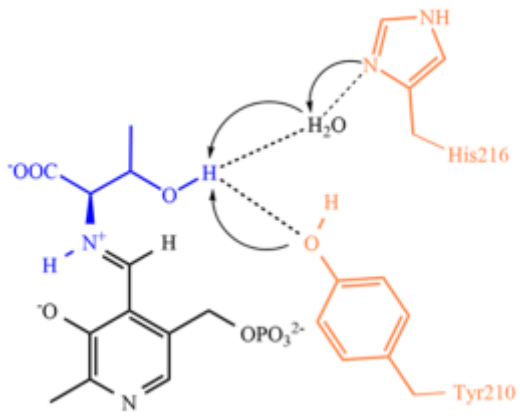
(5) Goldberg et al., Org. Process Res. Dev. 19, 1308–1316 (2015)

(6) Hirato et al., Acta Cryst. F79, 31-37 (2023).

(7) Uhl et al., PLOS ONE. PDB ID: 4V15 (2015)

Fig. 1. Candidates of catalytic residues for withdrawing the proton of β-OH (blue, substrate; black, PLP)

Fig. 1


NH
N
His216
H2O
-OOC
O
H
N+
H
H
O
H
-O
OPO3 2-
N
Tyr210

P2-15

Enzyme Proximity Sequencing: A Deep Mutational Scanning Workflow for Studying and Engineering Protein Catalysts

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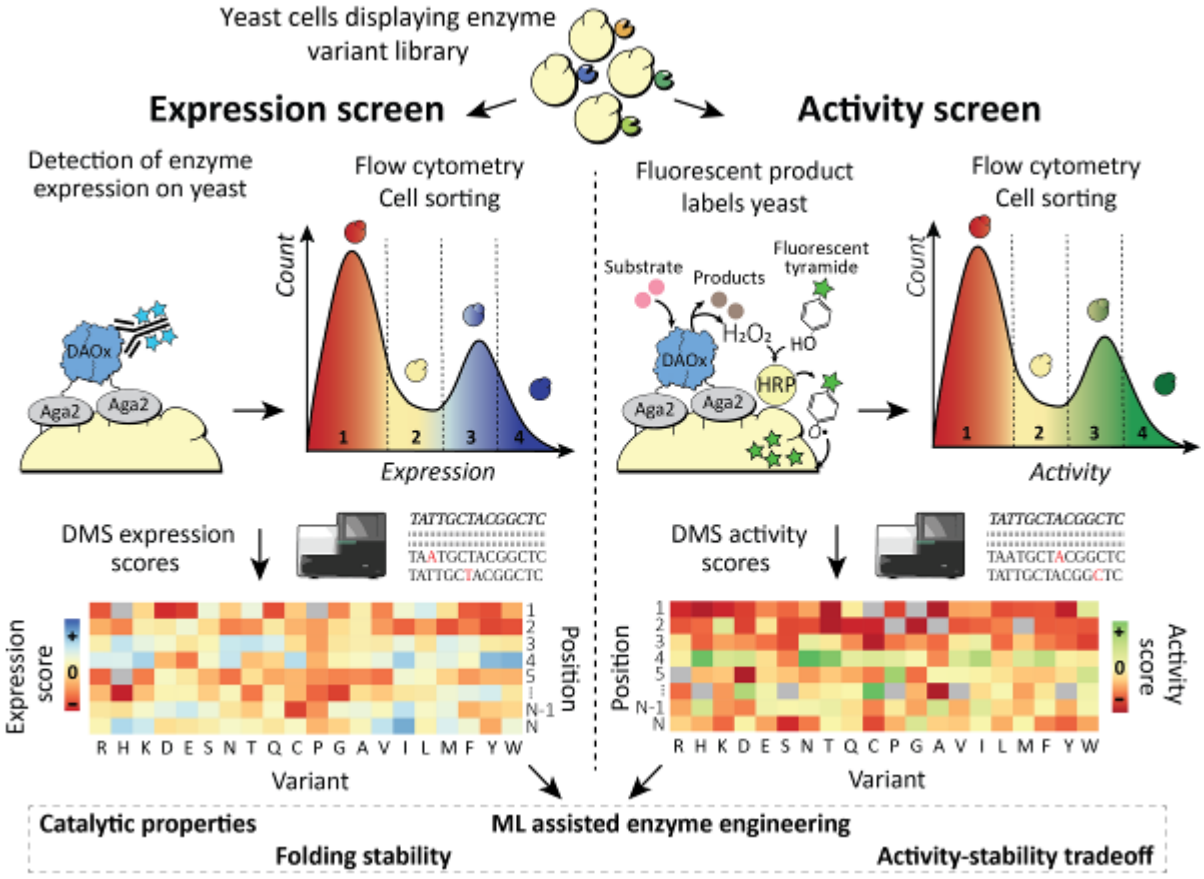
Understanding the complex relationships between enzyme sequence, folding stability and catalytic activity is crucial for applications in industry and biomedicine. However, current enzyme assay technologies are limited by the inability to simultaneously resolve both stability and activity phenotypes and to couple these to gene sequences at large scale. Here we present enzyme proximity sequencing (EP-Seq), a novel deep mutational scanning method that combines enzyme proximity labeling with next-generation DNA sequencing to assay both expression and catalytic activity of thousands of variant enzymes in a single experiment [1]. EP-Seq leverages yeast surface display to measure the expression levels and folding stability of each enzyme variant via a pooled cell sorting-sequencing experiment. In parallel, phenoxyl radical-based cell surface proximity labeling links enzyme activity to a fluorescent signal, which is then quantified by sorting and sequencing [2-4] (**Fig. 1**). Applying EP-Seq to D-amino acid oxidase from *Rhodotorula gracilis* reveals rich biophysical insights into the enzyme, identifying protein regions where catalytic activity acted as an evolutionary constraint on folding stability and enabling the prediction of mutations that enhance catalysis [1]. EP-Seq can be employed to study several enzyme classes whose activity can be linked directly or indirectly (via enzymatic cascade) to the production of peroxide. Moreover, due to its scalability, our approach finds applications in generating training data for machine learning algorithms, which represents a major challenge for catalytic enzymes. EP-Seq can contribute to a better understanding of evolutionary processes in natural enzymes, help in identifying functional allosteric sites, and be used to evolve protein catalysts for industrial and biomedical applications.

Fig. 1. EP-Seq workflow. (Top) A pooled library of enzyme variants is displayed on yeast. (Left) The cell population is sorted into bins based on the expression level of the displayed enzyme. (Right) The pooled variant library is assayed for DAOx activity using a cascade peroxidase-mediated proximity labeling reaction with single cell fidelity and sorted into bins. The genetic composition of cells in the sorted bins is quantified via high-throughput sequencing and the distribution of each variant along the expression and activity axes is converted into a fitness score. Joint analysis of the two independent datasets provides insights into the effects of mutations on folding stability and activity of the enzyme.

[1] **R. Vanella**, C. Küng, A.A. Shoepfer, V. Doffini, J.Ren, M.A. Nash. Nature Communications **15**, 1807 (2024).
[2] **R. Vanella**, G. Kovacevic, V. Doffini, J.F. de Santaella, M.A. Nash. Chemical Communications 2022 58 (15), 2455-2467
[3] **R. Vanella**, A. Bazin, D.T. Ta, M.A. Nash. Chemistry of Materials 2019 31 (6), 1899-1907
[4] C. Küng**, R. Vanella**, M.A. Nash. Reaction Chemistry & Engineering 2023, 8, 1960-1968

Fig. 1


Yeast cells displaying enzyme variant library
Expression screen
Activity screen
Detection of enzyme expression on yeast
Flow cytometry Cell sorting
Count
Expression
DAOx
Aga2
Aga2
1
2
3
4
Fluorescent product labels yeast
Substrate
Products
Fluorescent tyramide
H_2O_2
HO
HRP
Flow cytometry Cell sorting
Count
Activity
DMS expression scores
DMS activity scores
TATTGCTACGGCTC
TAATGCTACGGCTC
TATTGCTACGGCTC
Expression score
Position
Activity score
R H K D E S N T Q C P G A V I L M F Y W
Variant
Catalytic properties
Folding stability
ML assisted enzyme engineering
Activity-stability tradeoff

P2-16

Heterologous expression and characterization of a GDS(L)-like hydrolase from *Pleurotus sapidus,* which is highly stable at alkaline conditions and contains an unusual SGNH motif in the active site

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1. Introduction

The use of residual biomass for the production of daily products, food and feed is the key in the circular economy, and simultaneously partially fulfills the UN Sustainable Development Goals. However, the depolymerization of renewable agro-industrial residues is a challenge that can only be overcome by efficient biomass hydrolysis. A crucial step for the leap to technological applications is the production and use of efficient enzymes, which evolution has already tailored to degrade and modify biopolymers efficiently. Especially fungi, as natural cell factories with their unique enzymes, can degrade organic lignocellulosic biomass into biogenic raw materials, which are then available as feedstocks. The great biodiversity of the enzymes of the phylum Basidiomycota, the pillar fungi, often referred to as "higher fungi", has not yet been sufficiently characterized.

2. Objectives

To get more insights over these enzymes, one potential GDS(L), which was found in the secretome of the Basidiomycota *Pleurotus sapidus* grown on rape straw was cloned and characterized.

3. Results

The GDS(L)-like lipase (PSA_Lip) was heterologously expressed using *Trichoderma reesei* with an activity of 350 U L^{-1} using *p*-nitrophenyl-(pNP)- octanoate as substrate. The novel PSA_Lip showed only 23.8-25.1%, 25.5%, 26.6% and 28.4% identity to the previously characterized GDSL-like enzymes phospholipase, plant lipase, acetylcholinesterase and acetylxylan esterase, from the carbohydrate esterase (CE) family 16, respectively. Therefore, the enzyme was purified from the culture supernatant and the catalytic properties and the substrate specificity of the enzyme were investigated using different assays to reveal its potential function. While no phospholipase, acetylcholinesterase and acetylxylan esterase activities were detected, studies on the hydrolysis of methyl feruloate and feruloylated carbohydrates showed low conversions of these substrates. The highest activity was determined for medium chain-length pNP-octanoate at 65 °C and a pH value of 8, while almost no activity was detected for pNP-hexanoate. The enzyme is highly stable when stored at pH 10 and 4 °C for at least 7 days. Moreover, using consensus sequence analysis and homology modeling, we could demonstrate that the PSA_Lip does not contain the usual SGNH residues in the actives site, which are usually present in GDS(L)-like enzymes.

4. Conclusions

The information presented in this study is useful for identifying potential functions of the PSA_Lip and similar basidiomycetous enzymes due to their unusual SGGI motif. Their exact mechanism has to be further characterized with the help of structural determination, molecular dynamics and mutagenesis of different amino acid residues in the near future. Perhaps, these enzymes could be classified into a new CE family and will be applied at slight alkaline conditions due to their high stability at this pH range.

P2-17

Structure and function of a novel PU depolymerase

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Recycling and upcycling of enzyme-degraded plastics is an environmentally friendly method to deal with the increasing plastic pollution, and many methods have been used in PET plastic processing in recent years; However, due to the lack of effective enzymes, the processing of other plastics such as PU is still limited. Through sequence analysis and high-throughput screening, we discovered a novel esterase from *Bacillus Marianotherm*, which has three enzymatic activities: esterase, amidase and urease, it also exhibits activity towards PU plastic ,and has the potential to improve the efficiency of PU recovery and upcycling.

The hydrolysis paths of Tmca9 substrates and different short chain molecules containing benzene rings were analyzed. We analyzed the crystal structures of wild-type and inactivated mutants with substrate complexes and elucidated their substrate binding patterns. The catalytic chains and residues that make up the substrate entrance cavity were determined by scanning the small molecule alanine in the 5 Å range of the complex structure, and the functions of some key residues were determined by mutagenesis. The hydrolytic activity of its mutant R96A on BMC was 1.8 times higher than that of WT. PU substrates can be catalyzed by coupling this enzyme and TfCut from *Thermobifida* fusca KW3. The dual-enzyme system completely hydrolyzed PU membrane within 72h. Moreover, for polyether PU treated by alcoholysis, 0.1mg Tmca9 can achieve a conversion rate of more than 80% in 24h. This method provides feasibility for the recovery of polyurethane.

P2-18

_Gt_HNL catalyzes oxidative C=C bond cleavage

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*Gt*HNL is a hydroxynitrile lyase that was successfully utilized for the synthesis of cyanohydrins (Scheme 1B) and nitro aldol products, in batch and in flow [1,2,3]. *Gt*HNL has moderate similarity (35%) with *Tm*1459 and the same catalytic metal ion, Mn(II) (Scheme 1A). Enzymes display high selectivity for one type of reaction. Therefore, lyases are not expected to catalyze oxidative C=C bond cleavage reactions as *Tm*1459 does (Scheme 1C) [4]. However, examining the structure of *Gt*HNL there is no obvious reason why it should not catalyze the oxidative C=C bond cleavage, as described for *Tm*1459. Equally a hydroxynitrile lyase activity for *Tm*1459 cannot be ruled out.

Scheme 1: A) Superimposition of *Gt*HNL (PDB code: 4BIF; blue) with Mn(II) in grey coordinated by 5 amino acids and *Tm*1459 (PDB code: 1VJ2; green) with Mn(II) coordinated by only 4. B) Cyanohydrin formation. C) Oxidative C=C bond cleavage.

*Gt*HNL was employed under condition earlier successful for *Tm*1459 [4]. *tert*-butyl hydroperoxide (TBHP) was used as oxidizing reagent and *Gt*HNL satisfactorily catalyzed the oxidative cleavage of a number of styrene derivatives. Enzyme and reaction engineering led to improved selectivities and yields. On the other hand *Tm*1459 displayed only modest hydroxynitrile lyase activity without selectivity [5]. These results question our concepts of enzyme selectivity and at the same time open new routes to novel enzyme activities.

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[1] I. Hajnal, et al, *FEBS J*., **2013**, *280*, 5815–5828.

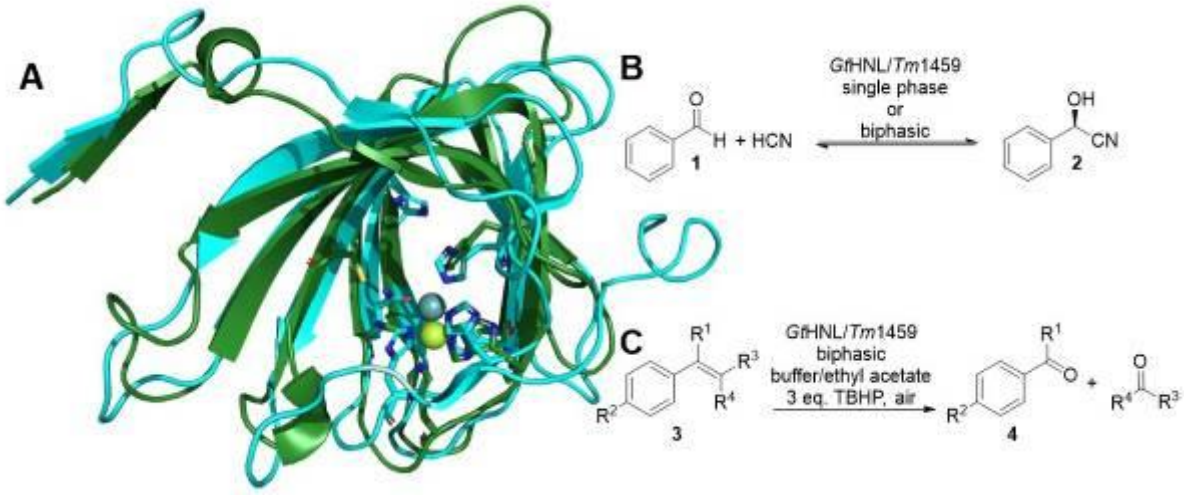
[2] J. Coloma, et al, *Catal. Sci. Technol*., **2020**, *10*, 3613–3621.

[3] J. Coloma, et al, *Catalysts* **2022**, *12*, 161.

[4] I. Hajnal, et al, *Adv. Synth. Catal*. **2015**, *357*, 3309 –3316.

[5] J. Coloma, et.al, *ACS Catal*. **2023**, *13*, 11182−11194.

Fig. 1



P2-19

Functionally Diverse Peroxygenases by AlphaFold2, Design, and Signal Peptide Shuffling

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Unspecific peroxygenases (UPOs) are secreted fungal enzymes. They are able to perform versatile oxyfunctionalization reactions, similar to cytochrome P450 monooxygenases. But in contrast to P450s, they use pre-reduced H2O2 and do not need a complex electron transfer chain.[1] The challenging functional expression in heterologous hosts is one of their largest drawbacks. Previous studies showed that changes in the signal peptide through the introduction of mutations[2] or exchange of the signal peptide[3-4] are promising ways to enable and improve the expression. Another approach is increasing the stability of the enzymes. We combined both ways to functionally express new unspecific peroxygenases derived from plant pathogen fungi (Figure 1). We performed stability-design calculations using the tool PROSS[5]. This algorithm relies on structural information, which is a challenge for UPOs as there are only a few crystal structures available. We used the power of AlphaFold2 to model the structure of 10 UPOs and used the predictions for PROSS design. For each UPO, three different PROSS designs were subjected to signal peptide shuffling. We accomplished a high success rate of 90 %. During Screening seven of the enzymes were found to be active against at least one test substrate (ABTS / DMP). In contrast, only one out of seven wild-type UPOs showed activity against the test substrates. Three of the new UPOs are derived not from the kingdom of fungi, as all previously described UPOs, but from oomycetes. They are therefore more closely related to algae than to fungi. On my poster, I present a quick and versatile way to produce new unspecific peroxygenases.

Figure 1: Workflow of the project. I) Sequence selection of UPOs from plant-pathogen fungi and structural modelling using AlphaFold2. II) PROSS stability design and selection of three models per UPO. III) Signal peptide shuffling using 17 signal peptides. IV) Screening towards activity (ABTS / DMP) and expression (split-GFP). V) Enzyme characterization and exploration of substrate scope.

[1] J. Münch, P. Püllmann, W. Zhang, M. J. Weissenborn, *ACS Catalysis* **2021**, *11*, 9168-9203.

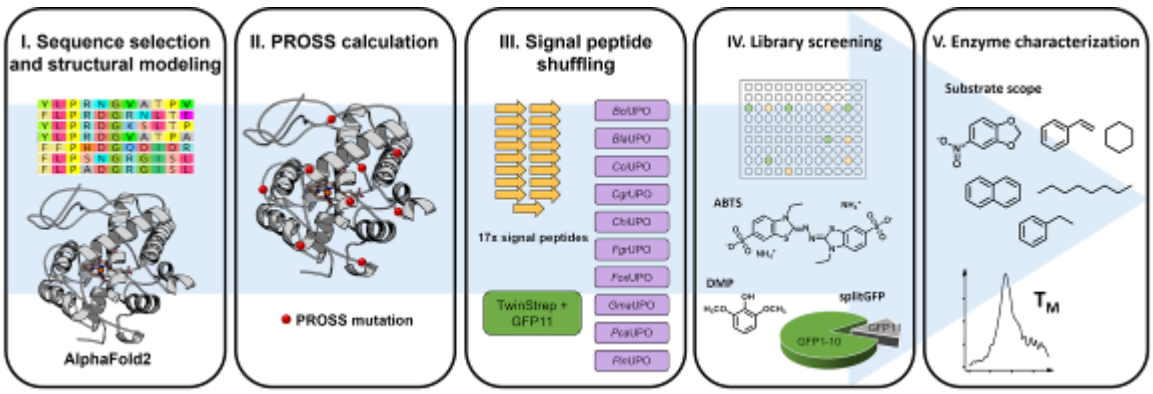
[2] P. Molina-Espeja, E. Garcia-Ruiz, D. Gonzalez-Perez, R. Ullrich, M. Hofrichter, M. Alcalde, *Applied and Environmental Microbiology* **2014**, *80*, 3496-3507.

[3] P. Püllmann, A. Knorrscheidt, J. Münch, P. R. Palme, W. Hoehenwarter, S. Marillonnet, M. Alcalde, B. Westermann, M. J. Weissenborn, *Communications Biology* **2021**, *4*.

[4] P. Püllmann, M. J. Weissenborn, *ACS Synthetic Biology* **2021**, *10*, 1360-1372.

[5] A. Goldenzweig, M. Goldsmith, S. E. Hill, O. Gertman, P. Laurino, Y. Ashani, O. Dym, T. Unger, S. Albeck, J. Prilusky, R. L. Lieberman, A. Aharoni, I. Silman, J. L. Sussman, D. S. Tawfik, S. J. Fleishman, *Mol Cell* **2016**, *63*, 337-346.

Fig. 1



P2-20

Machine learning guided directed evolution of unspecific peroxygenases

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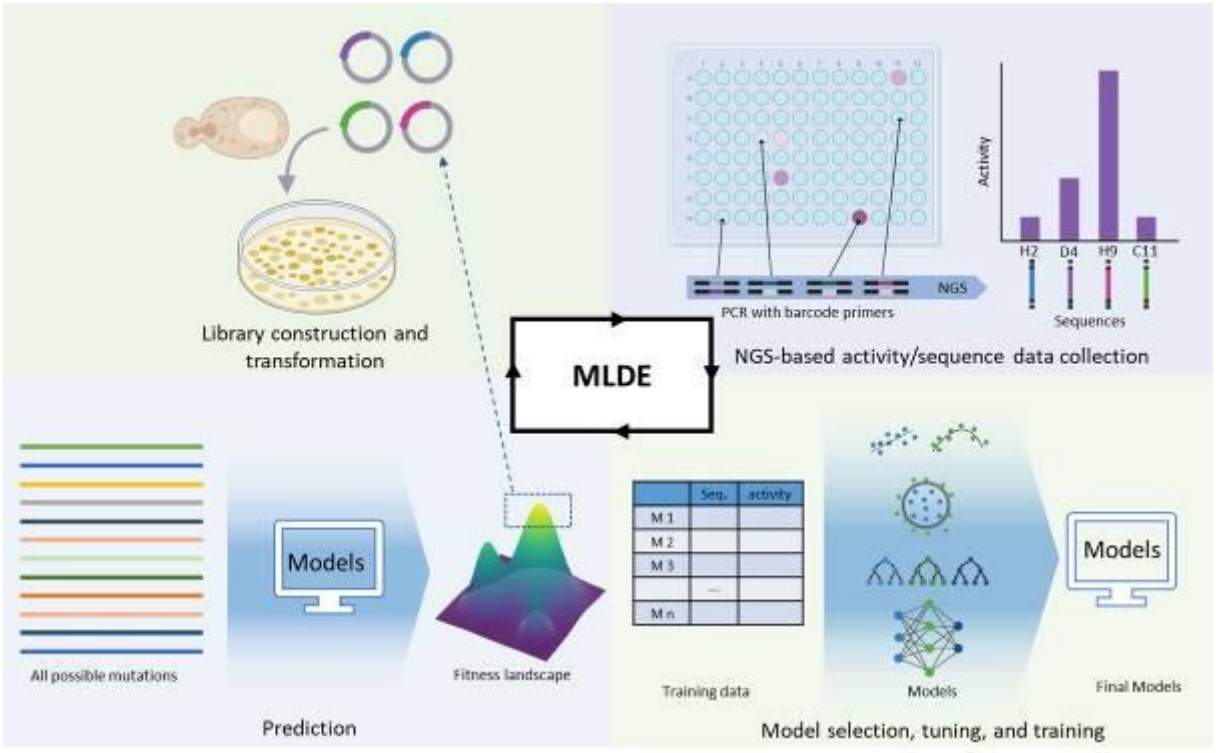
Epistasis, characterized by the interdependence of effects among various mutated positions, is frequently encountered in directed evolution (DE) campaigns, especially when employing multiple-site combinatorial mutagenesis libraries.[1] This phenomenon was notably observed in our prior work, which focused on engineering the unspecific peroxygenase (UPO) from *Myceliophthora thermophila* (MthUPO) for the enantiospecific hydroxylation of β-ionone.[2] To tackle the complexities introduced by epistasis and strive for a global optimum in DE campaigns, we have employed a data-driven approach, leveraging machine learning-guided directed evolution (MLDE).

A diverse library of mutants undergoes assay and sequencing to generate input data for training machine-learning models. These models are refined using various assessment metrics to accurately rank mutant activities. The most effective models guide the selection of additional mutants for assay, contributing to iterative model refinement. This process continues until predictions for untested mutants are consistently lower than those of the most active known mutants, indicating convergence. The final model's efficacy is confirmed through its accurate prediction of mutant activity levels. This approach showcases machine learning's capacity to enhance UPO engineering by effectively addressing epistasis challenges, leading to increased enzyme activity.

[1] a) N. C. Wu, L. Dai, C. A. Olson, J. O. Lloyd-Smith, R. Sun, *eLife* **2016**, *5*, e16965; b) S. Biswas, G. Khimulya, E. C. Alley, K. M. Esvelt, G. M. Church, *Nat Methods* **2021**, *18*, 389-396; c) C. M. Miton, K. Buda, N. Tokuriki, *Curr Opin Struct Biol* **2021**, *69*, 160-168.

[2] a) J. Münch, J. Soler, N. Hünecke, D. Homann, M. Garcia-Borràs, M. J. Weissenborn, *ACS Catalysis* **2023**, *13*, 8963-8972; b) A. Knorrscheidt, J. Soler, N. Hünecke, P. Püllmann, M. Garcia-Borras, M. J. Weissenborn, *ACS Catal* **2021**, *11*, 7327-7338.

Fig. 1



P2-21

Enzymatic bio-construction: diversity-oriented synthesis of building blocks

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Ene-reductases (ERED) are known to be robust and have a broad substrate scope. Some of our recent work has allowed to reduce cyclic ene-based derivatives, such as cyclopropene compounds with high enantioselectivity (>90%ee) and complementary stereoselectivities based on more traditional metal catalysis approach. By using these enzymes, *trans*-saturated compounds were isolated in good yield and excellent enantiomeric excess[i]. A wide range of ester- and ketone-substituted optically active derivatives were obtained with a variety of tolerating cyclic substituent patterns.

Few EREDs also catalyze the reverse desaturation reaction of ketones into enones. This reaction was assessed in the desaturation of **2** into ***1*** as a surrogate of a chemical desaturation reaction but the wild type ERED tested did not show any traces of activity despite a broad substrate scope. In the present work we aim to develop variants of the wild type ERED able to perform the desaturation of *2*. To do so, computational methods have been employed, including Molecular Dynamics simulations in combination with Shortest Path Map from the Compbiolab group[ii], as well as MSA (Multiple Sequence Alignment) together with AlphaFold 2 (AF2) to model the resulting variants.

In another example, we have succeeded in the selective bioreduction of a dehydroaminacid-alkene derivative. We carried out a screening test of twenty ene reductases (EREDs) Seqenzym. Within the exclusion of two enzyme candidates, all exhibited activity and the conversion rate during this first series of screening was moderate (around 30%) but still high degree of enantioselectivity were detected. Optimization of the reaction conditions is still on its way.

Finally, based on one selected Seqenzym ERED candidate, we engineered the reaction conditions in order to develop and manufacture an enzymatic process at commercial scale. As a result, we scaled-up an enzymatic process affording only 400 ppm of biocatalyst (in respect to substrate loading) within high reproducibility based on 52 batches.

[i] Results to be published - *in Press*.

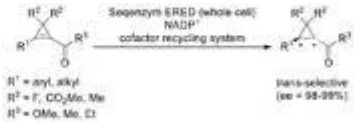
[ii] Osuna, Sílvia. « *The Challenge of Predicting Distal Active Site Mutations in Computational Enzyme Design* ». WIREs Computational Molecular Science, 2021, 11, 3. https://doi.org/10.1002/wcms.1502.

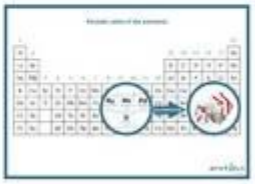
Fig. 1

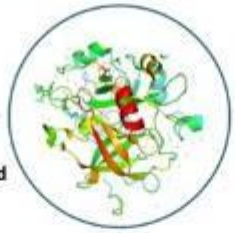
ERED for enzymatic desaturation

SELECTIVE

ERED for the bioreduction of cyclic-ene keto derivatives

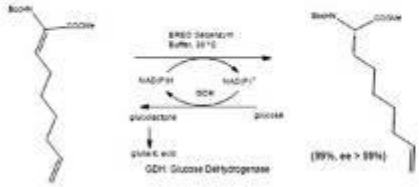






IMPROVEMENT

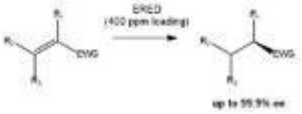
Bioreduction of dehydro-amino acid derivative with ERED Seqenzym



PERFORMANCE

Enzymatic manufacturing with only ppm of ERED biocatalyst

- By implementing the enzymatic process, we have favored atom economy process, compared to the alternative chemical process using stoichiometric amount of resolving agent.
- 52 batches were produced within a very high level of reproducibility. Average molar yield: 86%.



ECO FRIENDLY

EFFICIENCY

P2-22

Molecular modification of α-glucan phosphorylase during glucose metabolic processes

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Glucose-1-phosphate is an intermediate of glucose metabolism in organisms, which can enter the glycolytic pathway and provide energy for the organism. In addition, glucose-1-phosphate can directly serve as a nutritional enhancer and is also an important precursor of nutritional chemicals. α-Glucan phosphorylase (GP) can catalyze the synthesis of glucose-1-phosephate from inorganic phosphorus and starch.In this study, a new α-glucose phosphorylase was identified through database mining and its enzymatic properties was characterized. It was found that the optimal reaction temperature was 80 ℃ and the optimal reaction pH was 7.0. After being placed at 80 ℃ for 24 h, the enzyme could still maintain more than 50% of the initial enzyme activity, making it a super thermophilic enzyme. The minimum substrate for this enzyme was maltotriose, and the specific enzyme activity gradually increased with the increase of substrate chain length. The expression level of the truncated protein ΔGP obtained by truncating the non-catalytic domain at the C-terminus was 3.76 times higher than that of GP, and the specific enzyme activity was 1.3 times higher than that of GP. The thermal stability of ΔGP was comparable to that of GP, and the enzyme activity was higher. Through molecular modification, the catalytic efficiency and thermal stability of α-glucan phosphorylase have been improved. This study provides a reference for the efficient expression and application of α-glucan phosphorylase, and lays the foundation for further engineering optimization of its performance.

Reference

[1] Amin K, Tranchimand S, Benvegnu T, et al. Glycoside hydrolases and glycosyltransferases from hyperthermophilic archaea: Insights on their characteristics and applications in biotechnology[J]. Biomolecules, 2021, 11(11): 1557.
[2] Zhou W, You C, Ma H W, et al. One-pot biosynthesis of high-concentration α-glucose 1-phosphate from starch by sequential addition of three hyperthermophilic enzymes[J]. Journal of Agricultural and Food Chemistry, 2016, 64(8): 1777-1783.

P2-23

Engineering Formaldehyde Dehydrogenase from *Pseudomonas putida* and *Burkholderia multivorans* Towards Increased Direct Electron Transfer Capabilities

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In today's highly industrialised society, CO_2 is one of the most abundant greenhouse gases. The reliance on fossil energy and the burning of fossil fuels contribute to the high levels of CO_2 in the atmosphere and increase the effects of global warming. One promising idea to establish an efficient CO_2 circular economy is to use CO_2 as a carbon source for the production of high value bulk chemicals. Reversing the natural methanol utilization pathway to convert CO_2 into methanol has shown promising results in a number of studies. However, the bottleneck of the reaction has been shown to be the formaldehyde dehydrogenase ($F_{ald}DH$), which catalyses the reduction from formate to formaldehyde. The $F_{ald}DH$ (*P. putida* and *B. multivorans*) belongs to the family of zinc-containing medium-chain alcohol dehydrogenases and naturally oxidises formaldehyde to the non-toxic formate using NAD+ as a cofactor. The enzyme suffers from a rather low activity for the reverse reduction reaction, which reduces the overall yield of the reaction and is dependent on the expensive cofactor NADH.

This research focuses on the application of the $F_{ald}DH$ in an electro-enzymatic system. In general, electrochemical systems have the advantage of a high efficiency with which electrons are transferred into a reaction (Faradaic efficiency). By enabling a direct electron transfer (DET) of electrons from an electrode directly to the active site of the enzyme, the reaction can be carried out independently of the expensive NADH cofactor. This eliminates the need for a cofactor regeneration system, simplifying the reaction. We propose, that by creating a favourable orientation of the enzyme on the electrode and using a variety of protein engineering techniques, the yield of reaction can be increased.

The main aim of these strategies is to create a small distance between the active site of the enzyme and the electrode surface, which promotes the feasibility of DET. The enzyme variants and fusion proteins are biochemically characterised, k_M values determined and tested for DET capability during cyclic voltammetry (CV) measurements. The different strategies are compared in terms of Faradaic efficiency.

Side-specific mutations in the active site alter the reduction activity of the $F_{ald}DH$. Also, CV measurements show that the orientation of the active site towards the electrode surface is highly important for efficient DET. The aim of this work is to understand the properties of the DET between the $F_{ald}DH$ and the electrodes in order to increase the yield of enzymatic formate reduction.

P2-24

Effect of the Loop Structure of Alanine Racemase from *Shewanella livingstonensis* Ac10 on Pressure Tolerance

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Alanine racemase is an enzyme which catalyses the interconversion of L-alanine and D-alanine and is responsible for the synthesis of D-alanine contained in the peptidoglycan of bacterial cell wall. *Shewanella livingstonensis* Ac10 (*S. livingstonensis*) is a psychrophilic bacterium, isolated from Antarctic seawater. Unlike *Shewanella violacea* (*S. violacea*), which is also in the genus *Shewanella*, it is not pressure tolerant. We have successfully crystallized alanine racemase from *S. violacea* (SvAlr) and analyzed its structure. The conformation of alanine racemase from *S. livingstonensis* (SlAlr) was predicted using AlphaFold2. Comparison of the steric structures of the alanine racemases of these two bacteria revealed that SlAlr has a loop structure not observed in SvAlr. In this study, we constracted a mutant enzyme (SlAlr´) in which the loop structure was removed, and examined its pressure tolerance.

To measure the pressure tolerance of the enzymes, SvAlr, SlAlr, and SlAlr´ were incubated at 50 MPa for 1 hour, and their residual activities were assayed. Relative activity to that at ambient pressure was 92% and 60% for SvAlr and SlAlr, respectively. While SlAlr´ was found to exhibit 90% activity.

This result indicated that the loop structure of SlAlr is the critical point for pressure tolerance.

P2-25

Directed evolution of tailored enzymes for the remediation of bioproduction wastes

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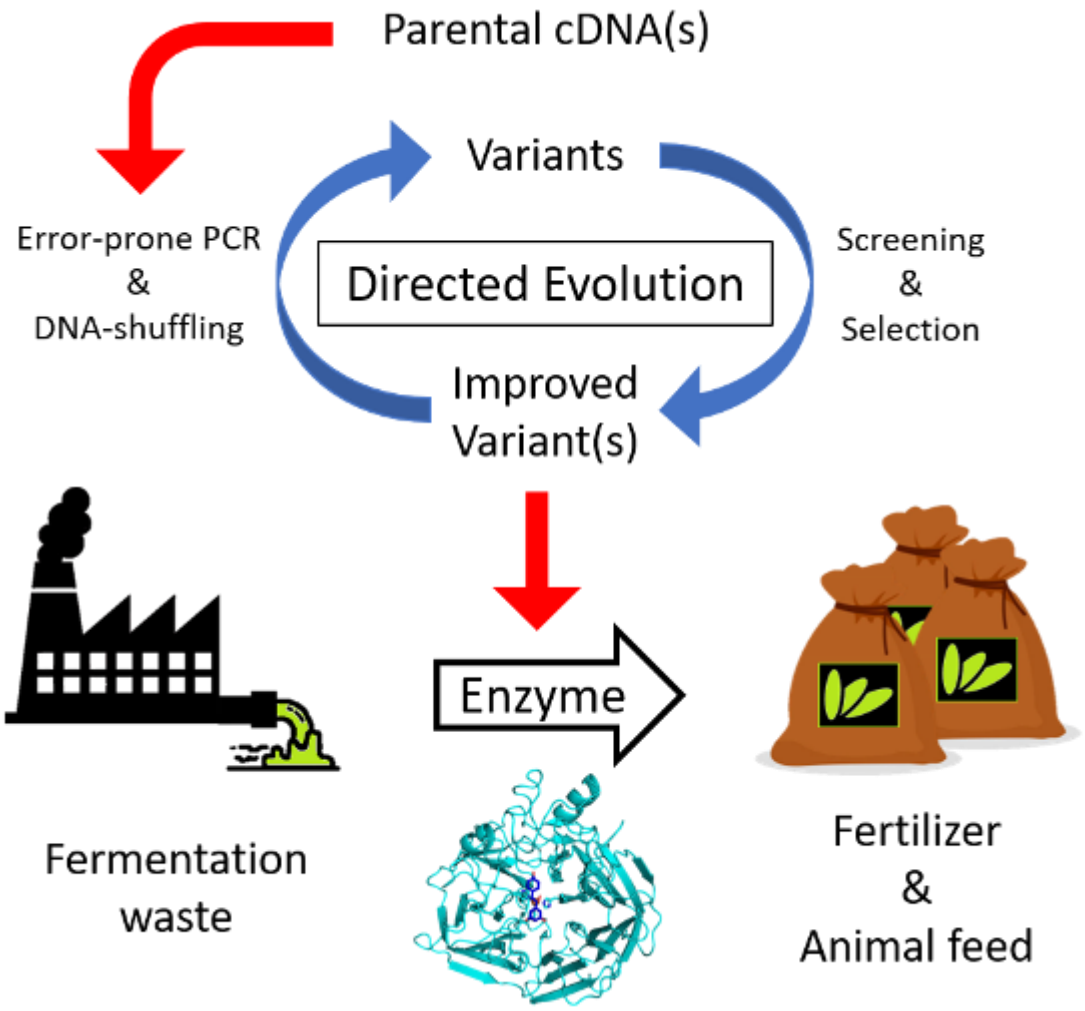
Science is becoming practical. Applied research is marching forward in order to find smart solutions to the modern world"s problems. Green house gasses are rising up to critical levels leading to global warming, so nowadays the challenge is finding a way to decrease the emission of CO_2 at least. One appropriate way to help the Earth healing is to make the production of economically relevant compounds environmentally sustainable as well. Employing fermentation and biocatalysis to replace organic synthesis, and upcycling wastes for the production of fertilizer and animal feed, would minimize the carbon footprint of many bioprocesses. This proof of concept will take part to the solution by making those wastes compostable. Especially drug residues in biomasses often pose a problem as they cannot be upcycled.

This biotechnological method for the mediation of bioproduction wastes, will employ directed evolution to cell-free synthetized enzymes in a multiwell microplate reader to facilitate screening activity. The cDNA(s) of the starting enzyme(s) will be amplified with error-prone PCR or DNA-shuffling, cloned into expression vector via Gibson assembly and transformed in *E. coli*. The resulting plasmids will be amplified via PCR colony and used as the substrate for the cell-free protein synthesis into the multiwell. Each well then will be topped with the assay solution to detect the activity of the novel enzymes and screened into the microplate reader. The best performing enzyme(s) will be the substrate for the next round of error-prone PCR or DNA-shuffling.

Once the system is established, it will be applied for the development enzymes suited for the digestion of any contaminant present in the industry wastes such as glyphosate, resveratrol or nanoplastics such as PET just to mention some. This will enable their upcycling for agriculture and animal husbandry, contributing towards carbon neutrality. The CO_2 emission of the companies will be compensated not only by throwing less biomass in the garbage, but also by supplying the ones producing animal feed and fertilizers with their starting material to cut their production energy cost. This represents a win-win situation for all those companies which connection will decrease cost and carbon footprint of their productions.

This proof of concept is committed in making the greatest possible contribution to reduce the environmental impact of industrial bioproduction. This will further develop sustainable biotechnological approaches to decrease the environmental impact of bioprocesses.

Fig. 1


Parental cDNA(s)
Variants
Error-prone PCR
&
DNA-shuffling
Directed Evolution
Screening
&
Selection
Improved
Variant(s)
Enzyme
Fermentation
waste
Fertilizer
&
Animal feed

P2-26

Hot Enzymes for Cool Reactions: sp³ C-H Functionalization of Pharmaceutical Building Blocks Using a Thermostable Enzyme

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[1]RWTH Aachen University, Institute of Applied Microbiology, Aachen, Germany
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1. Introduction

Tetrahydrocarbazole derivatives represent a cornerstone in the quest for new pharmaceutical compounds, due to their prevalence as core structures in drugs targeting a vast array of conditions, including cancers, neurodegenerative diseases, diabetes, allergies, and viral infections. The synthetic elaboration of these molecules, however, is hampered by the complexity of their sp^3 C-H functionalization, a critical step in diversifying their therapeutic potential. Traditional methods face significant challenges in efficiency and selectivity, necessitating the exploration of innovative synthetic pathways.

2. Objectives

Our research aimed to:

- Develop new methodologies for the hydroxylation and halogenation of sp3 C-H carbons in tetrahydrocarbazole derivatives, to overcome existing synthetic limitations.
- Employ genome mining to discover novel alpha-ketoglutarate dependent halogenases/hydroxylases (AKGHs) capable of facilitating these chemical transformations.
- Characterize and engineer a thermostable AKGH for enhanced chemoselectivity towards halogenation, thereby providing a scalable, efficient route to synthesize tetrahydrocarbazole derivatives.

3. Results

Through genome mining, we identified nine novel putative AKGHs, with a focus on enzymes demonstrating promising activity on tetrahydrocarbazole derivatives. Notably a halogenase from a thermophilic cyanobacterium was distinguished by its superior conversion rates.

Comprehensive characterization under various conditions revealed the enzyme's optimal catalytic environment and its preferential activity towards hydroxylation over halogenation. This specificity was attributed to substrate orientation within the active site.

Through site saturation mutagenesis targeting identified active site hotspots, we aim to alter the enzyme's chemoselectivity towards complete halogenation, showcasing the potential of protein engineering in refining enzymatic specificity for targeted synthetic applications.

4. Conclusions

Our study advances the field of pharmaceutical chemistry by introducing a novel enzymatic strategy for the synthesis of tetrahydrocarbazole derivatives. By harnessing the power of thermophilic AKGH enzymes, we have demonstrated a viable pathway for the efficient and selective functionalization of sp^3 C-H bonds. This not only addresses the synthetic challenges inherent in the production of these compounds but also opens up new avenues for drug discovery and development. The successful engineering of enzymes for improved halogenation activity further emphasizes the potential of biocatalysis in overcoming the limitations of traditional chemical synthesis, marking a significant step forward in the quest for new and effective pharmaceutical agents.

P2-27

Directed Evolution of Nylon Depolymerizing Enzymes

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Global polymer production is about to exceed 400 megatons yearly, and recycling methods enabling a climate-neutral, circular polymer economy are highly important to successfully address global challenges like environmental pollution and climate change. Polyamides (nylon-6, nylon-6,6) represent the third most-produced hydrolyzable polymer, behind polyurethanes and polyethylene terephthalate. The main challenges in developing enzymatic polyamide recycling processes are the limited number of reported polyamidases and the lack of screening systems that allow the employment of powerful directed evolution methods. Here we report the first validated high-throughput screening system to tailor polyamidases for their application in polyamide recycling. We successfully applied the screening system to detect nylon-6, nylon-6,6, and common polyurethane degradation products down to the nanomolar range in cell-free extract. In a semi-rational directed evolution campaign, we validated our screening system by improving the turnover frequency of the polyamidase NylCp2-TS by 6.9-fold (5.48 ± 0.2 s–1; NylC-HP), resulting in the highest-performing polyamidase to date. The implementation of this high-throughput screening system represents a significant step towards the engineering and characterization of process-relevant biocatalysts for polyamide recycling, thereby bolstering efforts towards a sustainable circular polymer economy.

P2-28

Mutants of the *pleurotus citrinopileatus* lipase for applications in cheese

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1. Introduction

The savory flavor of cheese variants such as Feta or Parmesan is mainly attributed to the release of volatile free fatty acids (vFFA). To achieve this, pregastric esterases (PGE) are added in the production process. During the ripening of the cheese PGE hydrolyze milk fat triglycerides and prevalently short to medium chain fatty acids (C4:0 to C10:0) are released. However, the application of animal-derived enzymes does not meet the requirements of vegetarian, kosher and halal diets.

2. Objectives

Identification of enzymes suitable for vegetarian, kosher and halal diets as PGE replacement as well as closer insights in Basidimycota derived lipases.

3. Results

A fungal lipase derived from the golden oyster mushroom *Pleurotus citrinopileatus* (PCI_Lip) has a similar activity profile as animal derived PGE. However, previous experiments have shown that wild-type PCI_Lip releases higher amounts of long-chain fatty acids (≥ C16:0) compared to PGE, which cause off-flavors during the ripening of the cheese. In docking experiments with six *p*-nitrophenyl(pNP)- fatty acid esters of different chain length, seven amino acid (AA) positions were identified to putatively influence the chain-length specificity. These positions are mainly located in the substrate channel but also in the lid domain and near the substrate channel oft he lipase. The AAs in these positions were swapped *in silico* with all other proteinogenic AAs and molecular docking was performed. Using protein engineering multiple variants of the PCI_Lip have been created to alter its chain-length specificity. The variants were subjected to photometric assays to determine the hydolysis profiles. Single mutants were used to proof the model of PCI_Lip by creating variants with putatively better and worse specificity. Combination variants were created by combining promising AA substitutions. The variants, which showed the best hydrolysis profiles in photometric assays were applied in the production of Feta-type cheese. Subsequently, vFFA of the cheeses were analyzed by SPME-GC-MS and the samples were evaluated in a sensory test. Six single point variants exhibited a hydrolysis profile with higher specificity for short- to medium-chain fatty acids and lower specificity for long-chain fatty acids. Variants predicted worse generally showed low activity with higher specificity for long-chain fatty acids. The cheese made with the double variant L302G+L305A showed a vFFA profile and sensory characteristics similar to cheese made with commercially available PGE. Using protein engineering a small, but smart library of a Basidiomycota derived lipase has been created. The variants showed altered chain length specificity.

4. Conclusion

Variants with improved properties for the application in cheese making could be created. In multiple rounds of mutagenesis lipase variants were created that achieve a similar flavor in cheese as PGE. In addition, the model of the enzyme could be fortified by the hydrolysis profiles of the variants predicted with worse specificity.

P2-29

Optimization of a microfluidics-based assay for the metagenomic screening of N-Acetylneuraminic acid synthase and aldolase

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[2]PROZOMIX LIMITED, Haltwhistle, United Kingdom
[3]Technische Universität Darmstadt, Chemistry department, Darmstadt, Germany

N-acetylneuraminic acid (Neu5Ac) is a 9-carbon α-keto acid with several possible residue modifications and whose derivatives are metabolically active molecules that participate in cell communication and recognition. Due to its structural and functional diversity, there is a growing need for the discovery of novel catalysts that synthesize Neu5Ac derivatives to produce pharmaceutical and nutritional compounds. Neu5Ac synthase performs the irreversible condensation of N-acetylmannosamine and phosphoenolpyruvate, releasing inorganic phosphate and Neu5Ac. Secondly, Neu5Ac aldolase performs the aldol condensation of N-acetylmannosamine and pyruvate into Neu5Ac in a reversible manner.

Conventional activity-based screening of metagenomes requires considerable use of resources and time. Therefore it is imperative to develop (ultra)high-throughput methods for a faster, more efficient, and affordable catalyst discovery in a timeframe compatible with industrial bioprocess development [1,2]. To this end, we have developed a droplet-based screening assay to discover Neu5Ac synthases and aldolases in the natural microbial diversity. For enzyme function and scope diversification, both assays were optimized for the natural substrate, as well as others (N-acetylmanose, N-acetylglucosamine, N-acetylxylose) producing Neu5Ac derivatives.

The aldolase assay entails the encapsulation of a single library clone per droplet, with a developing cascade reaction composed of a lactate dehydrogenase and a ketoreductase [3]. The Neu5Ac synthase assay consisted of the same cascade supplemented with Neu5Ac aldolase. Therefore, in the presence of an active clone, a fluorescent signal is formed, and the positive droplet can be detected and sorted using either FACS or fluorescence-assisted, on-chip sorting.The assay was developed by stepwise backward assembly of the developing cascade first in microtiter plate and subsequently in droplet. Then, the sensitivity of the assay was tested with a mock library and DNA was recovered by PCR or by direct transformation in *Escherichia coli* DH10B.

[1] Y. V Sheludko *et al.*, "Winning the numbers game in enzyme evolution – fast screening methods for improved biotechnology proteins," *Curr. Opin. Struct. Biol.*, vol. 63, pp. 123–133, Aug. 2020, doi: 10.1016/j.sbi.2020.05.003

[2] P. Jacques *et al.*, "High-throughput strategies for the discovery and engineering of enzymes for biocatalysis," *Bioprocess Biosyst. Eng.*, vol. 40, no. 2, pp. 161–180, 2017, doi: 10.1007/s00449-016-1690-x

[3] Y. C. Thai *et al.*, "Fluorogenic kinetic assay for high-throughput discovery of stereoselective ketoreductases relevant to pharmaceutical synthesis," *Bioorg. Med. Chem.*, vol. 26, no. 7, pp. 1320–1326, 2018, doi: 10.1016/j.bmc.2017.05.024

The work received funding from Programmes under Grant Agreement numbers 956631 and 101081957. Access to genetic resources for metagenomic library construction was granted under agreement ESNC107 (ABSCH-IRCC-ES-258964-1)

P2-30

Study of recombinant unspecific peroxygenases from *Candolleomyces (Psathyrella) aberdarensis* through crystallography and reaction analysis

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Fungal unspecific peroxygenases (UPOs) exhibit a high potential for selective C-H oxyfunctionalization reactions, positioning them as highly promising industrial enzymes. Indeed, UPOs are facing industrial demands and are getting closer to the global markets in terms of chemical synthesis and production of new pharmaceuticals. Despite these merits, the number of available UPOs is still scarce, due to a general poor functional expression in heterologous hosts (1). Among the recent new UPOs successfully expressed are two variants from *Candolleomyces (Psathyrella) aberderensis* (*Pab*UPOs), with which we achieved high titers in yeasts by adopting evolved secretion mutations (2). These recombinant UPOs are strongly robust against pH and temperature, and therefore are suited candidates for future directed evolution enterprises.

In this communication, we are presenting the crystal structures obtained at a high resolution for both enzymes in conjunction with soaking experiments and a detailed analysis of their oxidative pattern with a panel of representative substrates of industrial interest.

1. Monterrey, D. T., Menés-Rubio, A., Keser, M., Gonzalez-Perez, D. and Alcalde, M. (2023). Unspecific peroxygenases: The pot of gold at the end of the oxyfunctionalization rainbow?. *Current Opinion in Green and Sustainable Chemistry*. 41, 100786. doi.org/10.1016/j.cogsc.2023.100786

2. Gomez de Santos, P., Hoang, M.D., Kiebist, J., Kellner, H., Ullrich, R., Scheibner, K., Hofrichter, M., Liers, C. and Alcalde, M. (2021). Functional expression of two unusual acidic peroxygenases from *Candolleomyces aberdarensis* in yeasts by adopting evolved secretion mutations. *Applied & Environmental Microbiology.* 87: e00878-21

P2-31

Directed evolution of a fungal peroxygenase from *Daldinia* sp. EC12 for functional expression in yeast and synthesis of indigoids

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1. Introduction

Indigoids are a class of bis-indoles with an important commercial presence since the textile and denim industries rely heavily on indigo as a vat dye, and they have projection as pharmaceuticals and semiconductors [1]. Indigoids can be obtained from natural sources (plants, mollusks), but they are mainly produced by chemical synthesis generating harsh pollutants in the process [2]. A great amount of research has been carried out into developing more sustainable processes with genetically modified organisms and enzymes [3]. However, the turnover numbers of these processes do not reach still industrial standards [1,3]. The easiest way to obtain indigoids by enzymatic methods is hydroxylating indole to form 2-oxindole (indoxyl), which spontaneously oxidizes and dimerizes into indigo [3]. In this regard, fungal unspecific peroxygenases (UPOs) are oxygen-transfer biocatalysts with outstanding potential for industrial application: They are stable, extracellular enzymes that perform selective and efficient oxyfunctionalization reactions by using hydrogen peroxide as the only oxygen donor and electron acceptor, overcoming the use of complex cofactors or living cells as classical cytochrome P450 monooxygenases do need [4].

2. Objetives

Engineering a novel UPO (*Daldinia* sp. EC12 UPO, *Dsp*UPO) to increase its expression titers in yeast and improve the enzymatic synthesis of indigo and 6,6'-dibromoindigo (Tyrian purple) from different indoles.

3. Results

In the first place, two rounds of directed evolution driven by error-prone PCR disclosed a triple mutant (*Dsp*Triple) with significant improvements in functional expression. Five amino acids surrounding the heme cavity were selected based on docking simulations with indole, and subjected to a new round of saturation mutagenesis (Fig. 1). From these libraries emerged mutants that outperformed the previous variants for indigoid synthesis. These variants were purified and characterized in terms of thermostability, pH-range of activity and kinetics.

4. Conclusion

UPOs are versatile biocatalysts with remarkable potential in synthetic chemistry and industrial bioprocesses. Despite they are difficult to express in non-native hosts, directed evolution is an efficient vehicle to circumvent this shortcoming. Variants of the novel *Dsp*UPO exhibit increments in the production of different indigoids such as the valuable Tyrian purple. These variants are studied in terms of competing with current chemical and enzymatic methods for indigoid production.

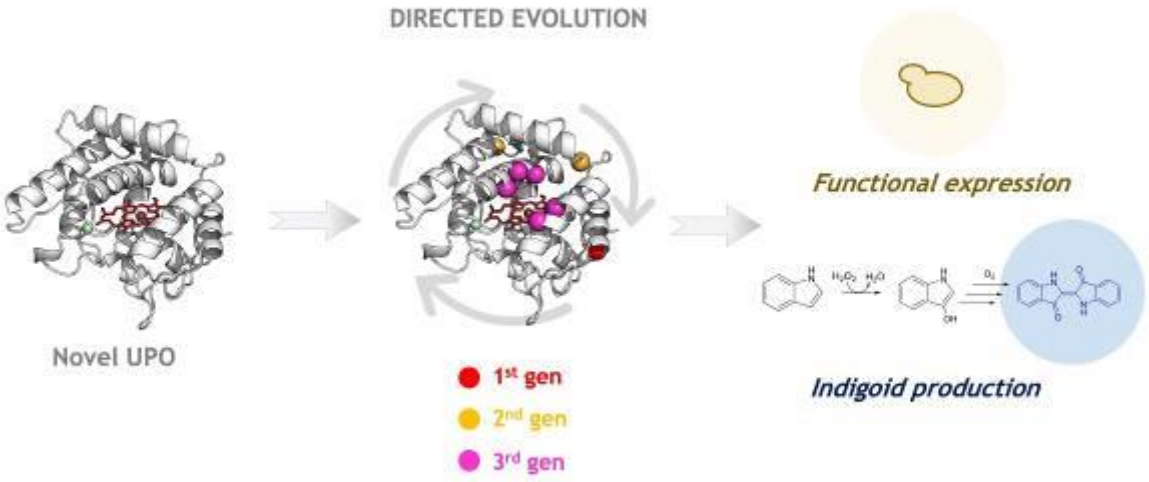
[1] Kong, F. et al., *ChemCatChem* (2022), e202201151.

[2] Kaplan, G., Seferoglu, Z., *Curr. Org. Synth.* 20 (2023), 361-364.

[3] Fabara, A. N., Fraaije, M. W., *Appl. Microbiol. Biotechnol.* 104 (2020), 925-933.

[4] Beltran-Nogal, A. et al., *Curr. Opin. Struct. Biol.* 73 (2022), 102342.

Fig. 1


DIRECTED EVOLUTION
Novel UPO
1st gen
2nd gen
3rd gen
Functional expression
Indigoid production

P2-32

Chiral alcohols from alkenes and water - Evolution of a "dream catalyst"

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The asymmetric addition of H_2O to unactivated C=C double bonds to produce chiral alcohols is a major challenge in organic chemistry[1,2]. This transformation is often referred to as a "dream reaction" since alkenes and water are cheap starting materials for the synthesis of enantiopure alcohols which are valuable precursors for chemical, agrochemical as well as pharmaceutical industries. The corresponding reaction thereby promises 100 % atom economy. In classical synthesis only unselective hydration is established under rather harsh reaction conditions, while a chemo-catalyst for stereoselective hydration of olefins is currently out of reach[1–4].

Our research group has lately identified and engineered a fatty acid hydratase originating from *Marinitoga hydrogenitolerans* (OhyMhy) for the asymmetric addition of water to simple alkenes. The engineered enzyme shows excellent enantioselectivity for hydration of styrenes to yield chiral 1-arylethanols (>99:1 e.r.). Despite the high stereoselectivity, the overall enzyme activity is still low[3].

Here we present our progress in further optimizing synthetically useful catalysts that allow easy access to a wide range of enantiopure alcohols and discuss current limitations. We focus on studies to transfer beneficial mutations to homologs via site directed mutagenesis. Thereby we aim to shed light on the prediction of transferability based on spatial proximity within a sequence similarity network. Complemented by mechanistic studies, we aim to develop an enzyme platform capable of carrying out the asymmetric hydration of a broad range of alkenes.

References

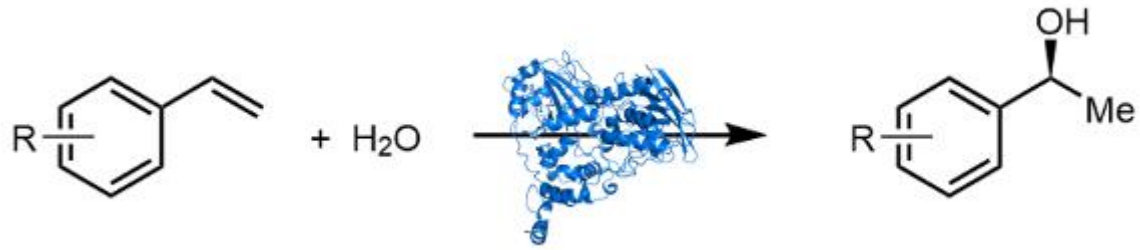
[1] J. L. Kennemur, R. Maji, M. J. Scharf, B. List, *Chem. Rev.* **2021**, *121*, 14649–14681.

[2] Y. Zhang, B. E. Eser, P. Kristensen, Z. Guo, *Chinese J. Chem. Eng.* **2020**, *28*, 2051–2063.

[3] M. Gajdoš, J. Wagner, F. Ospina, A. Köhler, M. K. M. Engqvist, S. C. Hammer, *Angew. Chem. Int. Ed.* **2023**, *62*, e202215093.

[4] H. Sato, W. Hummel, H. Gröger, *Angew. Chem. Int. Ed.* **2015**, *54*, 4488–4492.

Fig. 1



P2-34

Efficient and scalable enzyme engineering by computational design of combinatorial mutant libraries in an iterative manner

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Classical directed evolution is a powerful but often laborious method to improve enzyme properties, as it relies on iterative small steps in sequence space, typically leading to small incremental increases in performance (Fig. 1A).[1] Large steps in sequence space can be achieved by introducing multiple mutations in parallel, potentially enabling larger increases in activity and selectivity (Fig. 1B). However, such combinatorial libraries are huge, leading to massive screening efforts as most of the variants are non-functional.[2] For instance, the simultaneous randomization of an enzyme active site with twelve amino acids generates a mutant library with 4.1×10^{15} variants. Here, we report how *in silico* pre-screening of such large libraries can significantly improve the process of directed enzyme evolution when performed iteratively. In particular, we applied different computational tools[3,4] to identify potentially stable and functional protein sequences and thus condense large combinatorial mutant libraries to an experimentally trackable number of 50 to 4000 variants (Fig. 1C). This approach was used in an iterative manner to optimize *S*-adenosyl-l-methionine (SAM)-dependent methyltransferases (MTs) for regioselective methylation of *N*-heteroarenes (unpublished data). After three rounds of evolution, dozens of variants with more than 200-fold increase in activity were discovered. As high activities and selectivities could be determined for many substrates, this approach increases not only the speed but also the scale of a directed evolution campaign. The final variants contain up to eleven mutations in the first and second shell and build a small artificial enzyme family with high activity and selectivity on many different substrates.

Figure 1 : Classical directed evolution and iterative computational enzyme library design. Larger areas of the sequence space can be covered in fewer rounds of evolution using computational tools.

References

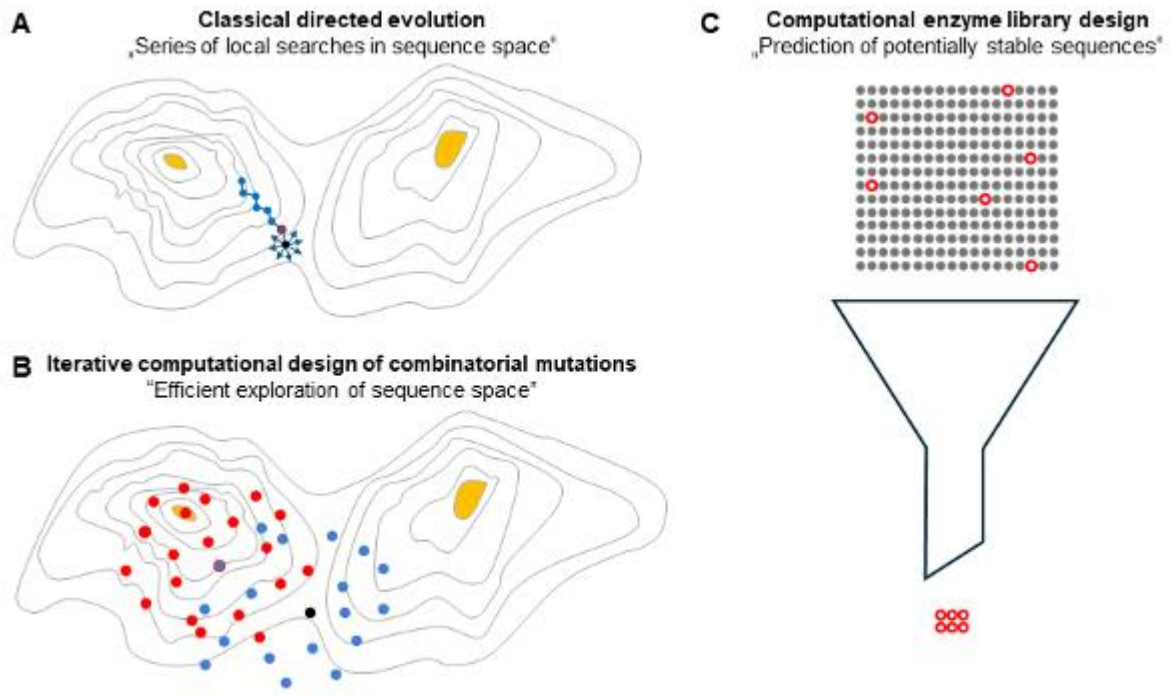
[1] Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K. Engineering the Third Wave of Biocatalysis. *Nature* **2012**, *485* (7397), 185–194.

[2] Goldsmith, M.; Tawfik, D. S. *Enzyme Engineering by Targeted Libraries.* Methods Enzym. **2013**, 523, 257–283.

[3] Khersonsky, O.; Lipsh, R.; Avizemer, Z.; Ashani, Y.; Goldsmith, M.; Leader, H.; Dym, O.; Rogotner, S.; Trudeau, D. L.; Prilusky, J.; Amengual-Rigo, P.; Guallar, V.; Tawfik, D. S.; Fleishman, S. J. Automated Design of Efficient and Functionally Diverse Enzyme Repertoires. *Mol. Cell* **2018**, *72* (1), 178-186.e5.

[4] Patsch, D.; Eichenberger, M.; Voss, M.; Bornscheuer, U. T.; Buller, R. M. LibGENiE – A Bioinformatic Pipeline for the Design of Information-Enriched Enzyme Libraries. *Comput. Struct. Biotechnol. J.* **2023**, *21*, 4488–4496.

Fig. 1


A
Classical directed evolution
„Series of local searches in sequence space"
B
Iterative computational design of combinatorial mutations
"Efficient exploration of sequence space"
C
Computational enzyme library design
„Prediction of potentially stable sequences"

P2-35

Engineering of NAD-dependent dehydrogenases to change cofactor specificity towards NMN

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NAD/NADP cofactors play a central role in the redox activity of oxidoreductases. However, since many metabolic pathways rely heavily on these cofactors, it poses a challenge to execute biocatalytic reactions for the desired product without cross reactivity.[1] Therefore, the implementation of a non-canonical cofactor such as NMN is desirable to increase the atom economy of those pathways.[2-4] Therefore, the objective is to engineer NMN-dependent enzymes in orthogonal biotransformation pathways to completely exclude NAD and to increase the affinity for NMN. Here we show our efforts to switch the acceptance of the NAD-dependent dehydrogenase glyceraldehyde-3-phosphatdehydrogenase (GAPDH) from the *E. coli* glycolysis pathway.

Figure 1: Schematic overview of the desired cascade step of the *E.coli* glycolysis pathway catalyzed by the GAPDH. The goal is to alter the cofactor specificity from the native NAD to the noncanonical NMN.

First approaches in literature regarding this cofactor swap for GAPDH were already successfully reported, by increasing the acceptance for NMN compared to the wildtype, however a simultaneous increase of NAD activity was observed for those engineered variants.[2] Based on these first approaches, variants were designed which decreased the activity for NAD to up to 98% of the wildtype activity, and increased NMN activity two-fold.

Another approach is based on the hindrance of the AMP binding cavity of the NAD binding pocket via a structural similar decoy substrate.[5] In that way the activity with NMN should be improved without the engineering of the GAPDH. First results with adenine and adenosine show a decreased activity of the enzyme for NAD, resulting in a competitive inhibition. Next, the effect of different decoy substrates on the GAPDH activity and different ADHs with NAD and NMN are going to be investigated.

[1] D. Meng, M. Liu, H. Su, H. Song, L. Chen, Q. Li, Y. Liu, Z. Zhu, W. Liu, X. Sheng, C. You, Y.-H. P. J. Zhang, *ACS Catal*. **2023**, *13*, 1983-1998.

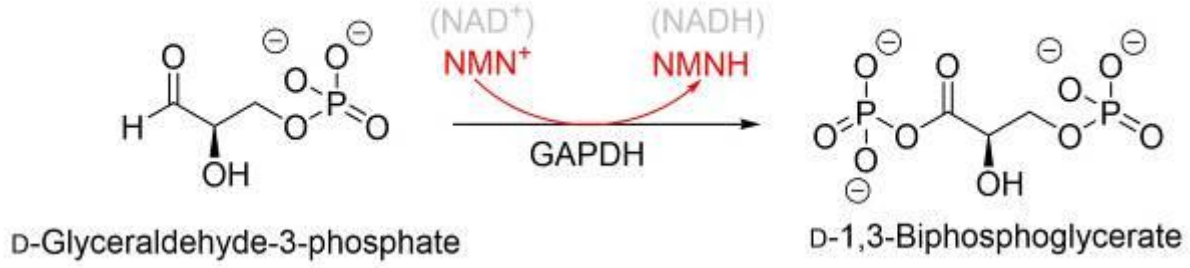
[2] E. King, *PhD Thesis*, **2021**, *University of California*.

[3] E. King, Y. Cui, D. Aspacio, F. Nicklen, L. Zhang, S. Maxel, R. Luo, J. B. Siegel, E. Aitchinson, H. Li, *ACS Catal.*, **2022**, *12*, 8582-8592.

[4] W. B. Black, L. Zhang, W. S. Mak, S. Maxel, Y. Cui, E. King, B. Fong, A. Sanchez Martinez, J. B. Siegel, H. Li, *Nat. Chem. Biol.*, **2020**, *16*, 87-94.

[5] S. Sicsic, P. Durand, S. Langrene, F. Le Goffic*, Eur. J. Biochem*., **1986**, *155*,403-407.

Fig. 1



P2-36

Improving the Hydrogen Peroxide Stability of $P450_{SP\alpha}$, a Highly Selective Peroxygenase for the α-Hydroxylation of Fatty Acids

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P450 Peroxygenases are capable of performing oxyfunctionalization reactions using hydrogen peroxide as the single electron and oxygen donor without the requirement for expensive cofactors or additional electron transfer systems.[1–3] While the use of hydrogen peroxide is preferable in terms of cost and process complexity, biocatalyst stability needs to be considered to avoid inactivation by the oxidant. Improving enzyme stability is nowadays commonly achieved through the use of computational tools like FireProt and PROSS, which introduce mutations to the target enzyme based on energy calculations.[4,5] To more directly address H_2O_2-stability, oxidation-sensitive amino acids can be targeted through rational design. This work aimed to increase the hydrogen peroxide stability of $P450_{SP\alpha}$, a highly selective peroxygenase of the CYP152 family, by generating enzyme variants through a combination of rational design and the use of computational tools.[6] Significant enhancements to the wildtype were not only achieved in terms of H_2O_2-stability, but also expressibility and activity for the α-hydroxylation of octanoic acid. To further elucidate the extent to which the enzyme was improved, preparative-scale biotransformations were conducted with the best-performing variants.

References:

[1] L. Hammerer, M. Friess, J. Cerne, M. Fuchs, G. Steinkellner, K. Gruber, K. Vanhessche, T. Plocek, C. K. Winkler, W. Kroutil, *ChemCatChem* **2019**, *11*, 5642–5649.

[2] S. Gandomkar, A. Dennig, A. Dordic, L. Hammerer, M. Pickl, T. Haas, M. Hall, K. Faber, *Angew. Chem., Int. Ed.* **2018**, *57*, 427–430.

[3] Y. Jiang, W. Peng, Z. Li, C. You, Y. Zhao, D. Tang, B. Wang, S. Li, *Angew. Chem., Int. Ed.* **2021**, *60*, 24694–24701.

[4] M. Musil, A. Jezik, J. Horackova, S. Borko, P. Kabourek, J. Damborsky, D. Bednar, *Brief. Bioinform.* **2023**, *25*, bbad425.

[5] A. Goldenzweig, M. Goldsmith, S. E. Hill, O. Gertman, P. Laurino, Y. Ashani, O. Dym, T. Unger, S. Albeck, J. Prilusky, R. L. Lieberman, A. Aharoni, I. Silman, J. L. Sussman, D. S. Tawfik, S. J. Fleishman, *Mol. Cell* **2016**, *63*, 337–346.

[6] K. Bangert, A. Swoboda, S. Vrabl, H. Rudalija, M. Lazzarotto, S. Payer, A. Glieder, C. A. M. R. Van Slagmaat, S. M. A. De Wildeman, W. Kroutil, *Green Chem.* **2024**, *26*, 3183–3189.

P2-37

Engineering Tunable Protein Nanoparticles for Biocatalysis

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Introduction

Nanobiotechnology-driven biocatalysis represents a promising technology in sustainable biomanufacturing for high-value products, particularly in the context of carrier-free enzyme immobilisation. Bacterial inclusion bodies (IBs), typically regarded as unfolded waste material, have been shown in recent studies to contain biologically functional proteins [1]. Catalytically active IBs (CatIBs) have gained attention as a cost-effective enzyme immobilisation technique due to their simplified production and purification processes, often resulting in improved enzyme activity and stability [2, 3].

Objectives

This study aims to harness the aggregating property of the p40 domain, initially identified within the multidomain β-mannanase from *Caldibacillus cellulovorans*. Prior attempts to produce p40 in *Escherichia coli* led to the formation of insoluble p40-IBs. The objective is to exploit this property to develop p40 as a platform for modular protein nanoparticles (PNPs) with diverse biotechnological applications. The study also involves designing novel modular structures with a functional biomolecule (e.g., enzyme, peptide, fluorescent protein) displayed on p40-PNPs.

Results

The suitability of p40 for generating functional CatIBs was investigated by fusing it with two industrially relevant enzymes, α-amylase and β-xylanase, resulting in Amy_{p40} and Xyn_{p40} IBs, respectively. Amy_{p40} and Xyn_{p40} expressed as CatIBs which retained not only their catalytic function against their respective substrate but also their original thermostability. Enzyme recycling was performed at the enzyme respective optimal temperature for activity. After 10 cycles, Amy_{p40} and Xyn_{p40} retained more than 50% of their initial activity. p40 incorporating the lytic peptide ZXR-2 was evaluated for antibacterial and cytotoxic activities. ZXR-2_{p40} was effective against selected Gram-positive and negative bacteria while cell viability assays showed cytotoxicity against human kidney HEK293T cells. The intracellular formation of p40 IBs in *E. coli* was studied by incorporating the fluorescent protein mCherry. *In vivo* expression kinetics of $mCherry_{p40}$ revealed the significant impact of production parameters such as temperature, induction time, and inducer concentration on IB properties. Further analysis of $mCherry_{p40}$ under varying production conditions was performed to determine their biophysical characteristics.

Conclusion

Based on the results obtained in this study, the aggregating propensity of p40 can be leveraged to generate active CatIBs and biologically functional p40-IBs, showing promise for applications across various biotechnological and biomedical domains.

References

1. García-Fruitós E et al (2005). Microb Cell Fact, 4:27.
2. Küsters K et al (2022). *Microb Cell Fact*, 21:108.
3. Jäger VD et al (2020). *Appl Microbiol Biotechnol*, 104:7313–7329.

P2-38

Enzyme exploration in the extreme environments of the Hellenic Volcanic Arc: the unveiling of a novel thermostable PL7 alginate lyase

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Introduction: In the last two decades global aquaculture of algae has experienced an increase, whereas the cultivation reached 36 million tons in 2020, making the production of macroalgae a 16.5 billion USD industry [1]. The high biomass of macroalgae can be exploited to produce food, feed, fertilizers, nutraceuticals, cosmetics, pharmaceuticals and biofuels [2–4]. Seaweeds are rich in polysaccharides and other high value products [5–7] and an efficient way to gain access to these products are polysaccharide-degrading enzymes (CAZymes) from the marine microbiome [8]. Macroalgal carbohydrates are new to industry and, therefore, there is a lack of such enzymes on this field [9].

Objectives: We focused our attention on discovering novel robust, thermophilic enzymes originating from the metagenome of marine microorganisms living in extreme environments. Via the collection of Hellenic Centre for Marine Research, we have access to metagenomic libraries from the Santorini volcanic complex of the Hellenic Volcanic Arc, Aegean Sea [10], which is characterized by high temperatures (of up to 220°C in hydrothermal vents), high concentrations of heavy metals and low pH values.

Results: We present a methodology on identifying CAZymes of industrial interest, which led us to the discovery of a new alginate lyase named KAlLy. Sequence analysis and biochemical characterization of KAlLy revealed a PL7 enzyme active on alginate and poly-mannuronic acid, at temperatures up to 60°C (56,1 ± 7,8 U/mg). Regarding the stability, circular dichroism (CD) and differential scanning fluorimetry (DSF) analysis showed a Tm of 54°C, while KAlLy has a half-life time 30 h at 50°C, which indicates a thermostable enzyme.

Conclusions: The approach of gene mining in metagenomic libraries derived of extreme habitants often seems to provide new enzymes with special characteristics. Thorough sequence data analysis including many parameters such as sequence alignment, sequence-activity relations, prediction of enzyme"s structure and functional domains as well as solubility and novelty, is required to manage a high number of candidate genes. In our case, we were led to the alginate lyase KAlLy, which has very good potential for further development of its stats through protein engineering and could be a good addition to the industrial"s enzyme toolbox.

References

[1] The State of World Fisheries and Aquaculture 2022, *FAO*, **2022**.

[2] Salido et al., *Algal Res.*, 77, **2024**, *103357*.

[3] Xie et al., *Food Res Int.,* **2023,** 1-36.

[4] Thakur et al., *Materials Today Sust.*, 18, **2022**, 100120.

[5] Arias et al., *Algal Res.*, 69, **2023**, 102954.

[6] Baghel, *Chem.* Engineering *J.*, 454(2) **2023**, 140177.

[7] Pradhan et al., *Biocatal. Agric. Biotechnol.* 39, **2022**, 102242.

P2-39

The structural insight into the SAM-dependent methyltransferase mechanism

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Methylation, catalyzed primarily by *S*-adenosylmethionine (SAM)-dependent methyltransferases (MTs), is a ubiquitous biochemical reaction. The structural and functional diversity of MTs is important for biocatalytic methylation of a wide range of substrates with high stereo-, regio- and chemoselectivity in pharmaceutical processes.[1] Large-scale application of MTs is challenging because of their requirement on expensive and unstable SAM as a stoichiometric methyl donor.[2] The potential solution to this limitation could involve the use of synthetic methyl donors, such as sulfate- and sulfonate-based compounds, for the *in situ* regeneration of SAM.[3] In this presentation we will discuss our efforts to understand the structural and dynamic basis for the remarkable ability of certain members of the thiopurine methyltransferase (TPMT) family to bind and activate these synthetic methyl donors.

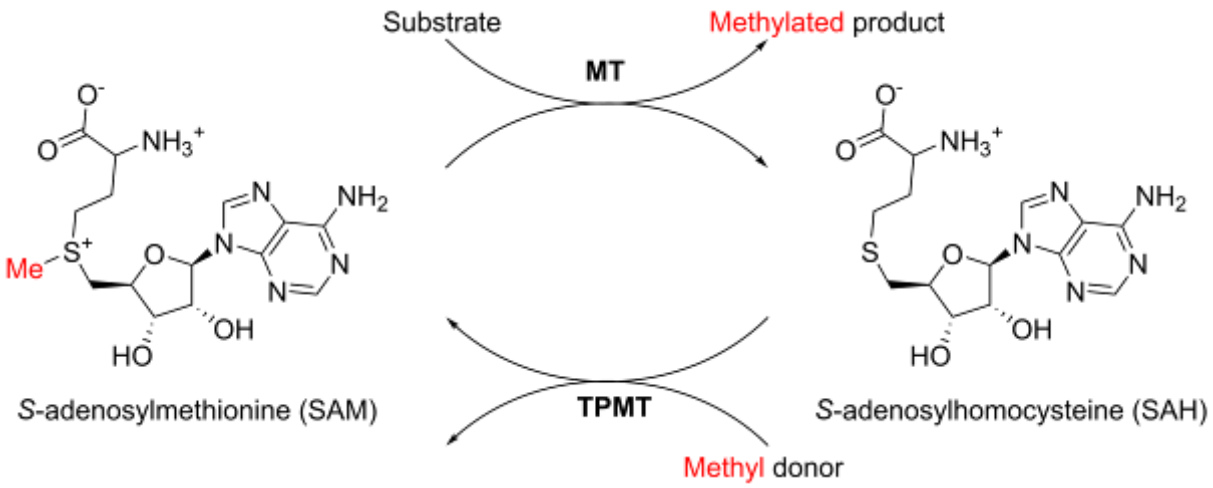
Fig. 1: Methyltransferase (MT)-catalyzed methylation of nucleophilic substrates. Thiopurine methyltransferase (TPMT) catalyzes methylation of S-adenosylhomocysteine (SAH) for *in situ* regeneration of SAM.

[1] Abdelraheem, E.; Thair, B.; Varela, R. F.; Jockmann, E.; Popadić, D.; Hailes, H. C.; Ward, J. M.; Iribarren, A. M.; Lewkowicz, E. S.; Andexer, J. N.; Hagedoorn, P.; Hanefeld, U. *ChemBioChem* **2022**, *23* (18), e202200212.

[2] Struck, A.W.; Thompson, M.L.; Wong, L.S.; Micklefield, J. *Chembiochem* **2012**, *13* (18).

[3] Wen, X.; Leisinger, F.; Leopold, V.; Seebeck, F. P. *Angew. Chem. Int. Ed.* **2022**, *61* (41).

Fig. 1



P2-40

The enzymatic synthesis of metaraminol – an unexpected hurdle race

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1. Introduction

The enzymatic production of metaraminol, an active pharmaceutical ingredient for the treatment of hypotension, has been investigated intensively in our working group. Here, a multi-step enzymatic process was established as a green alternative to the conventional chemical production route, potentially including the use of starting materials from renewable resources [1].

This enzymatic process comprises two steps, the decarboxylation of pyruvate and the subsequent carboligation with 3-hydroxybenzaldehyde toward (*R*)-3-hydroxyphenylacetylcarbinol by the pyruvate decarboxylase of *Acetobacter pasteurianus* (*Ap*PDC) followed by the transamination of the intermediate to the final product metaraminol catalyzed by the amine transaminase of *Chromobacterium violaceum* (*Cv*ATA). This last step is combined with *in situ* liquid-liquid extraction using 1-octanol as organic top layer and oleic acid as reactive extractant, thereby recovering metaraminol and shifting the equilibrium of the transamination reaction towards the product side. By applying the described *in situ* product removal to the enzymatic process, an overall metaraminol yield of 69 % was achieved [2]. Although the principle of the enzymatic metaraminol synthesis works very well, the enzymes needed in this process lack high operational stability and suffer from low metaraminol yields.

2. Objectives

To overcome this major hurdle, we aim to enhance the operational stability of the carboligase and amine transaminase by combining computer-aided rational design with random mutagenesis approaches and elucidating the overall inactivation mechanism of both enzymes.

3. Results

So far, the constraint network analysis of the *Ap*PDC suggested 142 weak spots which potentially lead to a destabilization of the enzyme. From this starting point, the 6 most promising mutations for stability enhancement are currently analyzed regarding their long-term stability against the substrate 3-hydroxybenzaldehyde.

Looking at the amine transaminase, during the characterization experiments it was observed that the yellowish color of the cofactor pyridoxal-5"-phosphate (PLP) vanishes in the presence of low metaraminol concentrations. Subsequently, the single compounds were analyzed, suggesting that the PLP is degraded in the presence of the product metaraminol. Detailed instrumental analyses are currently being carried out to elucidate the degradation process further.

4. Conclusion

To overcome the unexpected hurdles of low operational stabilities and low metaraminol yields, we need to understand the inactivation of both, *Ap*PDC and *Cv*ATA, upon exposition to each reaction compound. With that, we aim to create a sustainable and economically competitive process for metaraminol synthesis.

[1] Labib, M. *et al.* (2022), *ACS Sus. Chem. Eng*. 10 (16): 5117–5128

[2] Doeker, M. *et al.* (2022), *Green Chemistry*, 24(1), pp. 295–304.

P2-41

Harnessing Environmental Microbiota for the Discovery of Biocatalytic Enzymes Using Microbial Single-Cell Genome Sequencing

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Environmental microbiomes represent a vast, untapped resource for novel biocatalytic enzymes. However, efficiently sequencing genomes of environmental microbes remains challenging. We have developed a proprietary microbial genome database, bit-GEM, using a single-cell sequencing method called bit-MAP. This method enables the recovery of high-quality microbial genomes and genes from complex environmental microbiomes, such as soil, allowing us to rapidly expand the database in a cost-effective manner. bit-GEM now contains over 1.5 billion genes, with half being unique enzyme genes not present in public databases.

To harness this resource, we developed a computational pipeline called bit-QED to identify enzymes capable of executing specific reactions of interest. bit-QED primarily analyzes the active sites of enzymes, characterizing them based on surface features like charge, hydrophobicity, and hydrogen bonding potential. These features are quantified for each voxel within a three-dimensional lattice and then integrated and clustered by mapping into a two-dimensional space.

We have experimentally demonstrated that bit-QED is effective in identifying novel biocatalytic enzymes. In this presentation, we will showcase our studies on the discovery and engineering of various enzymes from the bit-GEM database.

P2-42

Investigation of Structural Features of Lipase from *Rhizopus Oryzae* and the Impact on Fatty Acid Selectivity in Vegetable Oils and Fats

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One of the indispensable applications of lipases in modification of oils and fats is tailoring the structure of triglycerides (TAGs), to meet specific requirements from various applications in food, nutrition, and cosmetic industries. The property of triacylglycerol is highly depending on the fatty acids" composition on the glycerol backbone, both the fatty acid types and where do they locate, which can be talored by using lipase with high regio-specificity and chemo-selectivity [1]. Lipase from *Rhizopus oryzae* (ROL) has been shown to be convincing lipase for this purpose due to its high sn1,3 selectivity. Two amino acid residues, Ala89 and Phe95, located at the substrate binding crevice of ROL were shown possible impact on influencing this substrate preference based on the screening of the ligand movement during 50 ns molecular dynamic simulation [2]. Two amino acid residues, Ala89 and Phe95, located at the substrate binding crevice of ROL were shown possible impact on influencing substrate chemo-selectivity based on the screening of the ligand movement during 50 ns molecular dynamic simulation. In this study, Ala89 and Phe95 were mutated into several different amino acid residues, following by swapping the lid region with the lid from a similar lipase which shows 33% sequence identity on the lid region. Our results showed that by doing the lidswapping, the fatty acid preference of ROL was to longer chain fatty acid compared with wildtype.

P2-43

Role of a conserved lysine residue in stabilizing the redox cofactors of a Mo-dependent formate dehydrogenase

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Metal-dependent formate dehydrogenases (Me-FDHs) are of high interest for biotechnological applications such as biofuel production because they catalyze the reversible oxidation of formate to CO_2 enabling the capture of atmospheric CO_2 and the storage of energy. Me-FDHs are potent catalysts that contain molybdenum (Mo) or tungsten (W) cofactor in the catalytic center to which electrons are transferred through an adjacent [4Fe-4S] cluster. A strictly conserved lysine (K) residue is located between the two redox-active cofactors but its possible interactions with these cofactors have not been investigated. To understand its role in the catalysis of Me-FDHs, we substituted the lysine 44 in a Mo-dependent FDH from *E. coli* (*Ec*FDH-H) with amino acids containing chemically and structurally diverse side chains. Kinetic analysis showed that elimination of the positive charge at site 44 by the replacement of lysine with alanine (A), glutamic acid (E), methionine (M) and glutamine (Q) resulted in complete inactivation of *Ec*FDH-H whereas the introduction of arginine (R) bearing a basic side chain preserved 17.6% of wildtype activity at saturating formate concentration. Nevertheless, the lysine to arginine substitution led to a ~31.7-fold increase in the *K*m value for formate and a 2.2 °C decrease in the melting temperature in comparison to the wildtype enzyme. Notably, a decreased occupancy of the [4Fe-4S] cluster was observed for all K44 variants, as evident from their UV-Vis absorption spectra. In silico structure analysis of *Ec*FDH-H variants K44R, K44E and K44M by molecular dynamics (MD) simulation indicated an increased residue mobility in the [4Fe-4S] cluster binding site and, in the case of K44R, a displacement of the Mo-cofactor. These results are consistent with the conserved lysine residue being essential for stabilizing the Mo-cofactor and [4Fe-4S] cluster in *Ec*FDH-H and thereby support Me-FDH engineering efforts aimed at the design of improved biocatalysts for CO_2 reduction.

P2-44

An amino acid position next to the active site histidine significantly affects enzyme activity and stability of a type IIa PETase

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Plastic waste management is a worldwide issue of increasing importance fostering research on efficient recycling strategies. Here, PETases (EC. 3.1.1) have gained increasing attention because they can efficiently degrade plastic waste at ambient temperatures and thus contribute to polyester degradation and recycling. A particular amino acid located next to the active site histidine in PETases has been described as an important modulator of polyester degradation activity. Introducing single amino acid exchanges at this site resulted in elevated enzymatic activity, but the reasons remained elusive.

We describe here a saturation mutagenesis at this position next to the active site histidine using as a model enzyme the novel type IIa PETase *Hoce*_PE-H produced by the marine bacterium *Halopseudomonas oceani*. The resulting variants which carried one each of 20 natural amino acids at position F265 were comparatively characterized *in vitro* and *in silico* regarding to their thermostability and pH optimum, as well as their hydrolytic activity towards different polyester substrates. The investigation of thermostability revealed deviations in the melting points of the variants of up to 6°C; pH optima were in a range from 8 to 11. Summarizing the activity analyses performed under slightly basic conditions, it appeared that small hydrophobic amino acid substitutions close to the active site create a hydrophobic patch, as well as a broader active site cleft.

*Hoce*_PE-H was computationally studied using its AlphaFold 3D structural model. Molecular docking studies provided binding modes of the different polyester substrates revealing mechanistic insights into the function of *Hoce*_PE-H. In addition, from constant pH molecular dynamics (CpHMD) simulations, pkA-values were predicted showing that a neutral charge at this site is the crucial factor for polyesterase activity.

Systematic mutagenesis of the position next to the active site histidine combined with bioinformatics analysis hence enabled novel insights into the structure-function relationship of PETases thus contributing to deeper understanding of the mechanism of polymer degradation.

P2-45

Computational-aided engineering of a selective unspecific peroxygenase towards enantiodivergent beta-ionone hydroxylation

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Introduction:

Unspecific peroxygenases (UPOs) are fungal enzymes known for their ability to perform oxyfunctionalization reactions using H2O2. Research has focused on engineering these enzymes for industrial applications, including heterologous expression, activity enhancement, and regioselectivity enhancements[2,3]. However, achieving targeted enantioselectivity for specific substrates remains a challenge. Several UPOs have demonstrated the conversion of α- and β-ionone4, yielding a diverse array of products. Despite progress, improving enantioselectivity persists as a challenge5.

Objectives:

This study aimed to engineer enantioselectivity in *Mth*UPO, derived from *Myceliophthora thermophila*, to selectively access C4-hydroxylated stereoisomers of β-ionone, a model substrate representing valuable compounds used in the fragrance industry and the synthesis of carotenoids and Vitamin A6,7.

Results:

A computational-aided engineering approach based on restrained molecular dynamic simulations identified near-attack conformations for selective hydroxylation and characterized binding modes of β-ionone. A small smart library was designed to modify the active site pocket of *Mth*UPO, directing oxyfunctionalization selectivity towards enantioselective *R/S* C4 hydroxylation. Engineered enzyme variants were expressed in *Saccharomyces cerevisiae* and screened using the Multiple Injection in a Single Experimental Run (MISER) GC-MS method8,9, developed by our group, which results in a significant gain in activity and regioselectivity. The MISER setup involves injecting 96 samples into the GC in a single experimental run, with product quantifications performed exclusively in the MS through different m/z ratios, eliminating the need for substrate/product separation. Rescreening of the best variants with a chiral GC-MS led to the determination of the enantioselectivities.

Conclusion:

After two rounds of enzyme evolution, activity increased up to 17-fold and regioselectivity reached 99.6% for 4-hydroxy-β-ionone. Enantiodivergent variants were identified with enantiomeric ratios of 96.6:3.4 (R) and 0.3:99.7 (S). In silico analysis provided insights into selectivity, highlighting the potential of engineered UPOs for tailored enzymatic transformations10.

References:

[1] Muench, J., et al. ACS Catal., 2021. 11(15), 9168-9203.

[2] Beltran-Nogal, A., et al. Curr. Opin. Struct. Biol., 2022. 73, 102342.

[3] Monterrey, D.T., et al. Current Opinion in Green and Sustainable Chemistry, 2023, 100786.

[4] Babot, E.D., et al. J. Agric. Food Chem., 2020. 68(19), 5375-5383.

[5] Urlacher, V.B., et al. Appl. Microbiol. Biotechnol., 2006. 70(1), 53-59.

[6] Beekwilder, J., et al. J. Biotechnol., 2014. 192, 383-392.

[7] Parker, G.L., et al. Tetrahedron, 2016. 72(13), 1645-1652.

[8] Knorrscheidt, A., et al. ChemCatChem, 2020. 12(19), 4788-4795.

[9] Knorrscheidt, A., et al. ACS Catal., 2021. 11(12), 7327-7338.

[10] Münch, J., et al. ACS Catal., 2023. 13(13), 8963-8972.

P2-46

Exploring the Substrate Promiscuity of Bacterial Pyrimidine Deaminases

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Nucleobase deaminases, integral to both purine and pyrimidine salvage pathways, contribute to the post-synthetic modification of RNA and DNA substrates, and can also be applied for targeted gene editing. These enzymes predominantly mediate the deamination of canonical nucleobases. Nonetheless, the bioactivity of these enzymes with an expanding repertoire of therapeutic nucleoside analogs—employed extensively as antineoplastic and antiviral (pro)drugs (e.g., 5-fluorocytosine, gemcitabine, cytarabine, azacytidine, Molnupiravir)—remains insufficiently elucidated. Our preceding investigations revealed that prokaryotic cytidine deaminases catalyzed nucleophilic substitution at the N4 position of N4-acyl-cytidines, N-alkyloxycarbonyl-cytidines, S4-alkylthio-uridines, and O4-alkyl-uridines, thereby yielding uridine derivatives and corresponding amides, amines, carbamates, thiols, or alcohols as leaving groups. The current study extends this research to assess the activity of metagenomically sourced cytosine and 8-oxoguanine deaminases against a spectrum of altered nucleobase substrates. Insights into the substrate promiscuity of bacterial nucleobase deaminases are anticipated to elucidate the metabolic fate of modified nucleosides in vivo and inform the rational design of novel antiviral and antitumor prodrugs. In addition, the primary screen of selected deaminases identified several enzymes that can utilize hydroxylamine as a nucleophile in the biotransformation reactions. The substrate scope of deaminases with other nucleophiles and modified nucleobase as substrates will be analyzed to gain more information about the chemical and biochemical determinants influencing the outcome of the biotransformation reactions. The study of enzyme catalytic promiscuity - a phenomenon in which enzymes enable unanticipated or synthetically induced reactions - is emerging as an important avenue for improving catalytic methodologies and developing innovative, environmentally benign synthetic protocols for modified nucleoside analogues.

P2-47

Molybdoenzyme mediates carbon-sulfur bond making and breaking

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Molybdoenzymes are proteins of ancient origins with diverse biological functions, utilizing specific cofactors with molybdenum or tungsten at their centers, commonly referred to as molybdopterin (MPT). Most known molybdoenzymes catalyze stereoselective and regioselective oxidation, dehydroxylation, hydration, and the formation and cleavage of C-O and X-O bond through two-electron transfer mechanism, and have seen potential use in organic synthesis.[1] We discovered MPT-dependent ergothioneine synthase (MES) as the first reported instance of molybdoenzyme catalyzing C-S bond formation, thereby adding it to the repertoire of enzymatic C-S bond formation.[2,3] MES from *Caldithrix abyssi* contains an N-terminal module related to tungsten-dependent acetylene hydratase and a C-terminal domain cysteine desulfurase. These two-module cooperates to transfer sulfur from cysteine onto trimethylhistidine. However, the complexity of producing molybdoenzyme constrained the preceding study to *in vivo* model.

This poster presents our latest efforts to understand the activity of this class of MPT-dependent enzymes *in vitro*. Our work encompasses the production and purification, biocatalytic activity *in vitro*, and an initial look of the MES structure.

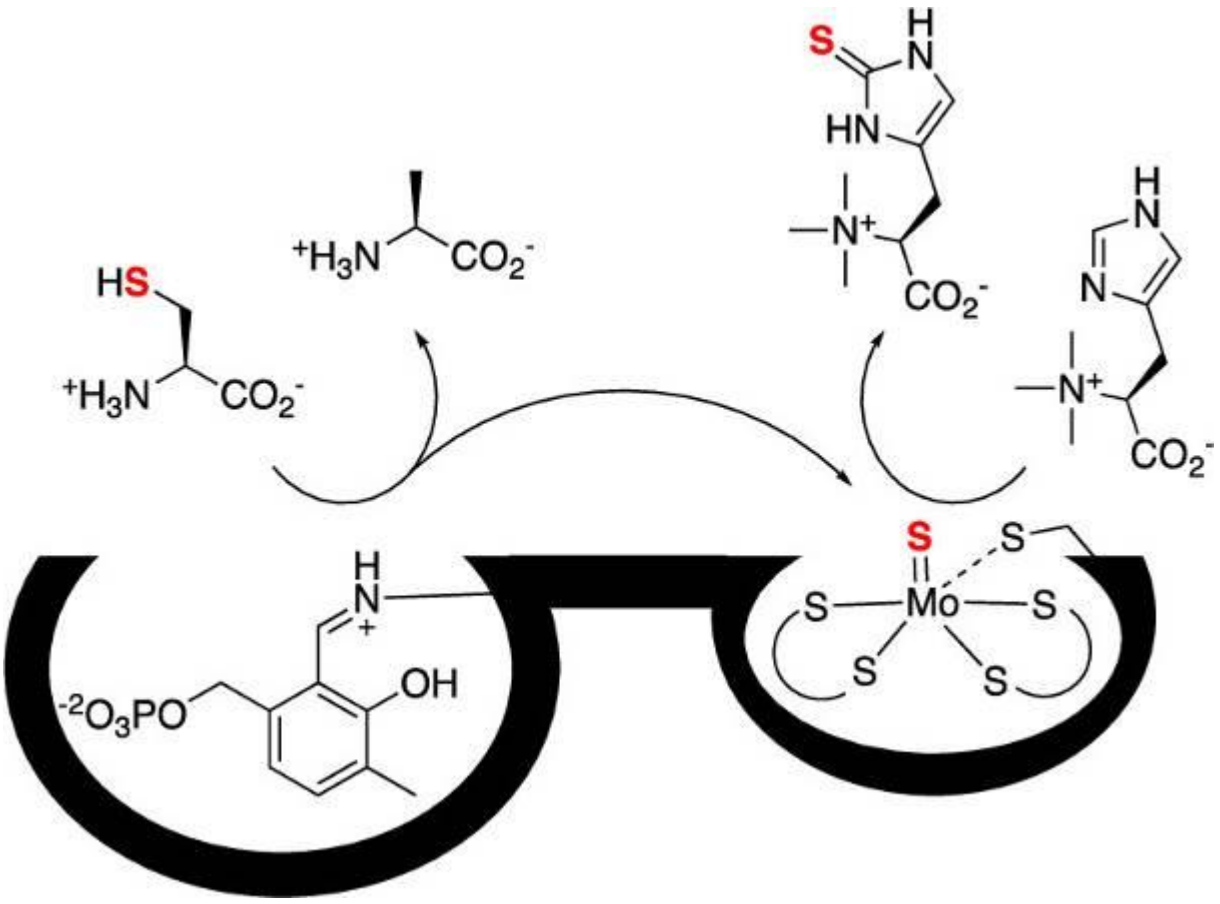
References:

[1] C. (Chip) Le, M. Bae, S. Kiamehr, E. P. Balskus, *Annual Review of Biochemistry* **2022**, *91*, 475–504.

[2] K. L. Dunbar, D. H. Scharf, A. Litomska, C. Hertweck, *Chem. Rev.* **2017**, *117*, 5521–5577.

[3] M. A. Beliaeva, F. P. Seebeck, *JACS Au* **2022**, *2*, 2098–2107.

Fig. 1


HS
+H3N
CO2-
+H3N
CO2-
S
H
N
HN
N+
CO2-
H
N
N
N+
CO2-
H
N+
-2O3PO
OH
S
S
Mo
S
S
S
S

P2-48

Improvement of the thermostability of the arylmalonate decarboxylase (AMDase) using ancestral sequence reconstruction

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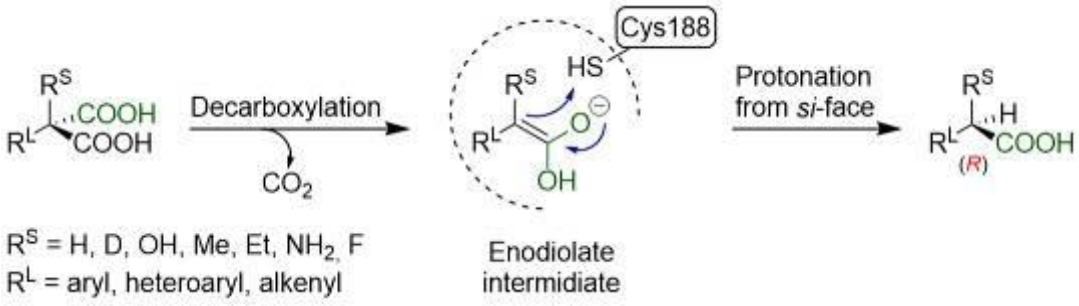
The arylmalonate decarboxylase (AMDase) from *Bordetella bronchiseptica* catalyzes a simple cofactor-independent single-step decarboxylation of prochiral arylmalonic acids. The broad substrate spectrum of arylmalonic acids that can be accepted by the AMDase in combination with its excellent stereoselectivity has already brought significant recognition.[1] However, some synthesis wise interesting variants known of bacterial AMDase are limited in terms of their substate scope and stability (storage stability with a half-life time of 1.2 h).[2] These challenges has been hindering its wider application in an industry setup, e.g., to produce optically pure propionates, which belong to the class of non-steroidal anti-inflammatory drugs (NSAIDs).[3] In our study, we demonstrate an approach to improve the stability of the AMDase based on ancestral sequence reconstruction (ASR) using *R* packages, with a combination of the "*Phangorn*" and "*APE"* algorithm.[4,5] We first focused on the thermostability aspect and based on the AMDase sequences known in literature, the most probable point mutations were computed for the phylogenetic tree to visualize the evolution of the enzyme to potentially thermostable variants. Using this method, ten ancestors of AMDase from different subfamilies were predicted and characterized regarding their catalytic performance (i.e., activity, stereoselectivity and thermostability) with arylmalonates and alkenylmalonates as the most prominent substrate classes.

Figure 1: Asymmetric decarboxylation catalyzed by AMDase.

References
[1] A. K. Schweiger, K. Miyamoto, R. Kourist, Front. Catal. 2021, 1, 1–19.
[2] M. Aßmann, C. Mügge, S. K. Gaßmeyer, J. Enoki, L. Hilterhaus, R. Kourist, A. Liese, S. Kara, Front. Microbiol. 2017, 8, 1–8.
[3] S. K. Gaßmeyer, J. Wetzig, C. Mügge, R. Kourist, ChemCatChem 2016, 8, 916–921.
[4] K. P. Schliep, Bioinformatics 2011, 27, 592–593.
[5] E. Paradis, K. Schliep, Bioinformatics 2019, 35, 526–528.

Fig. 1



P2-49

Bulky alkene reduction catalyzed by ene reductases

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Reducing "enes" while controlling the stereoselectivity is no easy task. Whereas classical chemical methods require complex homogeneous catalysts, enzymes should be straightforward to use (Lonardi et al., 2023). Ene-reductases (ERs) are well-known and described since decades. Notably, these Flavin-dependent enzymes can be employed in the fine chemistry with the production of key intermediates or products such as dihydrocarvone, dihydrocinnalmaldehyde or butanol (from pyruvate)(Toogood and Scrutton, 2018; Kumar Roy et al., 2022).

Usually, the substrates accepted by ERs present a relatively accessible ene – with at least one hydrogen and with an electron withdrawing group. The introduction of one stereocenter is often reported (Parmeggiani et al., 2022). Notably, (R/S)-carvone, 3-methyl-cyclohexenone or ketoisophorone are often used to characterize this enzyme class.

Our current research aims at studying the selective reduction of bulky enes, opening the possibility to introduce two stereo-centers (Scheme). 25 different ene-reductases have been produced, characterized and used for a screening of two model compounds. Enzyme activities and selectivity towards the different diastereoisomers obtained are discussed.

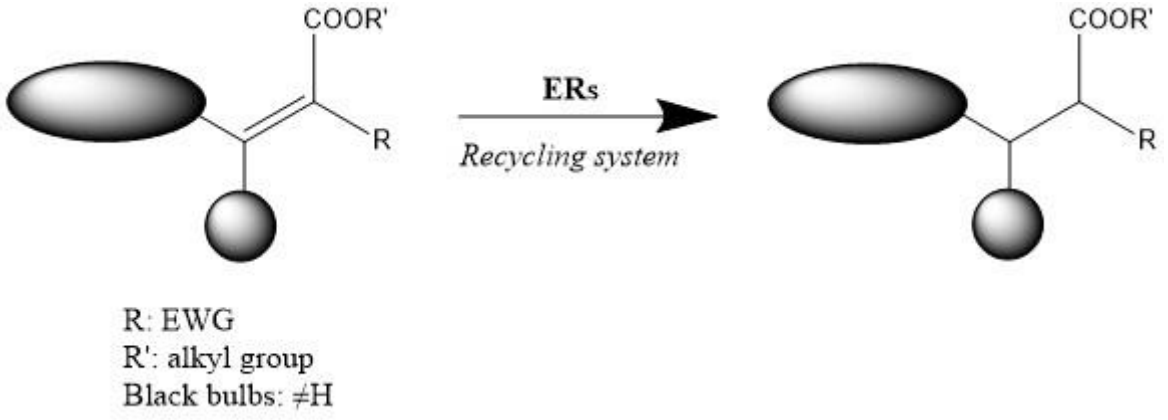
Kumar Roy, T., Sreedharan, R., Ghosh, P., Gandhi, T., and Maiti, D. (2022). Ene-Reductase: A Multifaceted Biocatalyst in Organic Synthesis. *Chem. – Eur. J.* 28, e202103949. doi: 10.1002/chem.202103949

Lonardi, G., Parolin, R., Licini, G., and Orlandi, M. (2023). Catalytic Asymmetric Conjugate Reduction. *Angew. Chem. Int. Ed.* 62, e202216649. doi: 10.1002/anie.202216649

Parmeggiani, F., Brenna, E., Colombo, D., Gatti, F. G., Tentori, F., and Tessaro, D. (2022). "A Study in Yellow": Investigations in the Stereoselectivity of Ene-Reductases. *ChemBioChem* 23, e202100445. doi: 10.1002/cbic.202100445

Toogood, H. S., and Scrutton, N. S. (2018). Discovery, Characterization, Engineering, and Applications of Ene-Reductases for Industrial Biocatalysis. *ACS Catal.* 8, 3532–3549. doi: 10.1021/acscatal.8b00624

Fig. 1



P2-50

Novel activities from an allogenic microbial glycosyltransferase

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Glycosylation is a function prevalent in eukaryotes, prokaryotes, and archaea, and is required for the formation of important glycoconjugates including cell surface glycans, glycosphingolipids, and glycoproteins.[1] The synthesis of such complexes is possible through the use of glycosyltransferases (GTs), enzymes ubiquitous in nature, which catalyse such glycosylations through the transfer of activated sugar donors, frequently nucleotide diphosphates (NDPs), to specific acceptors.[2]

GT family classification derives from sequence homology, with each preserving the structural fold and mechanism, and although their activities are substrate specific, families can be utilised with several donor-acceptor substrates.[3] Here, we report studies on a GT from the family 31 (GT31), sourced from the Gram-negative gut bacterium *Akkermansia muciniphilia*. After its expression in *Escherichia coli*, the enzyme displayed new activities not previously reported for this family.

Figure 1: HPLC chromatograms of GT31.9 reactions, including donor GDP-fucose (black), acceptor GlcNAc (blue), control reaction (red) and the recombinant reaction (green).

Figure 2: HPLC chromatograms of GT31.9 reactions, including donor UDP-GlcNAc (black), acceptor GalNAc (blue), control reaction (red) and the recombinant reaction (green).

The successful glycosylation of *N*-acetylglucosamine (GlcNAc) with fucose, and *N*-acetylgalactosamine (GalNAc) with GlcNAc indicate these new activities, with the predominant activity to be confirmed. Verifying this currently unestablished activity for the GT31 suggests many more activities could still be undiscovered in homologues of other families too. The potential to unlock a library of new activities for attainable, soluble GTs presents an alluring opportunity for the facile assembly of desirable enantiomerically pure glycoconjugates.

1. Ito T, Sadamoto R, Naruchi K, et al. Highly Oriented Recombinant Glycosyltransferases: Site-Specific Immobilization of Unstable Membrane Proteins by Using Staphylococcus aureus Sortase A. *Biochemistry*. 2010;49(11):2604-2614. doi:10.1021/bi100094g
2. Moremen KW, Haltiwanger RS. Emerging structural insights into glycosyltransferase-mediated synthesis of glycans. *Nat Chem Biol*. 2019;15(9):853-864. doi:10.1038/s41589-019-0350-2
3. Coutinho PM, Deleury E, Davies GJ, Henrissat B. An Evolving Hierarchical Family Classification for Glycosyltransferases. *J Mol Biol*. 2003;328(2):307-317. doi:10.1016/S0022-2836(03)00307-3

Fig. 1

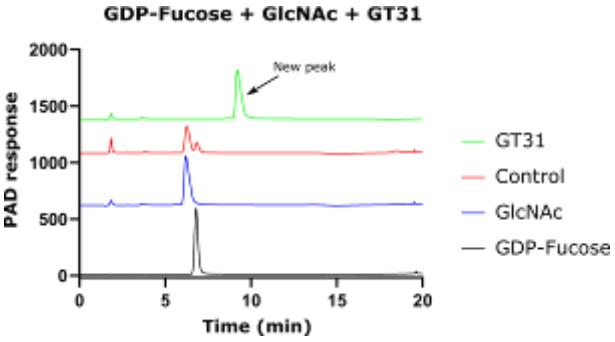
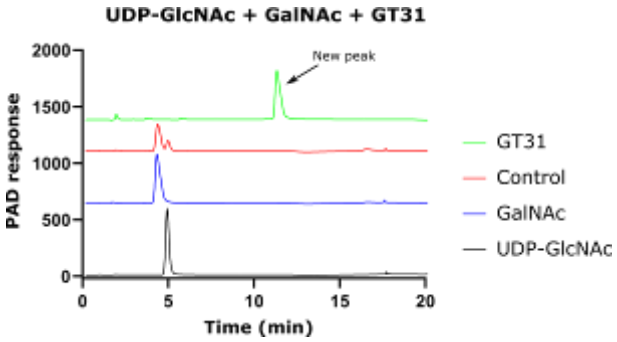


Fig. 2


UDP-GlcNAc + GalNAc + GT31
New peak
PAD response
2000
1500
1000
500
0
0
5
10
15
20
Time (min)
GT31
Control
GalNAc
UDP-GlcNAc

P2-51

Biotechnological Potential of a Novel Psychrophilic Phage-Type Single Subunit RNA Polymerase from a Low-Temperature Consortium Metagenome

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Introduction. Advancements in genome sequencing has led to a vast repository of bacterial genome sequences, many of which contain prophages derived from temperate bacteriophages. This has boosted the discovery of novel enzymes, including some essential for gene expression such as the single-subunit DNA-directed RNA polymerases (ss-RNAP, EC 2.7.7.6), pivotal for RNA synthesis from DNA templates. These enzymes stand out as versatile tools in biotechnology due to their structural simplicity and efficiency.

Objectives: Aiming to identify novel enzymes adapted to cold environments, we sequenced the metagenome of a psychrotolerant microbial consortium cultured from an environmental sample collected from a Southern Ice Fields glacier. Our objective was to find a new ss-RNAP active at low temperatures, and explore its potential applications, particularly in in-vitro transcription and cell-free protein synthesis systems (CFPS). Here, we report the identification, recombinant expression, and preliminary characterization of a novel prophage-derived ss-RNAP obtained from this extreme environment.

Results: Several consortia containing psychrophilic/psychrotolerant bacteria and viruses were obtained through culturing. Sequencing of the metagenome from one of these cultures, followed by thorough mapping of the sequencing reads against the IMG/VR virus database and subsequent bioinformatic analysis, resulted in the discovery of a novel ss-RNAP gene fragment within a prophage sequence. The identified gene (fb-RNAP) was synthesized, cloned, and recombinantly expressed in insoluble form in Escherichia coli. The enzyme was successfully recovered in fully functional form from inclusion bodies using 8M urea.

Preliminary characterization of the purified fb-RNAP revealed its remarkable properties, exhibiting activity over a wide range of temperature (20-60 °C) and pH (4.0-10.0), with optimal activity at 37 °C and pH 8.0. Furthermore, fb-RNAP exhibited higher activity compared to the well-studied T7 RNA polymerase. Through molecular modeling and rational design, we engineered a promoter-unspecific mutant (fb-RNAP_mut), showing promising results both in-silico and in-vitro. Molecular docking and dynamics simulations studies further elucidated the binding affinity of wild-type and mutant fb-RNAP to different promoters, which was also experimentally evaluated by cell-free expression of the green fluorescent protein.

Conclusion: Due to its unique properties and versatility, fb-RNAP represents an interesting discovery in the field of RNA polymerases. The enzyme's potential applications span various fields, including ribosensors, in-vitro transcription, and CFPS, making it a promising candidate for further research and biotechnological use. This study underscores the importance of continuing to explore extreme environments for the discovery of novel enzymes and the potential of microbes inhabiting cold environments as a source of valuable biocatalysts.

P2-52

Bioremediation of TNT by Novel Antarctic Bacteria: Genomic and Transcriptomic Analysis Unveils Enzymatic Arsenal for Pollutants Biotransformation

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Introduction. Environmental contamination by xenobiotic compounds derived from military activities, such as the explosive 2,4,6-trinitrotoluene (TNT), poses a significant threat to ecosystems and human health. Traditional physical/chemical remediation methods are often inadequate to deal with the persistence and toxicity of such contaminants. In contrast, bioremediation, which harnesses microbial metabolism to degrade contaminants, offers a promising alternative to mitigate environmental contamination from this and other sources. In this context, the exploration of extreme environments, such as Antarctica, in search of microorganisms with unique biocatalytic capabilities becomes crucial for the development of effective bioremediation strategies.

Objective. Our study aimed to isolate microorganisms from Antarctic environments capable of degrading xenobiotics, with a specific focus on TNT, and to elucidate the enzymatic mechanisms involved in their biotransformation. By integrating genomic and transcriptomic analyses with experimental results, we sought to identify key enzymes responsible for TNT biotransformation, shedding light on their catalytic properties and potential applications in bioremediation.

Results. Through screening Antarctic environmental samples, we isolated ten TNT-biotransforming bacterial strains, among which Pseudomonas sp. TNT3 exhibited exceptional tolerance, growth, and TNT biotransformation capabilities, which were superior compared to previously characterized strains. Genomic analysis revealed a diverse array of several xenobiotic and TNT degradation pathways in this bacterium, including genes encoding Old Yellow Enzymes, Nitroreductases, and Azoreductases. Notably, transcriptomic profiling during TNT exposure unveiled significant upregulation of a novel Azoreductase gene (> 1000-fold), suggesting a critical role in TNT biotransformation. In addition, in-silico studies using molecular docking and dynamics simulations were used to assess the affinity of this enzyme for different nitroaromatic substrates, including TNT and others, such as azo dyes. The aim of this evaluation was to assess its potential as a bioremediation agent for various families of pollutants, with promising results.

Conclusion. Our study showcases the successful isolation of Antarctic microorganisms, in particular Pseudomonas sp. TNT3, with remarkable TNT-biotransforming capabilities that have been experimentally demonstrated. Comprehensive genomic and transcriptomic analyses unveiled a diverse enzymatic arsenal involved in xenobiotic degradation, with the identification of a novel highly upregulated Azoreductase gene representing the most significant finding. Overall, this study contributes valuable insights into TNT biotransformation mechanisms and underscores the potential of Antarctic microorganisms for bioremediation applications.

P2-53

Crystal Structure and Substrate Spectrum of α-Ketoacid *C*-Methyltransferases SgvM and MrsA

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Enzymatic asymmetric methylation is a powerful approach in organic synthesis that utilizes enzymes to catalyze the addition of a methyl group (CH_3) to a substrate molecule. *S*-adenosyl methionine (SAM)-dependent methyltransferases enable the creation of chiral products with exact stereochemical precision. These enzymes transfer a methyl group from the cofactor SAM to a specific site on the substrate molecule.[1]

A few examples of *C*-MTs have been described to methylate enolizable β-branched α-ketoacids, leading to precursors of non-proteinogenic amino acids.[2,3] SgvM from *Streptomyces griseoviridis* and MrsA from *Pseudomonas syringae* pv. syringae catalyze the *R*-selective methylation of 4-methyl-2-oxovalerate and 5-guanidino-2-oxovalerate, respectively, with enantioselectivities exceeding 99%.[4,5] Here we report the substrate spectrum of MrsA can be extended to accept α-ketoacid substrates of SgvM with uncharged and lipophilic β-residues based on structure-guided rational design.

We discovered the catalytic mechanism similarity among α-ketoacid converting C-MTs and a certain type of dimethyltransferase found in polyketide synthase (PKS) pathways, like AprA,[6,7] which results in the loss of chiral centers. We propose to capitalize on this finding by redesigning the active pockets of SgvM and MrsA to enhance their ability to facilitate dimethylation of α-ketoacids. This approach presents a promising avenue for achieving the enzymatic introduction of *tert*-butyl groups.

Figure 1. A: Active site of MrsA wild type in complex with its native substrate 5-guanidino-2-oxovalerate (dark grey) and Mg2+ ion (green). **B**: Active site of SgvM and AprA with substrates, keto leucine (gray) and dimethyl malonic acid (blue), respectively.

[1] A.-W. Struck, M. L. Thompson, L. S. Wong, J. Micklefield, *ChemBioChem* **2012**, *13*, 2642–2655.

[2] C. Mahlert, F. Kopp, J. Thirlway, J. Micklefield, M. A. Marahiel, *J. Am. Chem. Soc.* **2007**, *129*, 12011–12018.

[3] A. W. Schultz, D.-C. Oh, J. R. Carney, R. T. Williamson, D. W. Udwary, P. R. Jensen, S. J. Gould, W. Fenical, B. S. Moore, *J. Am. Chem. Soc.* **2008**, *130*, 4507–4516.

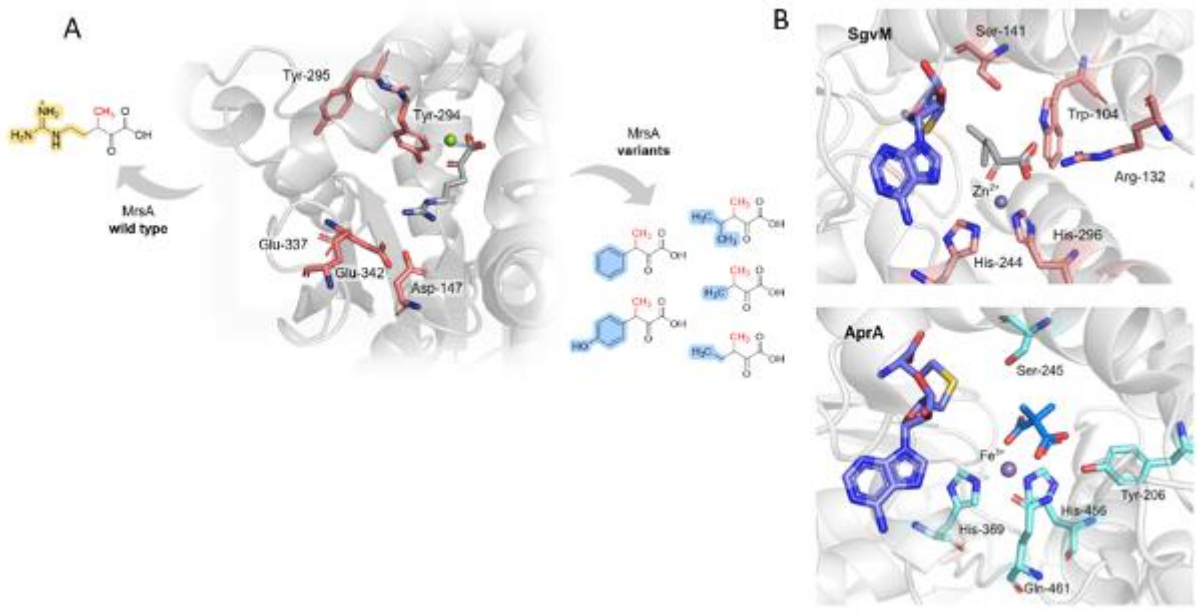
[4] Y. Xie, B. Wang, J. Liu, J. Zhou, J. Ma, H. Huang, J. Ju, *ChemBioChem* **2012**, *13*, 2745–2757.

[5] Braun S. D., Hofmann J., Wensing A., Ullrich M. S., Weingart H., Völksch B., Spiteller D., *Appl. Environ. Microbiol.* **2010**, *76*, 2500–2508.

[6] Y. Lao, M. A. Skiba, S. W. Chun, A. R. H. Narayan, J. L. Smith, *ACS Chem. Biol.* **2022**, *17*, 2088–2098.

[7] M. A. Skiba, A. P. Sikkema, N. A. Moss, C. L. Tran, R. M. Sturgis, L. Gerwick, W. H. Gerwick, D. H. Sherman, J. L. Smith, *ACS Chem. Biol.* **2017**, *12*, 3039–3048.

Fig. 1


A
Tyr-295
Tyr-294
Glu-337
Glu-342
Asp-147
MrsA
wild type
MrsA
variants
B
SgvM
Ser-141
Trp-104
Arg-132
Zn2+
His-296
His-244
AprA
Ser-245
Fe3+
Tyr-206
His-456
His-389
Gln-461

P2-54

Ancestral sequence reconstruction of unspecific peroxygenases

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Introduction

In the field of protein engineering, ancestral sequence reconstruction (ASR) emerges as an important strategy for acquiring novel enzymes with significant biotechnological potential (1,2). In this regard, ASR of unspecific peroxygenases (UPOs) holds crucial significance across multiple fronts. Firstly, it provides invaluable insights into the evolutionary trajectory and diversification of the UPO enzyme families, shedding light on the molecular mechanisms governing their unique catalytic properties and substrate specificities. Secondly, ancestral UPOs may harbour advantageous biochemical traits lost or altered in their modern counterparts.

Objective

In this study, we aimed our efforts at carrying out an ASR campaign to generate several ancestral nodes from short and long UPO type families.

Material and Methods

A multiple-sequence alignment was performed with the sequences obtained from gene bank. The curated alignments were introduced in a supercomputational cluster that generated phylogenetic trees. Then, the Bayesian approach Mr Bayes was used for the creation of a consensus tree. Ancestral nodes were selected based on probabilities and proximities to the reference sequences and were reconstructed using PMEL and amino acid substitution model.

The reconstructed nodes were expressed in *Pichia pastoris* and characterized in terms of substrate scope, pH profile and thermostability.

Evaluation of UPO activity was performed using various analytic techniques such as spectrophotometric analysis, HPLC and GC-MS.

Results

A reliable UPO phylogenetic tree was built, and 8 nodes were reconstructed and analyzed. Four ancestral UPOs were functionally expressed in yeast (Node 52 and 53 from *Mwe*UPO and Nodes 68 and 73 coming from *Dsp*UPO). The resurrected nodes showed distinct properties with respect to the modern UPO counterparts, in terms of expression, substrate scope, selectivity, pH profile and thermostability.

Conclusions

From the construction of a UPO phylogenetic tree, four ancestral UPO nodes were resurrected in yeast and characterized, showing diverse activities and biochemical properties.

1. Risso et al. (2018). Biotechnological and protein-engineering implications of ancestral protein resurrection. Curr. Opin. Struct. Biol., 51, 106–115.
2. Monterrey, D.T., et al. (2023). Unspecific peroxygenases: The pot of gold at the end of the oxyfunctionalization rainbow? Curr. Opin. Green Sustain. Chem., 41, 100786.

P2-55

Together or apart? Investigation of P450-CPR complex for flavonoids hydroxylation

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The biological activity of compounds exhibiting a range of health-promoting properties is determined by their structure, including the presence of hydroxyl groups [1]. Importance is attributed to the catechol motif, which determines effective scavenging of free radicals, inhibition of lipid peroxidation or complexation of metal ions [2]. However, regioselective enrichment of specific compounds with an additional hydroxyl group is still a challenge and requires the use of toxic reagents, without offering satisfactory selectivity of action [3]. Enzyme catalysis is an alternative to chemical methods for the regioselective hydroxylation of aromatic compounds.

The use of microorganisms or isolated enzymes as biocatalysts is a well-established strategy for the synthesis of biologically active natural compounds [4]. Most of the identified enzymes belong to the cytochrome P-450 monooxygenases, whose activity depends on a compatible reductase (CPR) [5]. The monooxygenases SbF6H (CYP82D1.1) from *Scutellaria baicalensis* [6] and GmF6H (CYP71D9) from *Glycine max* [7] expressed in eukaryotic cells demonstrated the ability to hydroxylate flavonoids at the C-6 position.

Figure 1. Scheme of (A) separated and (B) fused protein complex expression cassette.

Here, we present the results of work on the two P450s mentioned above. The eukaryotic proteins were co-produced with a compatible reductase or were generated as a P450-CPR fusion protein complex in *Escherichia coli* cells. Given the increasing interest in cytochrome P-450 monooxygenases and the typically low yields of reactions catalyzed by them, the development of an efficient enzymatic cascade based on recombinant fusion proteins could put targeted hydroxylation on a completely new and promising track.

Acknowledgments

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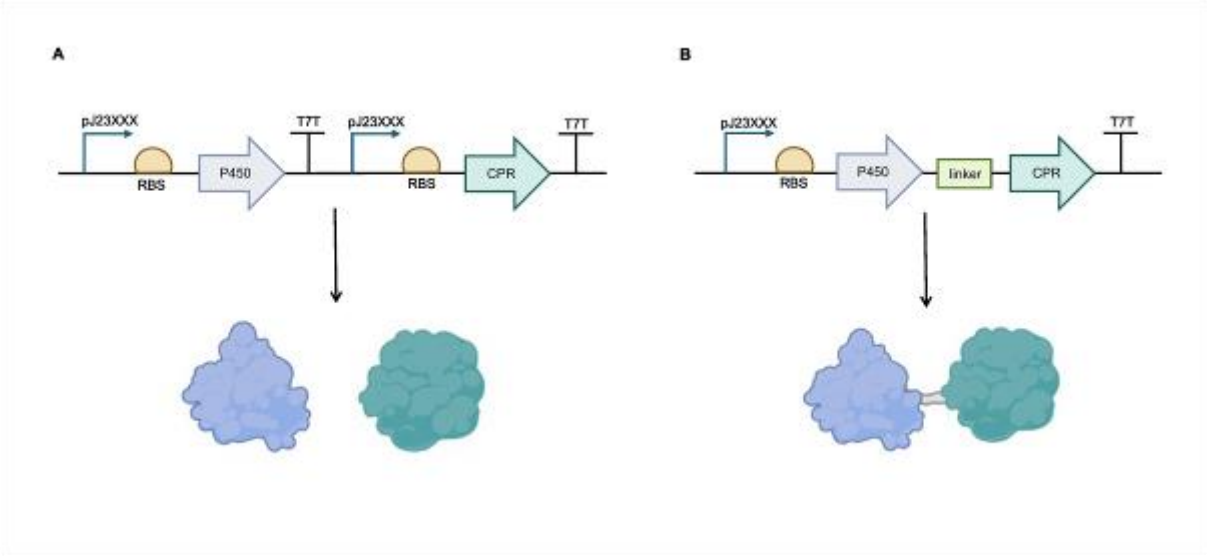
References:

1. Ren, W. et al. Medicinal Research Reviews 23, 519–534 (2003).
2. Heim, K. E. et al. The Journal of Nutritional Biochemistry 13, 572–584 (2002).
3. Massey, V. Journal of Biological Chemistry 22459–22462 (1994).
4. Chang, M. C. Y. et al. Nature Chemical Biology 3, 274–277 (2007).
5. Ziegler, D. M. Annual Review of Pharmacology and Toxicology 33, 179–199 (1993).
6. Zhao, Q. et al. Molecular Plant 11, 135–148 (2018).
7. Latunde-Dada, A. O. et al. Journal of Biological Chemistry 276, 1688–1695 (2001).

Fig. 1


A
pJ23XXX
RBS
P450
T7T
pJ23XXX
RBS
CPR
T7T
B
pJ23XXX
RBS
P450
linker
CPR
T7T

P2-57

Discovery of C-C phenol-coupling cytochromes P450 in *Crinum asiaticum*: a Machine Learning approach

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Plant metabolism has evolved a plethora of intricate pathways and specialized compounds to ensure survival and adaptation to diverse environmental conditions and actors, presenting promising potential outside of their natural roles. A notable example is the alkaloids of the Amaryllidaceae family, which encompass a range of biologically and pharmacologically active compounds, including Galantamine, the only FDA-approved natural product used in the treatment of Alzheimer's disease.

Central to amaryllidaceae alkaloid (AA) biosynthesis is the intramolecular C-C phenol coupling reaction of 4-O'-methylnorbelladine (4OMET), which yields three different alkaloid families with very distinct properties, depending on the stereoselectivity of the reaction. However, knowledge regarding AA biosynthesis downstream from the formation of 4OMET is scarce, hindering the determination of which characteristics of the CYP96T isoforms are able to assert stereoselectivity and determine the metabolomic profile of the plant.

This study aims to use a machine learning approach to identify putative cytochrome P450 genes within the transcriptome of *Crinum asiaticum*, capable of catalyzing an intramolecular C-C phenol coupling reaction over 4OMET. Subsequently, we seek to characterize the enzymatic activity and substrate specificity of the selected CYP450 isoforms to elucidate their probable role in AA biosynthesis.

Using a Support Vector Machine (SVM) algorithm, we identified 14 putative CYP450 transcripts in *C. asiaticum*, five of which were members of the CYP96 family, and nine of which spanned across the CYP74-92 families. Subsequent cloning and expression of selected transcripts enabled kinetic evaluation using microsomal protein fractions from *Nicotiana benthamiana* leaves. Preliminary kinetic assays with 4OMET and related substrates are underway to determine the kinetic parameters and substrate specificity of these enzymes.

Our findings highlight the potential of machine learning approaches in tackling the identification of enzymes involved in specialized metabolism. In addition, the characterization of the candidate CYP450 enzymes will shed light on the elusive molecular mechanisms underlying AA biosynthesis. This study not only contributes to expanding our knowledge of plant secondary metabolism but also underscores the utility of ML-based approaches for enzyme discovery and the prediction of enzyme promiscuity, instrumental aspects of rational metabolic engineering.

P2-58

Extension of PETase classes by newly identified alpha/beta hydrolases from the bacterial genus Halopseudomonas

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Introduction

Polyethylene terephthalate (PET) is one of the most abundant commercial plastic polyester. A number of enzymes have been identified that can depolymerize PET and potentially enable sustainable valorization of PET in a circular economy. Currently known PETases show high structure and sequence similarity to the canonical alpha/beta hydrolase fold and share highly similar substrate binding sites. Despite this similarity, discrepancies are observed in the number and position of disulfide bonds, the occurance of an extended loop flanking the active-site or the presence of specific amino acids at subsites. Based on these observations three classes of PETases have been defined, i.e. class I, IIa and IIb. Sequence-based searches have to be tuned for more diverse sequences if we want to identify novel PETases outside these classes.

Objective

In this work we aim to extent the sequence space of PETases by mining for novel enzymes in marine bacterial metagenomes using a profile hidden-Markov model (pHMM). We specifically selected also pHMM hits with lower bit-scores to diversify the test set.

Results

Target proteins were then produced by heterologous expression in *E. coli* and tested in agar-plate based screenings supplemented with PET nanoparticles. This led to the identification of three new PETase candidates. Hydrolytic activity of one candidate was further quantified in PET-coated well plate assays and with reverse-phase HPLC, which revealed a high salt tolerance. Further analysis showed that the three enzymes originate from metagenomes associated with bacteria in the genus of *Halopseudomonas*.

Conclusions

Further sequence analysis and comparison to *Is*PETase shows that the candidate PSW62-2 as well as some homologs are devoid of the typical pi-stacking clamp and have changes in the lipase-box motif. This group might be defined as the PETase class III. We believe that allowing for an increase in sequence diversity helps to find new PETases and engineer them to further biotechnological advances.

P2-59

Glucose oxidase converted into a C1 and C6 carbohydrate oxidase: Production of organic acids derived from glucose

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Recently, sugar oxidation has been tagged in many research due to the high quantity of application of oxidation products1, such as organic acid derived from monosaccharides. Organic acids derived from monosaccharides can be classified as aldonic (oxidation on C1), uronic (oxidation on C6), or aldaric (oxidation on C1 and C6) acid according to oxidized carbon on sugar molecules. One specific challenge to solve for producing organic acid from monosaccharides is the number of reactive groups present in sugar molecules, which can exhibit high reactivity under typical reaction conditions, resulting in several different products if the groups are not protected. This problem could be solved by an enzyme-assisted approach with suitable C1 and C6 carbohydrate oxidase. Y68W-GOx and F414K-GOx variants were previously reported for galacturonic acid oxidation[1], showing the differences in substrate specificity are due to substrate location on active site2. In this work, we evaluated several glucose oxidase variants for the oxidation process of glucose and its derived organic acid (table 1). The substrate specificity of variant Y68W variant was modified by a semi-rational design saturating the F414 amino acid. This approach resulted in modifying the catalytic promiscuity and the physicochemical properties of the final biocatalyst to produce four variants capable of catalyzing the oxidation of organic acids derived from glucose. The enzymatic performance of active variants was compared with wild-type GOx (Wt-GOx) and Y68W without mutation on the F414 position. The double modification allows obtaining variants capable of oxidizing glucose, gluconic acid, or glucuronic acid, achieving substrate conversion higher than 15% for C1 or C6 oxidation and 7-11% when the Y68W,F414C variant can oxidize on C1 and C6 position (table 1). The changes in substrate specificity of *A. niger*'s glucose oxidase could be explained by changes in the geometry and interaction potential in the active site of variants.

Acknowledgments the financial support from ANID Chile through FONDECYT 1220134

Reference

D. Concha, K. Rodríguez-Núñez, L. Castillo, R. Martínez, C. Bernal. Galactaric acid production by engineering substrate specificity in glucose oxidase from Aspergillus Niger Biochem. Eng. J., 187 (2022), Article 108646

Fig. 1

Table 1: Oxidation performance of GOx variants using glucose, gluconic acid or glucuronic acid as substrates

Variant	Oxidation position	Substrate Specificity	Substrate conversión (%)		
			Glucose	Gluconic acid	Glucuronic acid
Wt-GOx	C1	Glucose → Gluconic Acid	70	-	-
Y68W	C1	Galacturonic acid → galactaric acid	10	-	-
F414K	C1	Galacturonic acid → galactaric acid	5	-	-
Y68W, F414D	C6	Gluconic acid → Glucaric acid	3	15	-
Y68W, F414P	C1	Glucuronic acid → Glucaric acid	2	-	17
Y68W, F414C	C1, C6	Glucose → Gluconic acid Glucose → Glucuronic acid	56	7	11

The reactions were carried out by 6 hours, using potassium phosphate buffer 25mM pH 7.0 with substrate 10mM, in presence of catalase, at 30°C

P2-60

Manipulating Activity and Chemoselectivity of a Benzaldehyde Lyase for Efficient Synthesis of α-Hydroxymethyl Ketones and One-pot Enantio-complementary Conversion to 1,2-Diols

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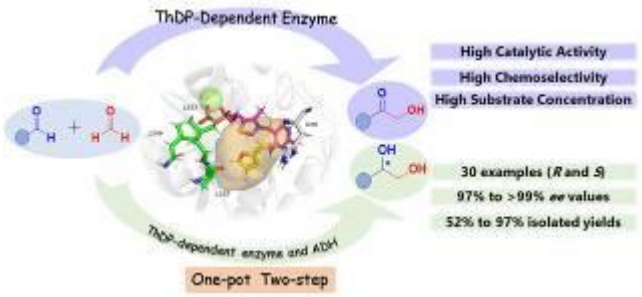
The α-hydroxymethyl ketone motif has been widely found in many pharmaceutically important molecules and used as a key intermediate for the synthesis of various fine chemicals and natural products. Hydroxymethylation of aldehydes with formaldehyde catalyzed by ThDP-dependent enzymes is considered a highly valuable approach in terms of atom-economics and sustainability, while low activity and chemoselectivity restricted its widespread application. In this study, a benzaldehyde lyase (BAL) with high activity and catalytic efficiency was acquired by enzyme screening and engineering, which catalyzed the hydroxymethylation of furfural at the concentration of 700 mM. Crystal structural analysis and molecular dynamics studies reveal that the activity and chemoselectivity of BAL was improved by reshaping the conformation of substrate entrance tunnel. Furthermore, a one-pot concurrent enzymatic process involving BAL-catalyzed hydroxymethylation of aldehydes and subsequent asymmetric reduction with carbonyl reductase was developed, offering a highly efficient way to produce chiral 1,2-diols from simple aldehydes, with excellent *ee* values (97-99%) in good to excellent yields (52-97%). The significantly improved activity and chemoselectivity demonstrated the potential of the engineered BAL in the industrial production of α-hydroxymethyl ketones, that could readily enable the synthesis of other high value-added chemicals from simple aldehydes and formaldehyde.

Fig. 1



P2-61

An engineered imine reductase for highly diastereo- and enantioselective synthesis of β-branched amines with contiguous stereocenters

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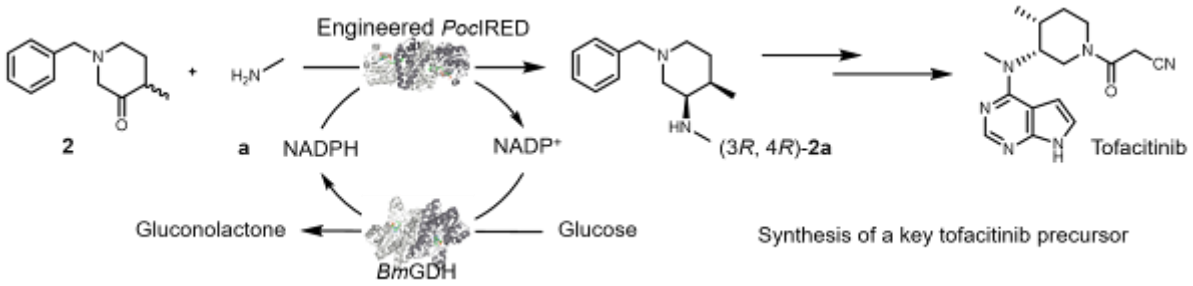
β-Branched chiral amines with contiguous stereocenters are valuable building blocks for preparing various biologically active molecules. However, their asymmetric synthesis remains challenging. Herein, we report a highly diastereo- and enantioselective biocatalytic approach for preparing a broad range of β-branched chiral amines starting from their corresponding racemic ketones. This involves a dynamic kinetic resolution-asymmetric reductive amination process catalyzed using only an imine reductase. Four rounds of protein engineering endowed wild-type *Poc*IRED with higher reactivity, better stereoselectivity, and a broader substrate scope. Using the engineered enzyme, various chiral amine products were synthesized with up to >99.9% *ee*, >99:1 *dr*, and >99% conversion. The practicability of the developed biocatalytic method was confirmed by producing a key intermediate of tofacitinib in 74% yield, >99.9% *ee*, and 98:2 *dr* at a challenging substrate loading of 110 g L^{-1}. Our study provides a highly capable imine reductase and a protocol for developing an efficient biocatalytic dynamic kinetic resolution-asymmetric reductive amination reaction system.

Fig. 1



P2-62

Reaction Mechanism and Regioselectivity of Uridine Diphosphate Glucosyltransferase RrUGT3: A Combined Experimental and Computational Study

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[2]Tianjin University of Science & Technology, College of Biotechnology, Tianjin, China
[3]Shandong University, State Key Laboratory of Microbial Technology, Qingdao, China
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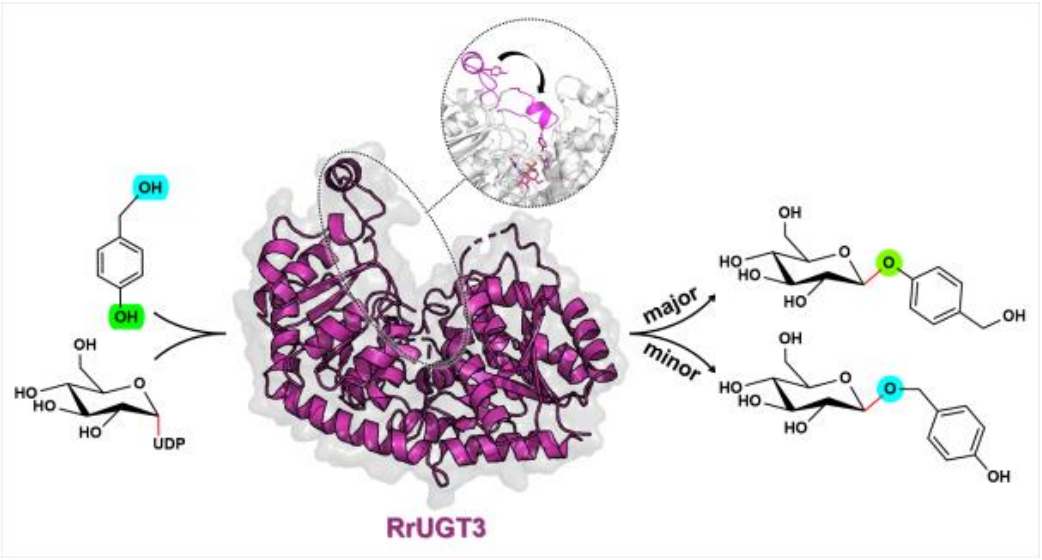
Question: The uridine diphosphate glucosyltransferase (UGT) from *Rhodiola rosea* (named as RrUGT3) shows high selectivity towards the phenolic hydroxyl group in the glucosylation of *p*-hydroxybenzyl alcohol (HBA). However, the detailed catalytic mechanism, including the substrate binding mechanism and the origins of the regioselectivity remain unclear.

Methods: In this work, we solved the crystal structure of the enzyme at 1.87Å resolution in space group *P*212121 with one molecule in the asymmetric unit forming one monomer (PDB ID code: 8YP7). Then, we performed the site-directed mutagenesis and the multi-scale computational studies which including the molecular dynamics, quantum mechanical/molecular mechanical (QM/MM) and quantum chemical (QC) calculations, to reveal the detailed reaction mechanism utilized by RrUGT3.

Results: Our molecular dynamics simulation results indicate that the active enzyme-substrates ternary complex can be formed only at the "closed" conformation of the RrUGT3, while the energy barrier of "open-to-closed" conformational change of the enzyme can be largely reduced by the binding of the substrates. The QM/MM and QC calculation results suggest that the protonation state of the pyrophosphate moiety of the uridine-5'-diphosphate-glucose (UDPG) significantly affects the catalytic activity of the enzyme, the single protonated pyrophosphate moiety of UDPG can lower the reaction energy barrier. Moreover, our calculations revealed that the enzyme environment can largely increases the p*K*a of the pyrophosphate moiety of UDPG by a preorganized binding pocket.

Conclusions: The conformational change of a loop region from the "open" to the "closed" state is crucial for the formation of the active enzyme-substrates ternary complex in RrUGT3, and the loop conformational change is favored by the substrate binding. The catalytic power of RrUGT3 is mainly originated from the preorganized protein environment that stabilizes the catalytically active state of the sugar donor uridine-5'-diphosphate-glucose (UDPG). The favored substrate binding and lower activation barrier of the phenolic glycosylation dictate the regiopreference of the enzyme.These results expand our knowledge on the catalytic mechanism of UGTs, and will facilitate the rational modification and design of highly active and regioselective UGT variants for the practical production of glycosides.

Fig. 1



P2-63

Determining the reaction mechanism of the flavin reductase ThdF from *S. albogriseolus*

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Two-component flavin dependent monooxygenases are of great interest as biocatalysts for the production of pharmaceutical and chemically relevant molecules, as they catalyze chemically important reactions such as hydroxylation, epoxidation and halogenation. The monooxygenase components are complemented by a flavin reductase, which provides the necessary reduced flavin cofactor. Among the flavin-dependent halogenases, the tryptophan halogenase Thal from *S. albogriseolus* is a well-characterized member that exhibits some limitations in terms of halogenation efficiency, which are also caused by unproductive enzyme-substrate complexes of Thal with reduced FAD in the absence of tryptophan. Since the reductase components have an important regulatory function for the activity and efficiency of the monooxygenase by controlling the supply of reduced flavin, we investigated the so far uncharacterized flavin reductase ThdF from the same gene cluster in *S. albogriseolus*, which possibly cooperates with Thal.

A crystal structure of ThdF in complex with FAD raised questions about the mechanism of flavin reduction. For this reason, steady-state enzyme kinetics with both substrates, FAD and NADH, and different inhibitors were performed to determine the mechanism. ThdF turned out to catalyze a sequentially ordered mechanism with FAD as the leading substrate that binds first and is released last after reduction (**Figure 1**). These results provide first insights into the interplay of the Thal-ThdF system and how the efficiency of Thal could be controlled by flavin reduction at the reductase ThdF.

Fig. 1

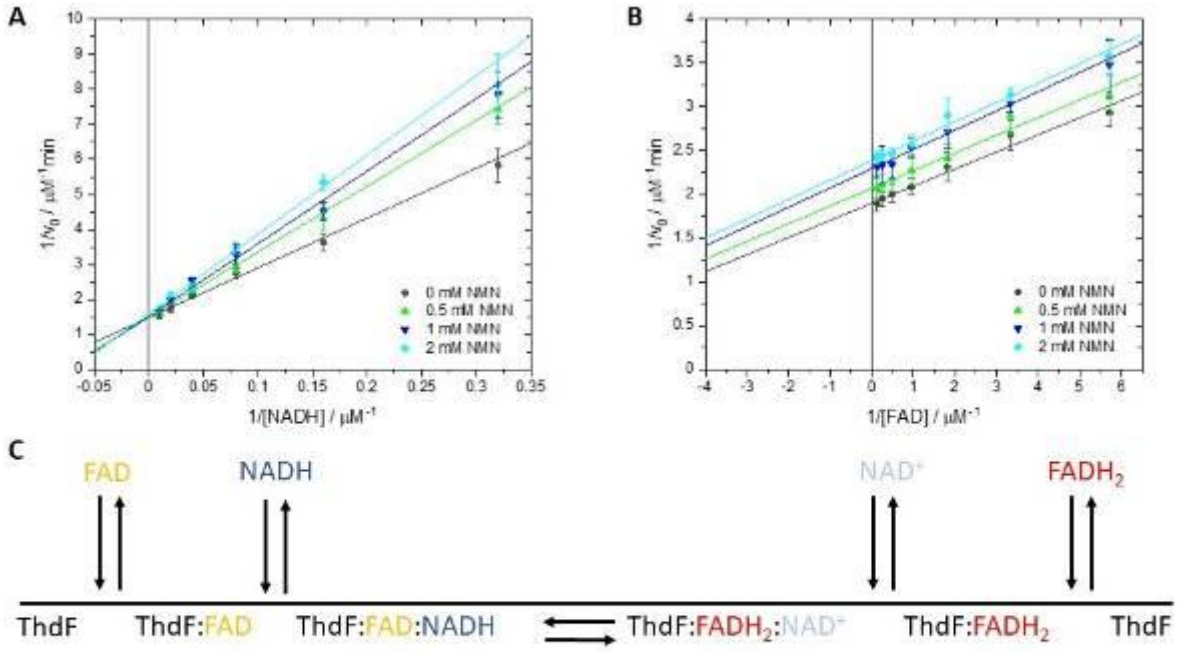

Figure 1: Steady-state initial velocity kinetics of ThdF with nicotinamide mononucleotide (NMN) as inhibitor. (**A**) NADH-dependent measurement at 1 µM FAD for different NMN concentrations, shown as a double reciprocal plot of initial velocity versus NADH concentration. The lines intersecting on the ordinate indicate competitive inhibition of NADH by NMN. (**B**) FAD-dependent measurement at 80 µM NADH for different NMN concentrations, shown as double reciprocal plot of initial velocity versus FAD concentration. The parallel pattern of the lines indicates uncompetitive inhibition of FAD by NMN, confirming FAD being the first substrate bound. (**C**) Reaction scheme of the ordered sequential mechanism of ThdF determined by steady state enzyme kinetics.

P2-64

Engineering of dimeric forms of the cysteine peptidase xylellain

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Oligomeric proteins make up almost half of the proteins in nature[1]. The abundance of oligomeric proteins can be explained by their functional and structural advantages over the monomeric variants, such as improved resistance to denaturation and degradation and refined control over the regulation of enzymatic activity.

Our research focuses on papain-like cysteine peptidases, primarily monomeric enzymes, with the aim of developing a method to convert them into stable homodimers. By integrating bioinformatics and protein engineering techniques, we aim to create recombinant dimeric enzymes with improved regulatory mechanisms and kinetic properties compared to natural enzymes.

Our approach involves engineering dimeric peptidases by optimizing protein surfaces to generate compatible interactions in terms of geometry and charge. Using site-specific saturation libraries and an in-house developed screening system, we have identified 25 potential homodimers of the bacterial papain-like cysteine protease xylellain. The mutants exhibit different amino acid mutations at the interface. Mutations expected to contribute to homodimerization were prepared in-silico, and the potential structures of the symmetric dimers were calculated using protein-protein docking. Experimental verification with purified recombinant proteins is ongoing.

In summary, our research highlights the utility of bioinformatics-based protein engineering in modulating enzyme activity and stability through oligomerization. Using a multidisciplinary approach that includes bioinformatic prediction and protein engineering techniques, we seek to find new ways to improve the utility of enzymes for various biotechnological applications.

[1] E. D. Levy, S. Teichmann. Structural, evolutionary, and assembly principles of protein oligomerization, Prog. Mol. Biol. Transl. Sci., 2013, 117, 25–51.

P2-65

Unveiling New Frontiers in Plastic Biodegradation: Novel Enzymatic Pathways for PET and Beyond

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The accumulation of plastic waste poses severe environmental and health risks globally. Conventional mechanical and chemical recycling methods fall short in efficiency and ecological impact, highlighting an urgent need for innovative solutions. This study introduces groundbreaking approaches to identify and utilize novel enzymes capable of degrading robust plastic polymers efficiently. Employing metagenomic analysis and high-throughput screening, we identified unique enzymes from diverse environmental samples that not only depolymerize PET but show promising activity towards other challenging polymers such as PA. Our investigation includes detailed enzymatic mechanism elucidation through structural biology and kinetic analysis, which guide the enzyme engineering phase to enhance plastic degradation under industrial conditions. Preliminary results demonstrate significant decomposition of plastic samples within controlled lab environments, suggesting potential for scalable biotechnological application. This research not only broadens the scope of enzymatic plastic degradation but also paves the way for more sustainable waste management practices and reduction of plastic pollution.

P2-66

Computational Identification of Nerve Agent Binding Proteins

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Organophosphorus (OP) compounds have been developed as pesticides and insecticides, yet, they have been posing a serious threat as chemical warfare agents due to their deadly effects on humans. These compounds, called nerve agents, inhibit acetylcholinesterase (AChE) by forming a covalent bond with the serine residue in the active site [1]. This leads to overstimulation of the nervous system, followed by respiratory failure and death. Increasing security, health and environmental concerns urge the development of green catalysts that will bind nerve agents stronger than AChE and break them down through an environmentally friendly decomposition way.

To develop green biocatalytic approaches for the decontamination of nerve agents it"s important to determine alternative proteins that can be phosphonylated by nerve agents. In this work [2], quantum mechanical active site models, called theozymes, were used to generate catalytic atom maps, which were screened in protein databases to identify proteins that can promiscuously bind nerve agents. Identification of nerve agent binding proteins provides new protein candidates into which hydrolysis activity can further be reengineered.

[1] Aroniadou-Anderjaska V., Apland J. P., Figueiredo T. H., Furtado M. D. A., Braga M. F., 2020, "Acetylcholinesterase inhibitors (nerve agents) as weapons of mass destruction? History, mechanisms of action, and medical countermeasures", Neuropharmacology, 181, 208298.

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P2-67

Discovery and engineering of biocatalysts for the synthesis of bioactive *C*-glycosides

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Natural product *C*-glycosyltransferases (*C*-GTs) are UDP-dependent enzyme biocatalysts capable of glycosylating small molecules such as flavonoids via a *C*-glycosidic linkage. The resulting glucosides display various health-promoting effects as well as outstanding chemical and biological stability [1,2]. Interestingly, most of the glycosylated natural products are formed via an *O*-glycosidic bond by *O*-GTs and only a small number of molecules are known to be *C*-glycosylated both *in vivo* and *in vitro*. Besides the rare occurrence and limited substrate scope, *C*-GTs show low catalytic rates. To pave a way for the industrial application of *C*-GT biocatalysts, enzyme discovery and structural studies are needed.

Our study aims to broaden the current understanding of *C*-GT biocatalysts by discovery of new enzymes, investigating their substrate preferences and identifying key determinants for substrate- and regio-specificities [3]. Besides that, our goal is to engineer the *C*-glycosylation specificity in selected enzymes using aloesone as a model substrate. The 8-*C*-glucoside of aloesone, called aloesin, is an important cosmetic compound which currently can only be obtained from *Aloe vera* in extremely low quantities since there is no efficient and more sustainable synthetic route established until now [4]. By using rational/semi-rational protein engineering techniques we aim to evolve a *C*-GT biocatalyst for the efficient production of aloesin.

[1] G. Tegl and B. Nidetzky, Biochem. Soc. Trans., 2020, 1583–1598.

[2] N. Putkaradze, D. Teze, F. Fredslund and D. H. Welner, Nat. Prod. Rep., 2021, 432–443.

[3] N. Putkaradze, V. Della Gala, D. Vaitkus, D. Teze and D. H. Welner, Biotechnol. J., 2023, 1–10.

[4] D. Yang, W. D. Jang and S.Y. Lee, JACS, 2021, 5364–5377.

P2-68

Engineering 2-Deoxy-d-ribose-5-phosphate Aldolase for *anti*- and *syn*-Selective Epoxidations of α,β-Unsaturated Aldehydes

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The enzyme 2-deoxy-D-ribose-5-phosphate aldolase (DERA) naturally catalyzes the reversible aldol addition between acetaldehyde and D-glyceraldehyde-3-phosphate to yield 2-deoxy-D-ribose-5-phosphate. Herein we describe the redesign of DERA into a proficient non-natural peroxygenase that promotes the asymmetric epoxidation of various *α,β*-unsaturated aldehydes. After performing only 5 rounds of directed evolution, the archetypical class I aldolase DERA was engineered into an efficient non-natural cofactor-independent peroxygenase. This repurposed aldolase, named DERA-EP, is able to utilize H2O2 to accomplish both *anti*- and *syn*-selective epoxidations of various α,β-unsaturated aldehydes to give the corresponding epoxides with good diastereoselectivity (d.r. up to 99:1) and excellent enantioselectivity (e.r. up to 99:1). Crystallographic analysis of DERA-EP in a substrate-free and substrate-bound state provides a structural context for the evolved activity, a clear explanation for the high enantioselectivity, and compelling evidence for catalysis via enzyme-bound iminium ion intermediates. The unprecedented *anti*-selectivity of DERA-EP with several α,β-unsaturated aldehydes is complementary to the *syn*-selectivity of previously reported enzyme-, metal- and organo-catalysts, which makes DERA-EP an attractive new asset to the toolbox of epoxidation catalysts.

P2-69

Construction of enzyme variants for optimised activity of AaeUPO-PaDaI towards aliphatic substrates

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Alkanes are inert to most chemical transformations due to the absence of functional groups. Traditionally, the main application for alkanes is the combustion and therefore the transformation of the chemical energy into thermal energy. Global climate change requires innovative approaches like alternative value generating production chains, for example direct use of alkanes as chemical starting materials. An example for this chemical use of alkanes is the synthesis of alcohols through hydroxylation. Alcohols can then be used as solvents or as intermediates for further synthesis of complex chemicals. Biocatalysts, such as unspecific peroxygenases (UPOs), offer a sustainable alternative to chemical catalysts for this hydroxylation, operating under mild conditions and using hydrogen peroxide as a co-factor. One of the best characterized UPOs was found in the fungus *Agrocybe aegerita* (AaeUPO).
This work aims to optimize the substrate affinity of the *Aae*UPO-PaDa-I mutant towards alkanes with short chain lengths. The *Aae*UPO-PaDa-I mutant is already optimized for expression in *Pichia pastoris*. *Aae*UPO can catalyse hydroxylation reactions with both aliphatic and aromatic substrates but shows a preference for aromatic compounds. This selectivity is hypothesized to be substantiated on the amino acids which are located in the reaction pocket of the enzyme. Part of these amino acids are aromatic. Especially the phenylalanines F69, F121 and F199 are of great interest. These amino acids are responsible for the positioning of the substrates in the reaction pocket. Phenylalanines are aromatic amino acids which could contribute to the preference towards aromatic substrates over aliphatic ones.
The hypothesis posits that substituting these three phenylalanines with aliphatic amino acids (alanine, glycine, isoleucine, leucine, and valine) will enhance the enzyme's affinity for aliphatic substrates. To test this, 15 *Aae*UPO-PaDa-I enzyme variants, each with a single amino acid substitution, were expressed in Pichia pastoris using a yeast surface display, allowing direct immobilization of the enzymes on the yeast cell membrane. The peroxidase and peroxygenase activity of these variants were detected. In addition, docking studies were performed to simulate protein-ligand dockings with four different substrates (NBD, cyclohexane, hexane and ethylbenzene) and calculate substrate affinity constants.

P2-70

EvoEnzyme in the Horizon Europe program: Engineering robust enzymes for healthcare monitoring, sustainable energy, green chemistry and plastic degradation

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WOUNDSENS (HORIZON-EIC2022-PATHFINDERCHALLENGE; https://woundsensproject.eu)

The main goal of WOUNDSENS project is to lead the development of a novel generation of smart bandage biosensors for early detection of infected non-healing wounds. EvoEnzyme is coordinating this project in which our specific objective is to engineer robust enzymes that resist the electrospinning aimed at integrating them as a detection biocatalyst inside conductive hollow fibers.

CirculH2 (HORIZON-CL6-2023-CIRCBIO; https://www.circulh2.com/) and W-BioCat (HORIZON-EIC-2023-PATHFINDEROPEN; https://www.wbiocat.com/)

Rapid transition toward the use of renewable, energy-efficient and recyclable resource is needed in industrial biotechnology to achieve sustainable production of chemicals. Efficient, scalable, selective and robust catalysts are needed to deploy hydrogen as a clean, circular and renewable reactant in industrial biotechnology. The aim of CirculH2 is to demonstrate the successful development of one or more highly robust and scalable hydrogenases for use of hydrogen that selectively drives biotransformations of bio-based materials to specialty and commodity chemicals in an industrial environment. Within that scope, EvoEnzyme is involved in engineering various hydrogenases in terms of stability and overall performance. Closely related to CirculH2, EvoEnzyme is part of W-BioCat consortium where the focus is put on engineering tungsten containing enzymes which catalyse challenging chemical reactions that are currently impossible to obtain economically and on scale to match industrial needs.

BLADE2CIRC (HORIZON-CL5-2023-D3)

BLADE2CIRC is exploring bio-based approaches to improve the sustainability of the wind energy sector. To this end, the project is developing bio-based materials to develop new biobased thermoset composites. Within BLADESCIRC, EvoEnzyme is participating in the engineering of new enzymes for degradation of this next generation of composites.

P2-71

Allozymes' microfluidic platform opens new perspectives for enzyme engineering.

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Allozymes is a cutting-edge biotechnology company specializing in the development and production of high-quality enzymes for various industries. Allozymes proprietary microfluidics technology is primarily designed around ultrahigh throughput screening. To engineer enzymes, we combine single site saturation mutagenesis followed by combinatorial libraries and screen millions of variants. Our in vitro strategy is supported by an in silico platform fed by a huge amount of data generating robust and reliable models. 2 recent success stories are presented including the engineering of an unspecific peroxygenase in Pichia pastoris for pharmaceutical application and a kinase, identified as a bottleneck enzyme in a metabolic pathway.

P2-72

Engineering of interfaces for screening of oxyfunctionalization targets catalyzed by heterogenized unspecific peroxygenases (UPOs)

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Unspecific peroxygenase (UPO; EC 1.11.2.1) represents a relatively new type of heme-thiolate enzyme with self-sufficient mono(per)oxygenase activity and has become a promising candidate for the oxyfunctionalization of a wide variety of substrates [1, 2]. However, the operational stability of UPO has been a limiting factor that prevents its applications from being used in technical setups. So far, the highest turnover number (TON) value was reported to be 909,000 in a biphasic system using an immobilized UPO [3, 4] in a rotating bed reactor [5]. We intend to cater to this challenge by site-specific immobilization of the new UPOs and novel variants [6, 7] to enhance stability, maximize re-usability, and allow continuous processing [4, 8, 9]. To achieve this, new UPO variants will be identified via data mining, and the crucial residues will be mutated to enhance the protein stability. The enzyme will be immobilized on polymer material (polystyrene, polypropylene) surfaces via Strep-tag and anchor peptides, designed via a structure-guided approach. Finally, the immobilization procedure will be verified for screening different enzyme variants and substrates using an in-house 3D-printed microfluidics system [10]. This approach aims to achieve a trailer-made and robust screening interface for oxyfunctionalization of novel substrates using UPO as a model system with multifaceted applications.

References:1. Hobisch, M., et al., Recent developments in the use of peroxygenases - Exploring their high potential in selective oxyfunctionalisations. Biotechnol Adv, 2021. 51: p. 107615.2. Beltran-Nogal, A., et al., Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases. Curr Opin Struct Biol, 2022. 73: p. 102342.3. De Santis, P., et al., A holistic carrier-bound immobilization approach for unspecific peroxygenase. Front Chem, 2022. 10: p. 985997.4. Meyer, L.-E., et al., A 3D printable synthetic hydrogel as an immobilization matrix for continuous synthesis with fungal peroxygenases. Reaction Chemistry & Engineering, 2023. 8(5): p. 984-988.5. Hobisch, M., et al., Peroxygenase-Driven Ethylbenzene Hydroxylation in a Rotating Bed Reactor. Organic Process Research & Development, 2022. 26(9): p. 2761-2765.6. Münch, J., et al., Functionally Diverse Peroxygenases by AlphaFold2, Design, and Signal Peptide Shuffling. ACS Catalysis, 2024: p. 4738-4748.7. Münch, J., et al., Computational-Aided Engineering of a Selective Unspecific Peroxygenase toward Enantiodivergent β-Ionone Hydroxylation. ACS Catalysis, 2023. 13(13): p. 8963-8972.8. Meyer, L.E., et al., Continuous oxyfunctionalizations catalyzed by unspecific peroxygenase. Catalysis Science & Technology, 2022. 12(21): p. 6473-6485.9. De Santis, P., L.-E. Meyer, and S. Kara, The rise of continuous flow biocatalysis – fundamentals, very recent developments and future perspectives. Reaction Chemistry & Engineering, 2020. 5(12): p. 2155-2184.10. Gkantzou, E., M. Weinhart, and S. Kara, 3D printing for flow biocatalysis. RSC Sustainability, 2023. 1(7): p. 1672-1685.

IL3-1

Biocatalysis @ scale

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Biotechnology can provide innovative and efficient solutions to complex problems. Specifically, biocatalytic conversions can be a complementary tool to produce fine and bulk chemicals in often very enantioselective manner. In white biotech research at BASF, we explore various multi-step, hybrid chemo-enzymatic routes by combinatory process development. In the presentation, a typical development workflow for a biocatalytic transformation is shown.

To define a technical solution for a target product, the whole pipeline is addressed: from biocatalyst production, formulation and recycling to downstream processing concepts. As one of the first steps, enzyme screenings and variant nomination are performed to determine biocatalyst activity and stability, which is accompanied by diverse parameter screenings to characterize suitable process conditions further. In this regard, model-assisted design-of-experiments (DoE) is a powerful tool for lab-scale process development. Key steps in this early project phase are initial cost estimations and definition of key performance indicators such as titer, specific yield and product specifications after downstream processing. Here, "starting with the end in mind" really becomes a daily mantra.

IL3-2

Self-assembled nanoparticle-enzyme clusters and substrate channeling – a developing system for *de novo* biosynthesis

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To combat the scourge of organic waste and other ills associated with traditional industrial chemical synthesis a new paradigm has emerged called Synthetic Biology. Here, scientists are trying to understand and harness how Nature performs 1000"s of different types of chemical reactions at the same time in a single cell with just a few begin starting materials such as salt, sugar, water, and light. There are many different subsets of Synthetic Biology such as engineering microbes or the use of cell free extracts to produce a desired product.[1] Each of these have their benefits but also liabilities such as toxicity issues and off target synthesis of undesired products. Our efforts have focused on using enzymatic cascades, theoretically from a variety of sources, in a plug-n-play fashion to synthesize a desired product. [2-4] What makes our approach unique is incorporating a nanoscaffold to bind different enzymes, bringing them in close proximity to each other, stabilizing their tertiary structure and enabling the formed nanoclusters to undergo "channeling".[5-8] Channeling occurs when the product of one enzyme is in close proximity of the next enzyme and is effectively "handed off" before product/substrate has a chance to diffuse away, increasing the overall flux. Channeling is the most efficient form of multienzyme catalysis. Our preferred prototypical nanoparticles (NP) are semiconductor quantum dots (QDs) due to their small size.[1,5,7] Enzymes that have been produced containing an N-terminal polyhistidine tail can be assembled onto the QD"s surface in a ratiometric fashion through metal-affinity coordination between the imidazole side chains of the histidine residues and the Zn^{+2} rich surface of the QD.[7] These self-assembled nanosystems allow for a high concentration of localized enzyme to access channeling phenomena.

There are, however, a lot of unknowns with how to develop multienzymatic nanoclusters for effective biochemical synthesis. Besides determining which enzymes are needed to achieve the synthesis of a desired product much research needs to be performed to address such questions as the optimal ratio of each enzyme present, optimal number of enzymes per NP, the order of assembly and other factors that influence the overall flux. To address these questions, we utilized the seven enzymes associated with oxidative glycolysis to convert the agricultural feedstock glucose to 3-phosphoglycerate. This system is tractable with the ability to monitor NADH formation over time as well as each enzyme in the pathway can be individually characterized through absorbance or fluorescence based protocols.[5] The apparent Michaelis-Menten kinetic characteristics of each enzyme free in solution or attached to a NP were determined and utilized to simulate the ratio of each enzyme to QD to achieve the maximum flux in nanoclusters. These ratios were tested and after two rounds of optimization a set of ratios were determined for the seven enzyme system. The NP shape, size, and concentration were also characterized to determine their influence on enzymatic nanocluster formation. Gel electrophoresis assays and TEM images were also performed/obtained to confirm the assembly of nanoclusters (**Figure 1**). By increasing the number of enzymatic nanoclusters formed while optimizing the ratio of individual enzymes in a cascade, we achieved an 800-fold increase in the 7-enzyme flux compared to enzymes free in solution (**Figure 2A**). The degree of enzyme packing or enzyme proximity that can be achieved as a result of NPs size and shape can also double the amount of NADH produced compared to when attached to QDs (**Figure 2B**). Insights from these experiments with the seven enzymes associated with glycolysis are now being applied to other systems to make useful products that cannot be achieved by cell-based synthetic systems. Our overall goal is to learn to utilize these nanomaterial assisted minimalist approaches to synthesize useful industrial and specialty chemistry products, which are currently produced by chemical plants, using agricultural feedstocks.[3,6,8] We will discuss how we are applying what was learned from the initial glucose studies to other systems.

Figure 1 A) Mobility assay of one enzyme assembled to a QD at increasing ratios. B) 7 enzymes sequentially attached to QD. Decreases in the shift of the QD indicated increased protein assembly. C) TEM analysis of enzyme attachment to QDs or planar nanoplatelets indicating the influence of QD size and shape on cluster formation.

Figure 2. (A) Increase in NADH formation when attached to a QD (red line) vs free in solution (blue line) followed by even more NADH formation when optimizing the ratio of each enzyme based on individual kinetics. (B) The influence of the nanoparticle shape and size on overall NADH formation due to larger enzyme nanocluster formation.

References:

1. Thakur, M., Breger, J.C., Susumu, K., Oh, E., Spangler, J.R., Medintz, I.L., Walper, S.A., Ellis, G.A. Self-assembled nanoparticle-enzyme aggregates enhance functional protein production in pure transcription-translation systems. *PLOS ONE* 17(3): e0265274. (2022).

2. Breger, J.C., Goldman, E.R., Susumu, K., Oh, E., Green, C.M., Hooe, S.L., Thakur, M., Medintz, I.L., Ellis, G.A. Enzyme Assembly on Nanoparticle Scaffolds Enhances Cofactor Recycling and Improves Coupled Reaction Kinetics. *Nanoscale* 15, 10159-10175 (2023).
3. Hooe, S., Breger, J., Dean, S., Susumu, K., Oh, E., Walper, S., Ellis, G.E., Medintz, I.L. Benzaldehyde Lyase Kinetic Improvements, Potential Channeling to Alcohol Dehydrogenase, and Substrate Scope when Immobilized on Semiconductor Quantum Dots. *ACS Applied Nano Materials* 5, 10900-10911 (2022).
4. Díaz, S.A., Choo, P., Oh, E., Susumu, K., Klein, W.P., Walper, S.A., Hastman, D.A., Odom, T.W., Medintz, I.L. Gold Nanoparticle Templating Increases the Catalytic Rate of an Amylase, Maltase, and Glucokinase Multienzyme Cascade through Substrate Channeling Independent of Surface Curvature. *ACS Catalysis* 11,627-638 (2021).
5. Breger, J.C., Vranish, J.N., Oh, E., Stewart, M.H., Susumu, K., Lasarte-Aragones, G., Ellis, G.A., Walper, S.A., Diaz, S.A., Hooe, S.L., Klein, W.P., Thakur, M., Ancona, M.G., Medintz, I.L. Self assembling Nanoparticle Enzyme Clusters Provide Access to Substrate Channeling in Multienzymatic Cascades. *Nature Communications* 14, 1757 (2023).
6. Hooe, S., Ellis, G.A., Medintz, I.L. Alternative Design Strategies to Help Build the Enzymatic Retrosynthesis Toolbox. *RSC Chem Bio* 3, 1301-1313 (2022).
7. Breger, J.C., Ellis, G.A., Walper, S.A., Susumu, K., Medintz, I.L. Implementing Multi-Enzyme Biocatalytic Systems Using Nanoparticle Scaffolds. *Methods in Molecular Biology* 2487:227-262 (2022). Special Issue: Multienzymatic Assemblies, Haris Stamatis Editor.
8. Ellis, G.A., Díaz, S.A., Medintz, I.L. Enhancing Enzymatic Performance with Nanoparticle Immobilization: Improved Analytical and Control Capability for Synthetic Biochemistry. *Current Opinion in Biotechnology* 71, 77-90 (2021).

Fig. 1

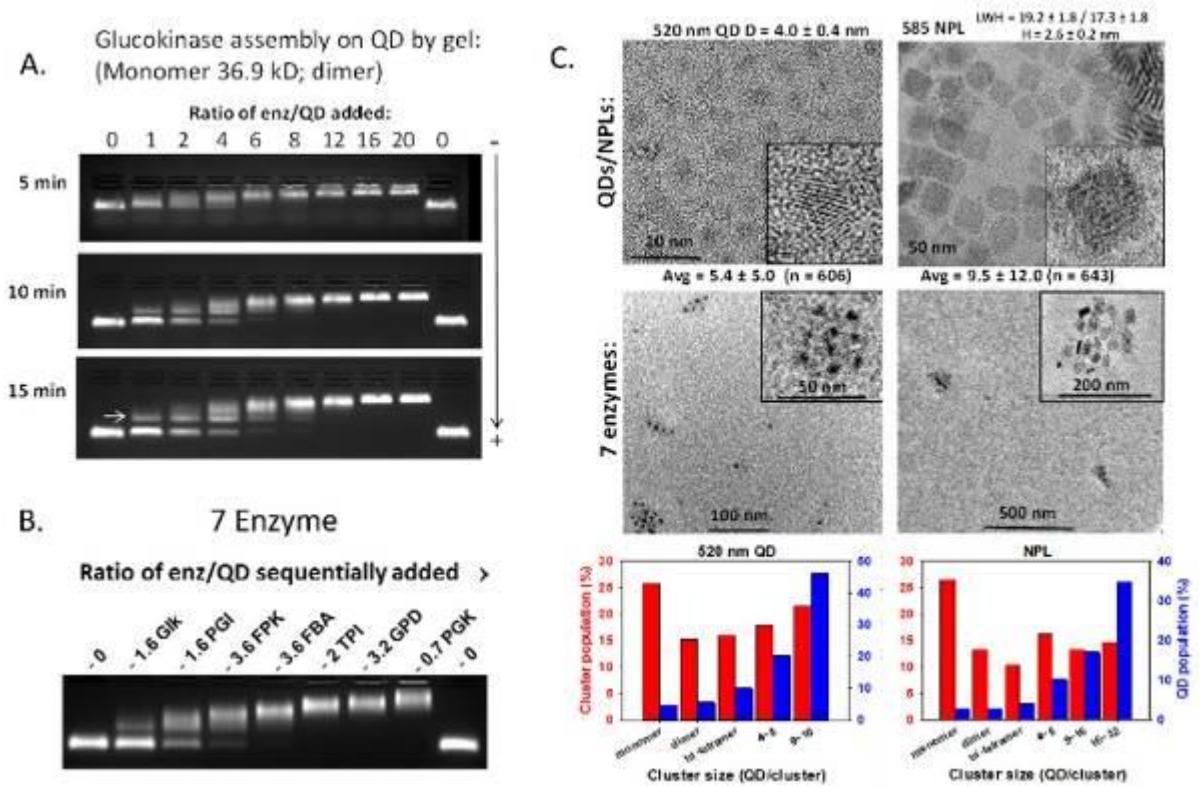
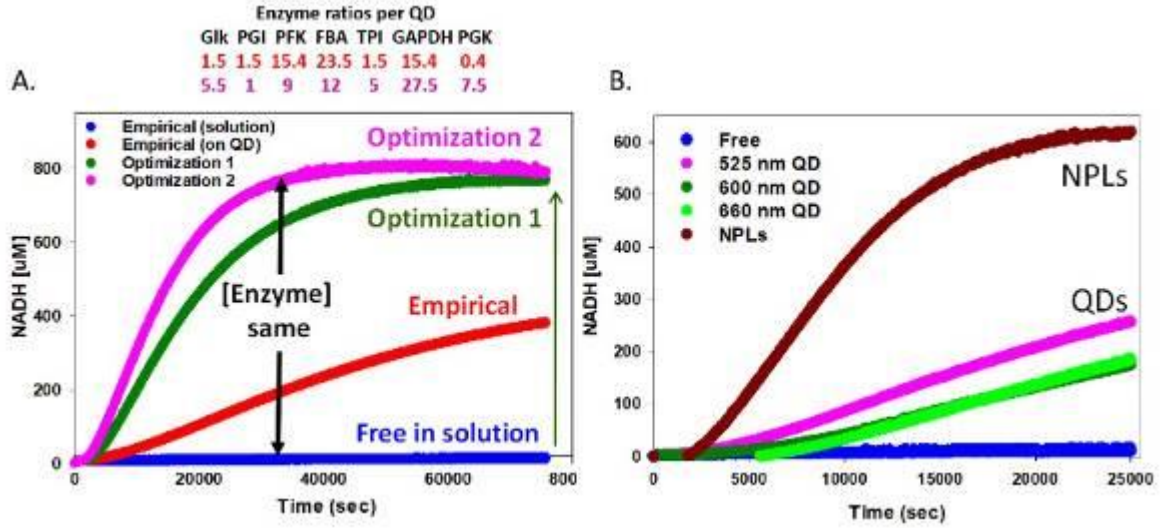


Fig. 2


A.
Enzyme ratios per QD
Glk PGI PFK FBA TPI GAPDH PGK
1.5 1.5 15.4 23.5 1.5 15.4 0.4
5.5 1 9 12 5 27.5 7.5
Empirical (solution)
Empirical (on QD)
Optimization 1
Optimization 2
Optimization 2
Optimization 1
Empirical
[Enzyme] same
Free in solution
NADH [uM]
Time (sec)
B.
Free
525 nm QD
600 nm QD
660 nm QD
NPLs
NPLs
QDs
NADH [uM]
Time (sec)

IL3-3

Engineering aliphatic halogenases

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Late-stage functionalization of complex molecular scaffolds offers an elegant route to create novel biologically active entities. In discovery chemistry, inactivated C-H bonds are regarded as particularly promising, if challenging, points of diversification as they allow to create new analogs without resorting to *de novo* synthesis. As a prerequisite for this approach, however, the reaction procedures must be compatible with already existing functional groups in the lead structural scaffold – a task still challenging most chemical methodologies. In this context, Fe(II)/α-ketoglutarate dependent dioxygenases, enzymes which are capable of halogenating and hydroxylating sp^3 carbons with high stereo- and regiocontrol under benign conditions, have attracted increasing attention. This enzyme family"s reported substrate scope, however, is often limited to natural substrates and their close analogues. By employing a combination of smart library design and machine learning assisted directed evolution, we engineered several Fe(II)/α-ketoglutarate dependent dioxygenases for the late-stage functionalization of molecules of pharmaceutical and agrochemical interest, ranging from non-natural amino acids to terpenes and bulky macrolides, hitherto not accepted substrates.[1-6] Notably, our enzyme engineering approach allowed us to rapidly identify more active enzyme variants increasing the apparent k_{cat} and the turnover number of the enzymes by orders of magnitude. Scaling up the biocatalytic reactions allowed the targeted analysis of the derivatized small molecule"s structure-function activity in biological assays.

1. Hegarty, E.; Büchler, J. and Buller, R., Halogenases for the Synthesis of Small Molecules, *Curr Opin Green Sustain Chem* 2023, 41: 100784.
2. Voss, M.; Hüppi, S.; Schaub, D.; Hayashi, T.; Ligibel, M.; Sager, E.; Schroer, K.; Snajdrova, R. and Buller, R. Enzyme Engineering enables inversion of substrate stereopreference of the halogenase WelO5*, *ChemCatChem* 2022, 14, e202201115.
3. Büchler, J.; Malca, S. H.; Patsch, D.; Voss, M.; Turner, N. J.; Bornscheuer, U. T.; Allemann, O.; Le Chapelain, C.; Lumbroso, A.; Loiseleur, O.; Buller, R. *Nat Commun* **2022,** *13* (1), 371.
4. Meyer, F.; Frey, R.; Ligibel, M.; Sager, E.; Schroer, K.; Snajdrova, R.; Buller, R. *ACS Catal* **2021,** *11* (10), 6261-6269.
5. Papadopoulou, A.; Meierhofer, J.; Meyer, F.; Hayashi, T.; Schneider, S.; Sager, E.; Buller, R. *Chemcatchem* **2021,** *13* (18), 3914-3919.
6. Hayashi, T.; Ligibel, M.; Sager, E.; Voss, M.; Hunziker, J.; Schroer, K.; Snajdrova, R.; Buller, R. *Angew Chem Int Ed* **2019**, *58*, 18535 - 18539.

L3-1

Holistic Understanding of Alcohol Dehydrogenase Catalysis in Deep Eutectic Solvents

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Biocatalysis has been shifting from traditional aqueous to non-aqueous media in the spirit of *Green Chemistry*. The use of oxidoreductases (EC1) in non-conventional media plays an indispensable role in the synthesis of a variety of value-added chiral compounds.[1a] Deep eutectic solvents (DESs) represent a new class of greener solvents that are highly tunable.[1b] Redox biocatalysis in DESs combines the two dominant assets of selectivity of enzymes and versatility of DESs.[1c, d] The rational design of redox biocatalysis in DESs requires a comprehensive knowledge of the effects of DESs on enzymes. Exemplarily, the impact of DESs on oxidoreductases has been holistically studied by assessing the catalytic performance of alcohol dehydrogenases (ADHs) in DES-water mixtures (e.g., choline chloride-glycerol, ChCl-Gly, 1:2) with the aid of experimental analysis and molecular dynamics (MD) simulations.[2a, b] It was revealed that enzyme activity is positively related to water activity due to solvation changes surrounding enzymes. In addition, the individual DES components showed discrepant effects, e.g., positive (in case of Gly) or negative (in case of ChCl), promoting the generation of an enzyme-compatible eutectic mixture by increasing the Gly fraction (ChCl-Gly, 1:9).[2c] Based on that, the Gly-based DESs with non-ChCl as HBAs (choline acetate and betaine), namely ChAc-Gly (1:2) and Bet-Gly (1:2), are envisioned to be more enzyme-friendly (**Fig. 1**).

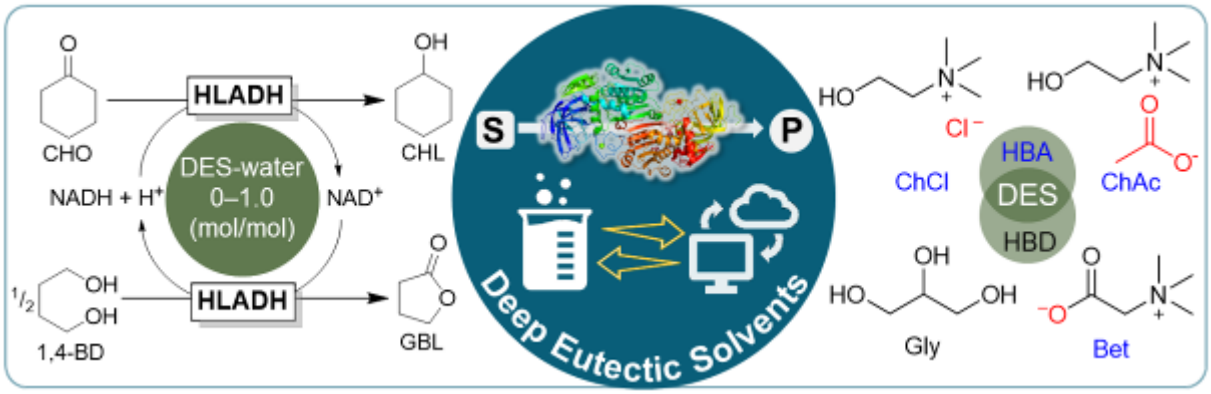
Expectedly, the selected enzyme, horse liver ADH (HLADH), exhibited highly improved stability in both DESs, especially in Bet-Gly. Further studies on different molar ratios and individual components of DESs were conducted to fully elucidate the relationship between the studied DESs and enzymes.

Fig. 1. Analysis of horse liver alcohol dehydrogenase in glycerol-based deep eutectic solvents containing various water content (0–100 vol.%) by combining experiments and simulations.

[1] a) M. van Schie, J. D. Sporing, M. Bocola, P. Domínguez de María, D. Rother, *Green Chem.* **2021**, *23*, 3191–3206. b) M. Pätzold, S. Siebenhaller, S. Kara, A. Liese, C. Syldatk, D. Holtmann, *Trends Biotechnol.* **2019**, *37*, 943–959. c) V. Gotor-Fernández, C. E. Paul, *J. Biotechnol.* **2019**, *293*, 24–35. d) N. Zhang, P. Domínguez de María, S. Kara, *Catalysts* 2024, *14*, 84.

[2] a) L. Huang, J.P. Bittner, P. Domínguez de María, S. Jakobtorweihen, S. Kara, *ChemBioChem* **2018**, *21*, 811–817; b) J. P. Bittner, I. Smirnova, S. Jakobtorweihen, *Molecules* **2024**, *29*, 703. c) J. P. Bittner, N. Zhang, L. Huang, P. Domínguez de María, S. Jakobtorweihen, S. Kara, *Green Chem*. **2022**, *24*, 1120–1131.

Fig. 1



L3-2

Photoenzymatic Asymmetric Hydroamination for Chiral Alkyl Amine Synthesis

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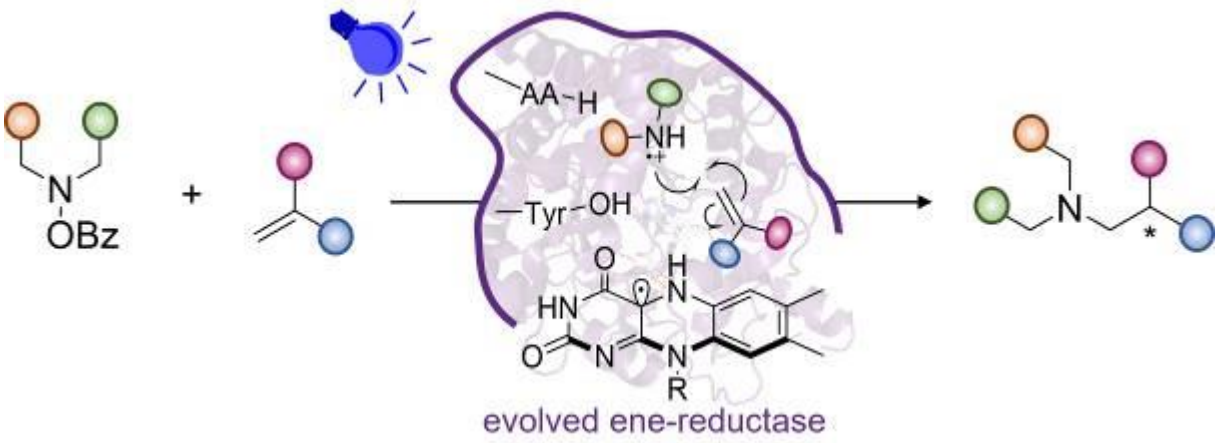
Chiral alkyl amines are common structural motifs in pharmaceuticals, natural products, synthetic intermediates, and bioactive molecules. An attractive method to prepare these molecules is the asymmetric radical hydroamination; however, this approach has not been explored with dialkyl amine-derived nitrogen-centered radicals since designing a catalytic system to generate the aminium radical cation, to suppress deleterious side reactions such as α-deprotonation and H-atom abstraction, and to facilitate enantioselective hydrogen atom transfer is a formidable task. Herein we describe the application of photoenzymatic catalysis to generate and harness the aminium radical cation for the asymmetric intermolecular hydroamination. In this reaction, the flavin-dependent ene-reductase photocatalytically generates the aminium radical cation from the corresponding hydroxylamine and catalyzes the asymmetric intermolecular hydroamination to furnish the enantioenriched tertiary amine, whereby enantioinduction occurs through enzyme-mediated hydrogen atom transfer. This work highlights the use of photoenzymatic catalysis to generate and control highly reactive radical intermediates for asymmetric synthesis, addressing a longstanding challenge in chemical synthesis.

Fig. 1



L3-3

Discovery and Application of Anthranilate-Glycosylating Enzymes, Utilising a UGT for Gram-Scale Production of Methyl Anthranilate Glucoside for Biological Studies

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Methyl anthranilate (MANT) is an ester-derivative of anthranilic acid (ANT) and is commonly used as a flavouring agent and in perfumery and cosmetics due to its distinct scent and taste (Luo et al., 2019) and as a bird and insect repellent (Avery et al., 1995). However, MANT is currently produced unsustainably using large volumes of acid catalysts while its precursor, ANT, is produced from a petroleum-derived precursor (Luo et al., 2019; Wiklund and Bergman, 2006). The cytotoxicity of MANT inhibits microbial production (Luo et al., 2019) while its volatility and poor water solubility limits its applications. Other ester-derivatives of anthranilic acid, such as butyl anthranilate, exhibit similar activities as MANT but are affected by an even lower water solubility.

In nature, compounds exhibiting similar adverse properties as the ester-derivatives of ANT are commonly solved through a glycosylation step catalysed by UDP-dependent glycosyltransferases (UGTs) (De Bruyn et al., 2015). Hence, we aimed to discover, characterise, and apply UGTs capable of glycosylating a panel of ANT ester-derivatives. This yielded the discovery of three UGTs which were able to efficiently catalyse the glycosylation of this class of compounds. Kinetic characterisation revealed UGT16 to be the most efficient in the glycosylation of MANT and was subsequently kinetically characterised with the other ANT ester-derivatives in the panel. This led to the discovery of a relationship between the increasing length of the alkyl chain of the ester-derivative and the catalytic efficiency. Investigating this property through molecular docking and site-directed mutagenesis led to the elucidation of the binding mode of ANT ester-derivatives in UGT16 along with the elucidation of two residues vital for activity. In addition, UGT16 exhibited extreme tolerance to high concentrations of MANT along with DMSO which facilitated the gram-scale production of 99 % pure (HPLC) MANT-glucose purified via flash chromatography at a 74 % yield. The purified MANT-glucose was subsequently used for *in vivo* studies and for testing its biological activity.

In conclusion, the first reported UGTs capable of glycosylating ester-derivates of ANT were discovered. The binding mode and important residues for activity were uncovered through mutagenesis and the chemo tolerance of UGT16 enabled the gram-scale production of MANT-glucose which, in return, enabled *in vivo* and biological studies of the purified compound.

Figure 1: The conversion of MANT to MANT-glucose via a UGT.

References

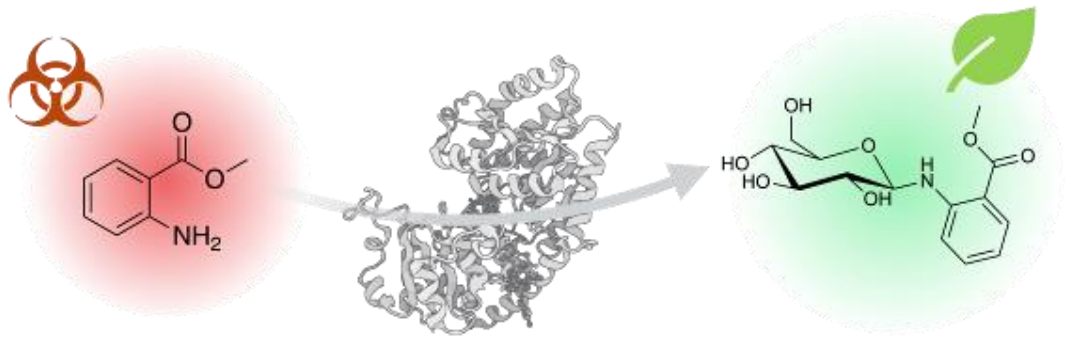
Avery, M.L., Decker, D.G., Humphrey, J.S., Aronov, E., Linscombe, S.D., Way, M.O., 1995. J Wildl Manage 59, 50.

De Bruyn, F., Maertens, J., Beauprez, J., Soetaert, W., De Mey, M., 2015. Biotechnol Adv 33, 288–302.

Luo, Z.W., Cho, J.S., Lee, S.Y., 2019. Proceedings of the National Academy of Sciences 116, 10749–10756.

Wiklund, P., Bergman, J., 2006. Curr Org Synth 3, 379–402.

Fig. 1



L3-4

A platform approach to manufacturing single stranded oligonucleotides by enzymatic assembly

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Traditionally, oligonucleotides have been synthesised using phosphoramidite chemistry on a solid support [1]. Whilst this approach has proven highly successful, issues persist around the scalability of the approach and the solvent usage across both the synthesis and subsequent chromatographic purification. GSK has developed a templated oligonucleotide assembly platform that takes advantage of engineered DNA ligases to make single stranded oligonucleotides [2]. The process eliminates the need for chromatography yet produces oligonucleotides with purity that exceeds that typically seen for solid supported synthesis. Data on application of this platform to different oligonucleotide types and progress on scale up will be presented.

[1] Sanghvi, Y. S. (2019). Large-scale automated synthesis of therapeutic oligonucleotides: A status update. In S. Agarwal & M. J. Gait (Eds.) Advances in nucleic acid therapeutics pp. 453–473

[2] WO2018011067A2

L3-5

Imine synthesis in gram scale by variant of D-amino acid oxidase

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Purpose: We created *S*-amine oxidases by modifying the structure of porcine kidney-derived FAD-dependent D-amino acid oxidase (pkDAO), and synthesized chiral (*S*)-α-methylbenzylamine (*R*-MBA) and (*R*)-4-Cl-benzhydrylamine, through deracemization reactions. α-Alkylamino acids were also synthesized by oxidative nucleophilic cyanation reactions. The reaction mechanism by which the amine captures highly reactive intermediate imine was elucidated. In this study, we investigated the catalytic ability of the variants pKDAO and synthesized various symmetric and asymmetric imines based on the reaction mechanism.

Purified variants pkDAO (Y228L/R283G) and pkDAO (I230A/R283G) were used under optimal conditions of pH 9.0 and 20°C, and the synthesis of various imines was confirmed by HLPC and GC-MS. Various substituted aromatic amines were used as substrates, and aliphatic primary amines were also used in the reaction.

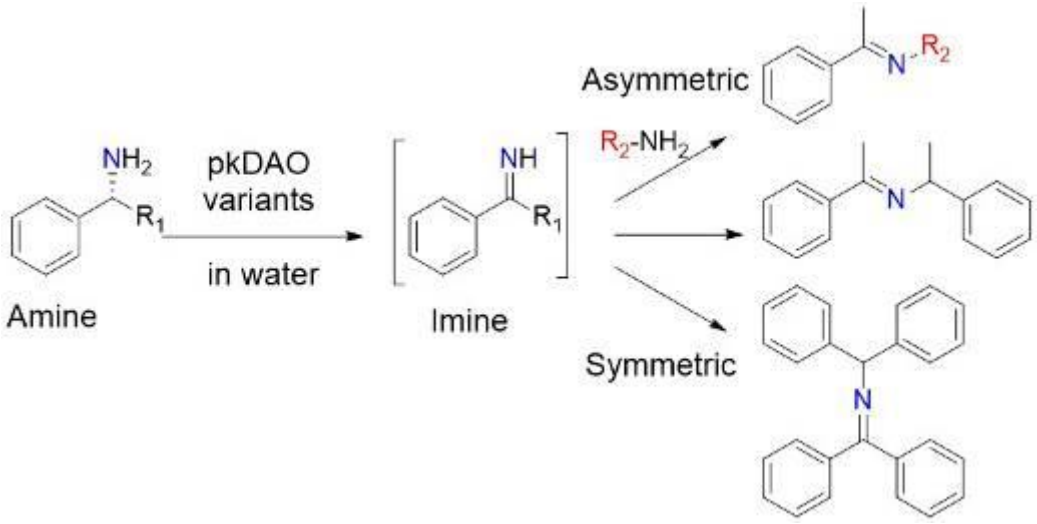
Results and Discussion

In the dimerization reaction, aromatic amines with electron-withdrawing substituents at the para-position were good substrates, and most of them could be synthesized in large quantities on the gram scale. Symmetric and asymmetric imines were synthesized using R-MBA as a substrate in the presence of n = 2-7 aliphatic amines, cyclopropylamine, or hexamethylenediamine, on which R-MBA does not act.

Thus, the relatively stable conjugated imine intermediate produced by the amine oxidase derived from D-amino acid oxidase can be used to synthesize imines without the use of special catalysts.

1. K. Yasukawa et al, *The Enzymes*, **47**, 117-136 (2020).
2. N. Kawahara et al, *Catalysts*, **12**, 511 (2022).
3. W. Khanghachit et al, in preparation.

Fig. 1



L3-6

Hybrid Technology as a Solution for Biomanufacturing Challenges

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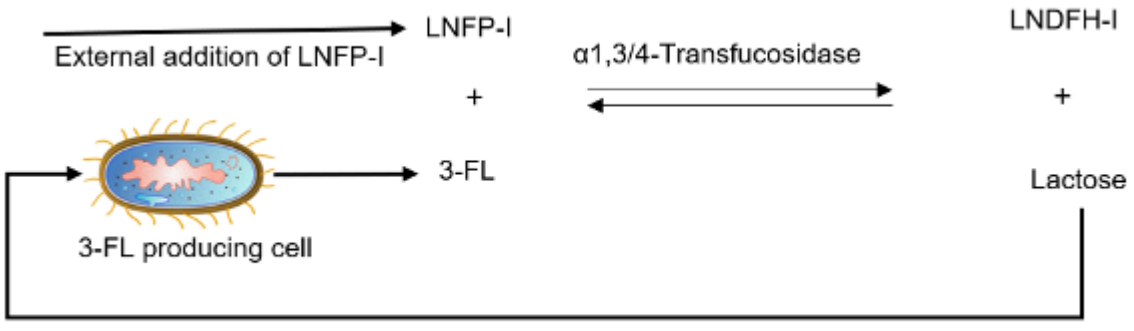
Major inherent limitations of enzyme catalyzed (*in vitro*) and whole-cell catalyzed (*in vivo*) biotransformation processes are the inability to change the reaction equilibrium position and ineffective product transportation out of cells, respectively. While *in-situ* product removal techniques can be used to shift a reaction equilibrium of an *in vitro* process it relies on substantial differences in chemo-physical properties between the substrate(s) and product(s), which is often not the case. Product export out of cells in an *in vivo* process can be achieved using transmembrane transporters. However, it is not trivial to find suitable transporters capable of exporting big and complex products. Moreover, they need to be very specific and not also export precursor molecules, which typically contain the same molecular structures recognized by the transporter binding sites. A novel technology that solves these inherent limitations was developed by integrating *in vitro* and *in vivo* approaches into a hybrid process. The hybrid technology was proven to be efficient for the biomanufacturing of several different complex human milk oligosaccharides (HMOs). Results from the hybrid production of a neutral lacto-N-difucohexaose I (LNDFH-I) and a charged sialyllacto-N-tetraose (LST-a) HMOs are presented in this abstract. Figure 1 shows a schematic illustration of the hybrid process for the production of LNDFH-I.

Figure 1: A schematic overview of hybrid process technology illustrating the production of LNDFH-I in a one-pot reaction by combining in vivo formation of 3-fucosyllactose (3-FL) and in vitro transfucosylation of lacto-N-fucopentaose I (LNFP-I) with in-situ recycling of the side-product lactose.

An *in vitro* synthesis of LNDFH-I led to 43% conversion of LNFP-I and producing a mixture at equilibrium consisting of 15% 3-FL,17% lactose, 35% LNFP-I and 33% LNDFH-I (wt./wt.). In comparison a hybrid process, which combined the *in vivo* 3-FL formation from lactose by fermentation and *ex vivo* transfucosylation of externally added LNFP-I into LNDFH-I achieved 99% conversion of LNFP-I and a final mixture consisting of 37% 3-FL, 0.7% lactose, 0.8% LNFP-I and 61% LNDFH-I (wt./wt.). The high conversion in the hybrid process could be achieved as the enzymatic reaction equilibrium was circumvented by *in-situ* recycling of the side-product lactose into the fucosyl donor 3-FL.

An *in vitro* LST-a process catalyzed by α-2,3-transsialidase using 3"-sialyllactose (3"-SL) as sialyl donor and lacto-N-tetraose (LNT) as acceptor achieved 57% conversion of 3"-SL and a mixture at equilibrium consisting of 18% lactose, 21% 3"-SL, 18% LNT and 43% LST-a (wt./wt.). A corresponding hybrid process using α-2,3-transsialidase combining the *in vivo* production of LNT from lactose with the enzymatic transsialylation of the formed LNT using externally supplied 3"-SL, achieved full conversion of the 3"-SL and a final mixture of 35% LNT and 65% LST-a (wt./wt.), with no residual lactose or 3"-SL.

Fig. 1



L3-7

Enzymatic synthesis of novel oligosaccharides from raffinose oligosaccharides

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[2]Technical University of Denmark, Department of Biotechnology and Biomedicine, Kongens Lyngby, Denmark
[3]Norwegian Institute of Doof, Fishery and Aquaculture Research, Ås, Norway

Introduction Pulses, such as peas, are commonly used as a base for plant protein concentrates and isolates which are a crucial ingredient in meat-alternative foods. However, they also contain a large amount of raffinose oligosaccharides (RFOs) that can reduce the protein percentage in protein concentrates and contribute to gut discomfort for some consumers. RFOs are classified as fermentable oligo-, di- and monosaccharides and polyols (FODMAPs), which can result in flatulence and gut-related symptoms.[1] To mitigate the discomfort of FODMAP, it is beneficial to make less fermentable carbohydrates or enhance their specificity for beneficial gut bacteria, as was previously demonstrated with RFOs and alternansucrases.[2] A similar approach is routinely performed in the production of human milk oligosaccharides (HMO) from lactose via enzymatic synthesis.[3]

Objectives In this study, RFOs were isolated in kilogram scale from pea and faba bean protein concentrates. The RFOs were further treated with invertase to obtain melibiose-based oligosaccharides that were used as building blocks in transglycosylation reactions with either α-galactosidase[4] or a potent β-galactosidase from HMO production[2] in the presence of lactose.

Results Novel oligosaccharides (tri-octamers) were obtained with a combination of alpha and beta-linked galactoses. It could be shown that the addition of melibiose to transglycosylation reactions with lactose and β-galactosidase improved not only the conversion of lactose, but also the observable rate of tranglycosylation compared to the hydrolysis reaction. Furthermore, low enzyme loadings (≤0.5 µM) and short reaction times (≤60 min) benefited transglycosylation.

Conclusion The hypothesized transglycosylations could be shown and the combination of alpha and beta-linked oligos in the product mixture was hypothesized to reduce fermentability in microbes commonly found in the upper intestinal tract, which in general have relatively simple carbohydrate degrading machineries. Upscaling onto a liter scale membrane reactor system is planned as a next step in order to produce sufficient amounts to investigate the fermentability behavior of the novel oligosaccharides and to demonstrate the reactions industrially suitability. Detailed compositional analysis will also be performed.

[1] Nyyssölä et al., *Enzymatic reduction of galactooligosaccharides content of faba bean and yellow pea ingredients and food products*, 2021, https://doi.org/10.1016/j.fufo.2021.100047

[2] Hernandez-Hernandez et al., *In vitro fermentation of alternansucrase raffinose-derived oligosaccharides by human gut bacteria*, 2011, https://doi.org/10.1021/jf202466s

[3] Zeuner et al., *Thermostable ß-galactosidases for the synthesis of human milk oligosaccharides*, 2016, https://doi.org/10.1016/j.nbt.2016.01.003

[4] La Rosa et al., *The human gut firmicute Roseburia intestinalis is a primary degrader of dietary beta-mannans*, 2019, https://doi.org/10.1038/s41467-019-08812-y

L3-8

Bio-oxidation of cellobiose using engineered *Gluconobacter oxydans* yields highly concentrated cellobionic acid

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Introduction

Cellobionic acid, a sugar-acid, can be produced by selectively oxidizing cellobiose, which is the building block of cellulose and widely accessible as a renewable source. Classified among aldobionic acids, cellobionic acid serves as a plant-derived vegan substitute for its stereoisomer lactobionic acid, which is commonly used in pharmaceuticals, cosmetics, and food manufacturing.

Objective

Acetic acid bacteria, like *Gluconobacter oxydans*, are established whole-cell biocatalysts for specific oxidations due to membrane-bound dehydrogenases. This method bypasses intracellular enzyme use, improving cell-specific conversion rates by avoiding transmembrane transport of substrates and products. To boost cellobiose oxidation rates in *G. oxydans*, the membrane-bound glucose dehydrogenase from *Pseudomonas taetrolens* was overexpressed in *G. oxydans* BP9, a multi-deletion strain lacking nine native dehydrogenases.

Results and conclusion

Biotransformation experiments were conducted using engineered *G. oxydans* resting cells in lab-scale stirred- tank bioreactors containing phosphate buffer (pH 6, 30°C, DO > 30% air saturation). Negligible substrate and product inhibitions were observed up to concentrations of 320 g L^{-1}. Modelling and simulation were utilized to optimize the bio-oxidation process. Increasing the initial cell density to 5.2 g L^{-1} dry cell mass in a simple batch process yielded final cellobionic acid concentrations exceeding 500 g L^{-1} with a product yield of >99% (mol/mol) within four days. Complete cellobiose conversion in a simple phosphate buffer enables efficient cellobionic acid isolation via crystallization or ethanol precipitation after post cell separation. Scalability of this bio-oxidation batch process was ensured by limiting the maximum oxygen transfer rate to 50 mM h^{-1} in the lab-scale studies. Scale-up of this whole-cell biotransformation process with engineered *G. oxydans* is presently under study, including the isolation of cellobionic acid.

References

Bieringer E, Garcia Vazquez U, Klein L, Bravo NM, Tobler M, Weuster-Botz D (2023): Bioproduction and applications of aldobionic acids with a focus on maltobionic acid and cellobionic acid. *Bioproc Biosyst Eng* **46**: 921–940.

Peters B, Mientus M, Kostner D, Junker A, Liebl W, Ehrenreich A (2013): Characterization of membrane-bound dehydrogenases from *Gluconobacter oxydans* 621H via whole-cell activity assays using multideletion strains. *Appl Microbiol Biotechnol* **97**: 6397–6412.

Achnowledgements

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L3-9

Asymmetric whole-cell bioreduction of (*R*)-carvone with elimination of host-mediated side reactivity

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The use of ene reductases for the asymmetric C=C bond reduction of (*R*)-carvone has been shown to be a powerful route for the synthesis of (2*R*,5*R*)-dihydrocarvone (DHC), an industrially applied building block[1]. High diastereomeric excesses (de) >99 % can be achieved with purified enzymes[2]. However, even for the recently developed NostocER1$_{\Delta 1\text{-}37}$L1,2a protein accepting the cheaper NADH instead of NADPH as a cofactor, the application as an *Escherichia coli* (*E. coli*) whole cell biocatalyst with internal cofactor recycling seemed to be the most economical way. While a promising cell-specific productivity of 2.8 mM (g_{CDW} h)$^{-1}$ was reported, the de dropped to 95.4%[1]. Besides an inevitable solvent-derived isomerization rate of -0.27 de% h^{-1} in aqueous systems, *E. coli* cells were reported to exhibit undesired isomerization activity towards the product leading to lowered final de[3].

Consequently, the aim of this work was to first investigate the molecular reason of this cell-mediated isomerization in order to purposefully develop biocatalytic routes leading to higher optical purity in a subsequent step. An activity (isomerization rate) guided fractionation process of *E. coli* host cells lysate was applied. The first step, dialysis, located the activity of interest in the regime of >3 kDa, hence no small molecule. Add-on treatment with fractionated ammonium sulphate precipitation gave strong hints that the compound of interest might be a protein. Anion- and cation exchange as well as size exclusion chromatography and clear native page reduced the sample complexity to the point that 18 remaining proteins could be identified by MALDI-TOF mass spectrometry. Accordingly, these candidates were cloned, expressed and analysed for their DHC isomerization activity. Unambiguously, YgiN could be identified to be the protein showing undesired isomerization activity. As the enzyme is non-essential, in the following a genomic ygiN-knockout cell line based on *E. coli* BW25113 with plasmid-based heterologous expression capabilities of NostocER1$_{\Delta 1\text{-}37}$L1,2a and a formate dehydrogenase was developed. This was employed in a high-cell density fed-batch process in order to prepare enough biocatalyst for the subsequent preparative biotransformation of 300 mmol (*R*)-carvone in a 1 L stirred tank reactor. In spite of a lower maximal cell-specific productivity of 1.95 mM (g_{CDW}h)$^{-1}$ a to date unmatched de of 99.12 % at 99.87 % conversion was achieved. The preparative extraction of the product revealed 35.28 g of product equivalent to 77.3% total yield.

To conclude, the identification of YgiN as the host cell component responsible for the undesired isomerization of (2*R*,5*R*)-DHC enabled the development of a biocatalyst capable of generating so far unmatched product qualities in whole cell biotransformations.

[1] C. Mähler; *Molecules*, **2019**, *24*, 2550.

[2] C. Mähler; *Advanced Synthesis&Catalysis*, **2019**, 361, 2505-2513

[3] K. Castiglione; *Biochemical Engineering Journal*, **2017**, 117, 102-111.

L3-10

Convergent biocatalytic mediated synthesis of siRNA

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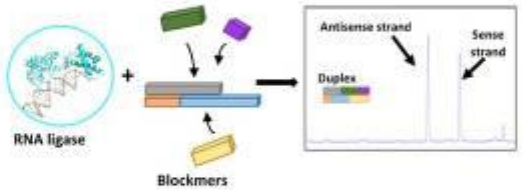
The application of biocatalysis in pharmaceutical development continues to grow with the strong technology base being applied to develop enzymatic methods for oligonucleotide synthesis resulting in improved yields and purity profiles compared to traditional methods. Biocatalytic approaches are alleviating the pressures on existing solid phase capacity and resulting in more convergent syntheses.

Herein we report a convergent biocatalytic synthesis strategy for an Alnylam model siRNA. The siRNA chemical structure includes several of the unnatural modifications and conjugations typical of siRNA drug substances. Using Almac"s 3-2-3-2 hybrid RNA ligase enzyme strategy that sequentially ligates short oligonucleotide fragments (blockmers), the target siRNA was produced to high purity at 1 mM concentration. Additional strategies were investigated including the use of polynucleotide kinase phosphorylation and the use of crude blockmer starting materials without chromatographic purification[1]. These findings highlight a path towards a convergent synthesis of siRNAs for large scale manufacture marrying both enzymatic liquid and classical solid phase synthesis.

1) Stephanie Paul, Darren Gray, Jill Caswell, Joshua Brooks, Wenjie Ye, Thomas S. Moody, Roumen Radinov, and Lubomir Nechev *ACS Chemical Biology***Article ASAP** DOI:10.1021/acschembio.3c00071

Fig. 1



L3-11

Extensive mapping of IRED performance reveals untapped potential for reductive amination at equimolar substrate concentrations

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Introduction

Asymmetric reductive amination catalysed by imine reductases (IREDs) and reductive aminases (RedAms) has quickly progressed from a proof of concept on laboratory scale to industrial implementation for the synthesis of pharmaceuticals. However, the general scope and limitations of this biocatalytic reaction are still insufficiently understood, as systematic screenings covering a representative diversity across all three dimensions of the reaction space (amine and carbonyl structure, and enzyme sequence) are lacking.

Objectives

We set out to fill this gap by evaluating the reductive amination performance of 175 IREDs against structurally diverse panels of 36 carbonyl and 24 amine substrates. To tackle this immense screening effort, we implemented an iterative screening strategy and leveraged machine learning for performance predictions based on the collected data. Throughout the workflow, we supplied the two coupling partners in equimolar concentrations (50 mM each), as our objective was to identify enzymes that catalyse reductive amination without the need for excess amine.

Results

Our results reveal a significantly larger potential for biocatalytic reductive amination at equimolar substrate concentrations than previous studies had indicated. In a first screening of all 175 IREDs against 54 substrate combinations, 15% of reactions were positive and 67% of the possible products could be formed by at least one enzyme. When the 100 best-performing IREDs were screened against a broader set of 185 target reactions, 27% were positives and again nearly two-thirds of the products (65%) could be accessed; among them, many were formed with high conversion and enantiomeric excess. The most proficient IREDs combined acceptance of ≥40% of the tested substrate pairs with specific activities of >10 U/mg for the most favourable combination. However, the screening also revealed generally challenging substrate motifs: the reductive amination of aromatic ketones with aniline derivatives systematically failed, requiring the development of an alternative strategy for accessing the corresponding amine products.

Conclusion

We have extensively mapped reductive amination performance across the IRED sequence space and have identified enzymes that combine broad scope with high activity and stereoselectivity. Moreover, we have developed an alternative strategy for preparing amines that are inaccessible by direct IRED-catalysed reductive amination.

L3-12

Ene reductase catalysed biosynthesis of delta-decalactone at high substrate concentration and high yield

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Delta-lactones are potent aroma compounds in food and are also used in many perfumery products. The individual lactones have tonalities from milky, creamy, fatty to sweet/fruity. Delta-decalactone can be obtained by the reduction of the double bond in α,β-unsaturated delta-decalactone (massoia lactone). The currently routinely used metal catalysts are often not environmentally friendly and face increasing costs. Here, we report the enzymatic reduction of C10 massoia lactone by ene reductases (EREDs) to obtain delta-decalactone. Ene reductases catalyse the biocatalytic reduction of activated alkenes such as enones, enals and enoates. They can be divided in different families and subgroups. During an extensive screening it was observed that ERED activity can be found in various families and subgroups. Since EREDs are NAD(P)H-dependent, a suitable cofactor regeneration system was identified as well.

After optimisation of the reaction conditions, the ERED catalysed reduction of C10 massoia lactone was performed at 50 g/L substrate concentration, achieving a yield of >90%.

P3-1

Non-Conventional Biocatalysis Strategies for Propyl Oleate Synthesis through the halophilic Lipase/Esterase *LipN* in *n*-Propanol Surfactantless Microemulsion and AOT/H_2O/Isooctane Reverse Micelles systems

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Lipases (E.C. 3.1.1.3) are versatile enzymes performing, hydrolysis, acidolysis, aminolysis, esterification, and transesterification, hence essential tools for biocatalysis [1]. Industrial environments encounter extreme conditions, thus extreme lipases such as those from the haloarchaeon *Natronococcus* sp. TC6, maintaining activity above 2M NaCl[2,3], are interesting operational stable candidates for industrial biocatalysis. This study provides insights into Surfactantless Microemulsions (SLM) and Reverse Micelles (RM) that mimic the physiological environment of halophile biocatalysts, as they can contain high NaCl concentrations. We hypothesize that these non-conventional strategies may preserve the active halophilic lipase conformation for their use as biocatalysts [4,5]. Here, we describe the use of *Natronococcus* sp. TC6 halophilic lipase/esterase *LipN* under RM and SLM in the presence of NaCl, as non-conventional strategies that may favor its native and active structure. *LipN* gene was cloned and expressed as inclusion bodies (IBS) in *Escherichia coli* BL21 using the vector pET28a and refolded using 8 M urea and further suspension in 0.8M L proline. The refolded IBS were employed to form RM and SLM in the presence of 2M NaCl to optimize the propyl oleate synthesis using a Central Composite Design (CCD), and the effects of 1-Propanol, H2O and oleic acid were analyzed. The highest initial specific propyl oleate synthesis rate was 266.80 mU/mg in the SLM system, while in the RM system was 177.04 mU/mg. The detriment of the activity in RM may be due to an AOT inhibition which exerts an electrostatic interaction with *LipN*. On the other side, the SLM system offers amphiphilic properties, where the n-propanol alkyl group acts as the "aliphatic tail" towards the organic solvent, and the hydroxyl group acts as the "polar head" interacting with the water/NaCl halophilic microenvironment, stabilizing *LipN* in its catalytically active conformation. Through the CCD model, the initial specific activity in the RM was restricted by water concentration. Meanwhile, in SLM, the highest initial specific activity was found with higher water concentrations. However, in both systems, the highest activities were found at > 200 mM oleic acid. In this work, we demonstrate that non-conventional strategies preserve the active halophilic lipase conformation, tailoring their polar content to increase the initial propyl oleate synthesis rate. In both systems a further increase of oleic acid concentration to yield higher propyl oleate productivity is possible, enabling the use of halophilic enzymes as potential industrial biocatalysts.

[1] Mateos-Díaz, J. C. *et al*. *J. Mol. Catal. B Enzym*. 2007, 49, 104.

[2] Pikuta, E. V., *et al*.*Crit. Rev. Microbiol*. 2007, 33, 183.

[3] Camacho-Ruíz, R. M., *et al*. *J. Ind. Microbiol. Biotechnol*. 2009, 36, 901.

[4] Reetz, M. T., *et al*. *European J. Org. Chem*. 2021 (46), 6224–6228.

[5] Márquez-Villa, J. M., *et al*. *Catalysts*, 2023, 13 (3).

P3-2

Selective Methylations and Alkylations using Methyltransferases

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Research into the utilisation of methyltransferase enzymes to perform selective methylations and alkylations in chemical synthesis is rapidly gaining momentum, in part due to the stringent substrate stereoselectivities and regioselectivities these biological catalysts offer [1]. In addition, these methyltransferases operate under mild conditions and avoid the use of toxic chemical reagents, such as methyl iodide and dimethyl sulfate [2,3], that would otherwise be used in equivalent traditional synthetic methods [4].

A range of *S*-adenosylmethionine (SAM)-dependent methyltransferases are being explored to assess their ability to selectively methylate an array of valuable small molecule substrates of pharmaceutical relevance, given the impressive enhancements in bioactivity of drugs commonly observed post-methylation [4]. Furthermore, an enlargement of the scope of non-methyl alkyl groups that these enzymes can transfer to substrates is being investigated, to probe the further expansion of chemical space in a selective manner. This is being carried out by the generation of SAM analogues that have their methyl group substituted for various alkyl moieties. Importantly, alkylations are performed in the context of a multi-enzyme *in situ* SAM analogue generation cascade that is necessary to ensure a continuous and immediate supply of these alkylation cofactors [5], which are relatively unstable molecules and expensive to procure. This cascade also ensures the efficient removal of the methyltransferase inhibitor *S*-adenosylhomocysteine (SAH) generated by SAM analogue dealkylation [6]. Furthermore, the properties of the methyltransferases and the accessory enzymes in this cascade are being enhanced by several approaches to improve activities.

Fig. 1: The scheme for the multi-enzyme methylation/alkylation cascade which generates SAM and its alkyl analogues *in situ*. MAT = methionine adenosyltransferase, MT = methyltransferase, MTAN = methylthioadenosine nucleosidase.

Fig. 2: The general structure of the SAM analogue, whereby R represents a range of alkyl groups.

[1] F. Subrizi, Y. Wang, B. Thair, D. Méndez-Sánchez, R. Roddan, M. Cárdenas-Fernández, J. Siegrist, M. Richter, J. N. Andexer, J. M. Ward and H. C. Hailes, Angew. Chem. Int. Ed., 2021, 60, 18673-18679.

[2] A. Sidana, A. Singh, N. Sawal, B. S. Chavan and R. Gupta, Indian J. Psychiatry, 2020, 62, 97-99.

[3] J. C. R. Rippey and M. I. Stallwood, Emerg. Med. J., 2005, 22, 878-879.

[4] P.d.S.M. Pinheiro, L.S. Franco, C.A.M. Fraga, Pharmaceuticals, 2023, 16, 1157.

[5] J. Siegrist, S. Aschwanden, S. Mordhorst, L. Thöny-Meyer, M. Richter and J. N. Andexer, ChemBioChem, 2015, 16, 2576-2579.

[6] R. Kumar, R. Srivastava, R. Kumar Singh, A. Surolia and D. N. Rao, Bioorg. Med. Chem., 2008, 16(5), 2276-2285.

Fig. 1

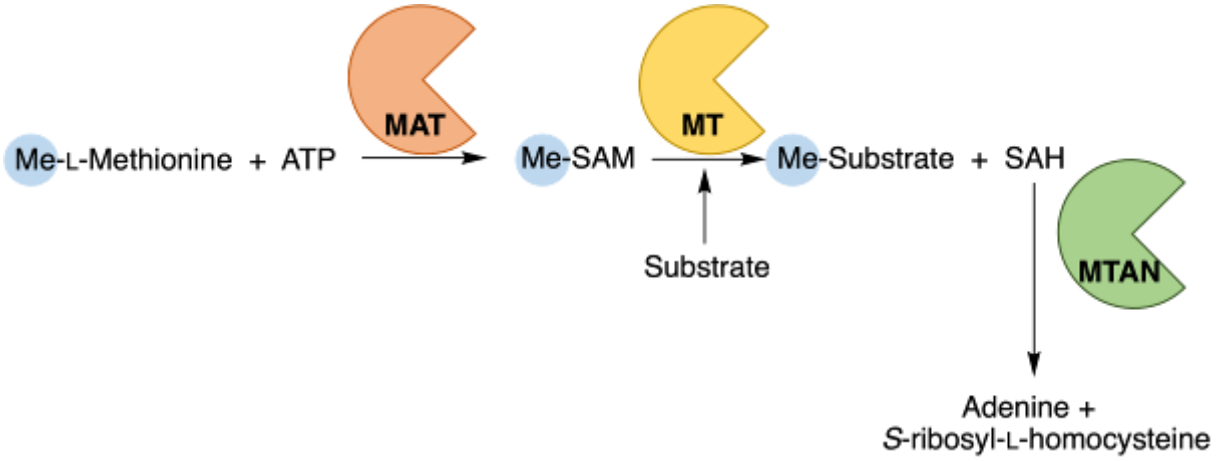
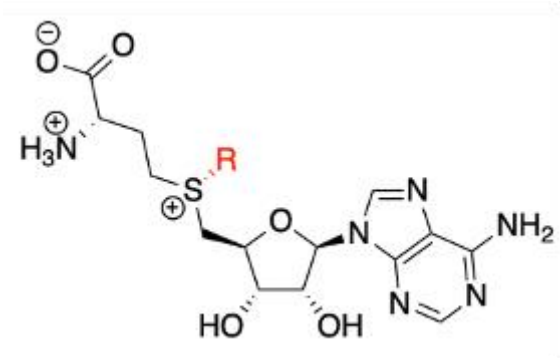

MAT
MT
Me-L-Methionine + ATP
Me-SAM
Me-Substrate + SAH
Substrate
MTAN
Adenine +
S-ribosyl-L-homocysteine

Fig. 2


O
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H3N
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P3-3

Strain- and process optimization of hydroxy-L-lysine production fueled by D-xylose using *Pseudomonas taiwanensis* VLB120

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Pseudomonas taiwanensis VLB120 is able to use the renewable carbon source D-xylose efficiently via Weimberg pathway, generating efficient supply of α-ketoglutarate (α-KG). This strain therefore offers a solution for developing sustainable bioprocesses. To demonstrate the suitability of the organism and Weimberg pathway for whole-cell biotransformations, we utilized non-heme Fe^{2+}/α-ketoglutarate-dependent lysine dioxygenases (KDOs) for stereo- and regioselective L-lysine hydroxylation.

To ensure efficient hydroxylation of L-lysine, we knocked out the three genes *davB*, *ldcC* and *aruH* of *P. taiwanensis* VLB120 L-lysine catabolism. Next, recently in-vitro tested KDO genes from different bacteria [1] were plasmid-based heterologously expressed in the generated triple mutant strain. The in-vivo activity of the constructs was tested with an initial concentration of 20 g L^{-1} D-xylose and various L-lysin concentrations. The strains were selected based on their achieved L-lysine conversion and hydroxy-L-lysine titers. Among them, the KDO from *Flavobacterium* sp. showed the most promising in-vivo L-lysin conversion, producing (4*R*)-4-hydroxy-L-lysine in the triple mutant *Pseudomonas* chassis. In 200 mL-scale stirred tank bioreactor cultivations with growing cells and initial substrate concentrations of 20 g L^{-1} D-xylose and 14.6 g L^{-1} L-lysine, we achieved hydroxy-L-lysine concentrations up to 8.8 g L^{-1} and a yield on L-lysine of approximate 80 % within 70 h. To further increase hydroxy-L-lysine production, a fed-batch cultivation was developed by feeding D-xylose and L-lysine, primarily focusing on D-xylose supply. Performing various D-xylose feeding strategies, we achieved a maximum final titer of 15.7 g L^{-1} of (4*R*)-4-hydroxy-L-lysine within 72 h.

We successfully designed an engineered *P. taiwanensis* VLB120 chassis, enabling a sustainable hydroxy-L-lysine bioprocess from readily available L lysine and D-xylose. Our work further established *P. taiwanensis* VLB120 as a versatile chassis organism for sustainable whole-cell biotransformations using D-xylose.

[1] Rolf, J.; Nerke, P.; Britner, A.; Krick, S.; Lütz, S.; Rosenthal, K., *Catalysts* **2021**, 11(9), 1038

P3-4

Unveiling Novel Carboligase Homologues for C1-C3 Aldehyde Conversion to Bioactive Hydroxy Ketones

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[3]acib – Austrian Center of Industrial Biotechnology, Graz, Austria
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Acyloins, valuable industrial chemicals, are typically produced via thiamine diphosphate (ThDP)-dependent carboligase-catalyzed reactions.[1] These reactions involve α-ketoacid decarboxylation and subsequent ligation with aldehydes to form acyloins. This study explores the production of short-chain aliphatic acyloins using newly discovered carboligase homologues of pyruvate dehydrogenase from *E. coli* (*Ec*PDH E1).[2] We demonstrate the condensation of formaldehyde (C1 aldehyde) with pyruvic acid to produce hydroxyacetone and with 2-oxobutyric acid to yield 1-hydroxybutan-2-one. Similarly, acetaldehyde (C2 aldehyde) condenses with pyruvic acid to form acetoin, while 2-hydroxypentan-3-one is the primary product when 2-oxobutyric acid is used. Finally, propionaldehyde (C3 aldehyde) condensation with pyruvic acid predominantly yields 3-hydroxypentan-2-one.[3] All these acyloins, except for 2-hydroxypentan-3-one, are listed as Generally Recognized As Safe (GRAS) by FEMA and find applications in the flavor, chemical, and pharmaceutical industries. Notably, 1-hydroxybutan-2-one is a crucial intermediate for the anti-tuberculosis drug ethambutol.[4] Our research focuses on novel carboligase homologues as promising whole-cell biocatalysts for α-hydroxy ketone synthesis. We investigate enzyme stability across pH ranges, determine kinetic parameters, and explore reaction intensification strategies.

Acknowledements: We thank the COMET center acib: Next Generation Bioproduction is funded by BMK, BMDW, SFG, Standortagentur Tirol, Government of Lower Austria and Vienna Business Agency in the framework of COMET - Competence Centers for Excellent Technologies. The COMET-Funding Program is managed by the Austrian Research Promotion Agency FFG. Additionally, this work was supported by the Slovak Research and Development Agency under the contract number APVV-20-0317.

[1] D. Gocke, T. Graf, H. Brosi, I. Frindi-Wosch, L. Walter, M. Müller, M. Pohl, J Mol Catal B Enzym 2009, 61, 30–35.
[2] S. R. Marsden, D. G. G. McMillan, U. Hanefeld, Int J Mol Sci 2020, 21, 1–15.
[3] H. Dobiasova, V. Jurkas, P. Both, M. Winkler, ChemCatChem 2024, DOI 10.1002/cctc.202301707.
[4] H. Lin, J. Xu, W. Sun, W. Hu, H. Gao, K. Hu, J. Qiu, B. Huang, L. Zhang, Catalysts 2021, 11, DOI 10.3390/catal11101184.

P3-5

Engineering the Fatty Acid Photodecarboxylase from *Chlorella Variabilis* to Catalyze C-C Bond Formations

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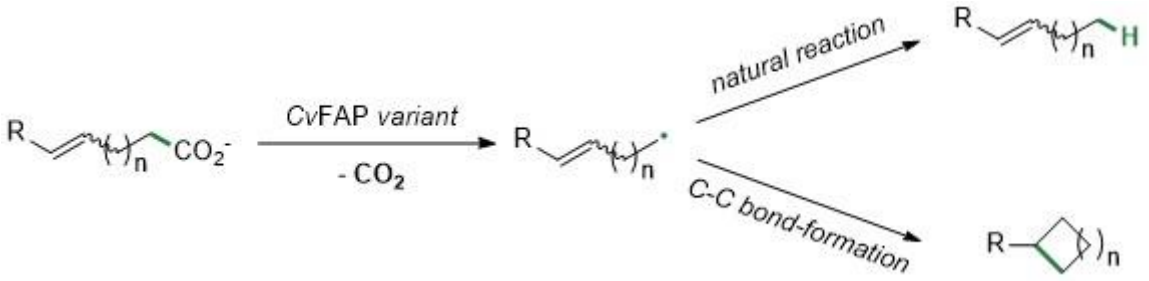
The fatty acid photodecarboxylase from *Chlorella variabilis* (*Cv*FAP) is capable to decarboxylate carboxylic acids in a light-dependent process[1] *via* a radical mechanism (Figure 1).[2] Since its first description, its potential as biocatalyst has been broadly explored, unraveling a large number of variants, catalyzing the decarboxylation of an extensive scope of carboxylic acids.[3]
Inspired by chemical photocatalysis[4] we investigated the option to utilize the intermeidate radical for C-C bond formation chemistry instead.
Using a range of structurally distinct substrate probes possessing functional groups capable to react with a radical and testing a small library of enzyme variants we were able to proof the desired bond forming reactivity. Directed evolution paired with further investigation of the substrates scope is expected to establish this novel reaction pathway as a new method for photobiocatalytic decarboxylative coupling reactions.

Figure 1: Natural Decarboxylation reaction and novel C-C bond-formation activity of CvFAP and its variants.

[1] D. Sorigue, B. Legeret, S. Cuine, S. Blangy, S. Moulin, E. Billon, P. Richaud, S. Brugiere, Y. Coute, D. Nurizzo, P. Muller, K. Brettel, D. Pignol, P. Arnoux, Y. Li-Beisson, G. Peltier, F. Beisson, *Science* **2017**, *357*, 903-907.
[2] D. Sorigue, K. Hadjidemetriou, S. Blangy, G. Gotthard, A. Bonvalet, N. Coquelle, P. Samire, A. Aleksandrov, L. Antonucci, A. Benachir, S. Boutet, M. Byrdin, M. Cammarata, S. Carbajo, S. Cuine, R. B. Doak, L. Foucar, A. Gorel, M. Grunbein, E. Hartmann, R. Hienerwadel, M. Hilpert, M. Kloos, T. J. Lane, B. Legeret, P. Legrand, Y. Li-Beisson, S. L. Y. Moulin, D. Nurizzo, G. Peltier, G. Schiro, R. L. Shoeman, M. Sliwa, X. Solinas, B. Zhuang, T. R. M. Barends, J. P. Colletier, M. Joffre, A. Royant, C. Berthomieu, M. Weik, T. Domratcheva, K. Brettel, M. H. Vos, I. Schlichting, P. Arnoux, P. Muller, F. Beisson, *Science* **2021**, *372*, eabd5687.
[3] J. Xu, Y. Hu, J. Fan, M. Arkin, D. Li, Y. Peng, W. Xu, X. Lin, Q. Wu, *Angew. Chem. Int. Ed.* **2019**, *58*, 8474-8478; J. Xu, J. Fan, Y. Lou, W. Xu, Z. Wang, D. Li, H. Zhou, X. Lin, Q. Wu, *Nat. Commun.* **2021**, *12*, 3983.
[4] N. A. Romero, D. A. Nicewicz, *Chem. Rev.* **2016**, *116*, 10075-10166.

Fig. 1



P3-6

Dimethylsulfoniopropionate lyase from *Pelagibacter ubique* HTCC1062 as a catalyst for β-amino acid synthesis by aza-Michael reaction

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The construction of C-N bonds is an important chemical transformation for the preparation of amines, amino alcohols and amino acids. These compounds have been widely employed as chiral building blocks in the pharmaceutical and agrochemical industries. Furthermore the presence of a chiral amine in active pharmaceutical ingredients is estimated to be around 40% and this percentage is larger when considering only the amino groups (chiral and achiral).1 Several strategies have been employed for the biocatalytic synthesis of C-N bonds, e.g., transaminases, iminoreductases and aza-Michael additions. Particularly, the biocatalytic aza-Michael addition reactions have been performed using promiscuous hydrolases (e.g., proteases, lipases, amylases) using alkyl acrylates, acrylonitrile and enones, and with specific ammonia lyases.2 However, the use of acrylic and methacrylic acids as Michael acceptors in biocatalysis is not documented. α,β-Unsaturated carboxylic acids are difficult substrates that must be efficiently activated to increase the electrophilicity.

In this communication we report the catalytic properties of dimethylsulfoniopropionate (DMSP) lyase from *Pelagibacter ubique* HTCC1062 (DddK) as catalyst for Michael type reactions. In nature, DddK catalyzed the cleavage of DMSP to dimethyl sulphide and acrylate. We envisaged that the enzyme can operated in the synthetic direction and catalyze both hetero C-X and C-C Michael additions using acrylic, methacrylic and other α,β-unsaturated carboxylic acids as acceptors. This study explores the catalytic potential of DddK for the aza-Michael addition of primary and secondary amines to acrylic and methacrylic acids (Scheme 1). Key parameters such as DddK activity, metal content, reaction conversion rates, and the isolated yield of the N-mono and N,N''-disubstituted β-amino acids are investigated. Furthermore, the stereochemical outcomes of the products originating from methacrylic acid are presented and discussed.

1. Breuer, M.; Ditrich, K.; Habicher, T.*, et al.*, *Angew. Chem. Int. Ed.* **2004,** *43*, 788-824.
2. (a) Steunenberg, P.; Sijm, M.; Zuilhof, H.*, et al.*, *J. Org. Chem.* **2013,** *78*, 3802-3813; (b) Torre, O.; Alfonso, I.; Gotor, V., *Chem. Commun.* **2004**, 1724-1725; (c) Dutt, S.; Goel, V.; Garg, N.*, et al.*, *Adv. Synth. Catal.* **2020,** *362*, 858-866; (d) Zhang, J.; Abidin, M. Z.; Saravanan, T.*, et al.*, *ChemBioChem* **2020,** *21*, 2733-2742.

This project has received funding from the European Union''s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 956631. Grant PID2021-122166OB-I00 funded by MCIN/AEI/10. 13039/501100011033 and "ERDF A way of making Europe".

Fig. 1

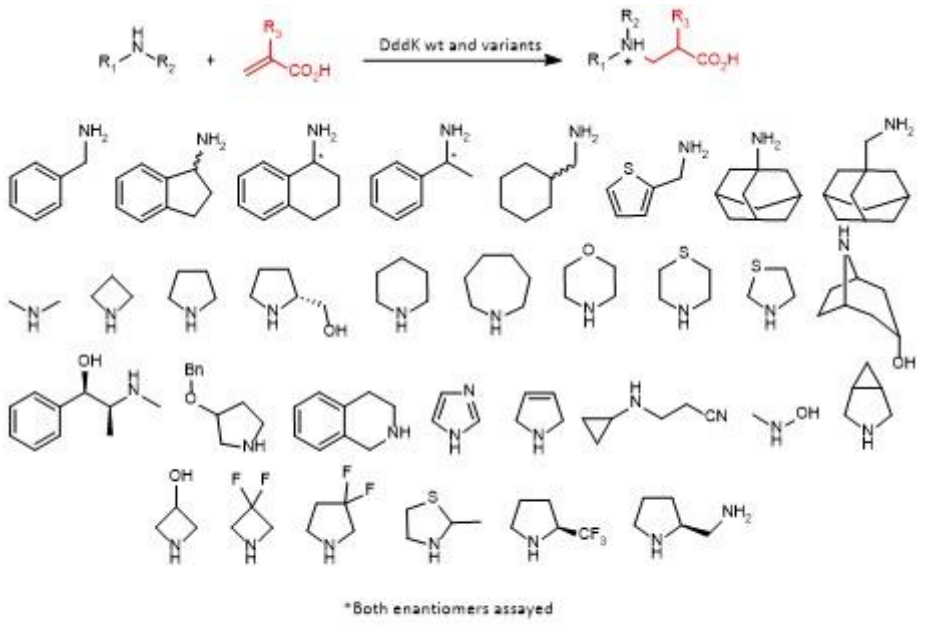

Figure 1. DddK catalyzed aza-Michael additions of primary and secondary amines to acrylic (R_3 = H) and methacrylic acids (R_3 = CH_3).

P3-7

Enantioselective Biocatalytic Reduction of Sterically challenging Pharmaceutically Relevant Chiral Amines Using whole cell biocatalyst

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[2]KBC North Maharashtra University, School of Chemical Science Organic Chemistry, Jalgaon, India

Question:

Enantioselective synthesis of chiral compounds holds significant importance in pharmaceutical and chemical industries due to the inherent chirality of many biologically active molecules. Sterically challenging chiral amines pose a particular synthetic hurdle, necessitating innovative approaches for their efficient synthesis. In this study, we explore the utilization of Baker's yeast as a biocatalyst for the enantioselective reduction of such challenging chiral amines. Chiral amines as key intermediates in the synthesis of a variety of biologically active molecules. In particular, thiazolidin-5-ylidene group is a privileged heterocyclic scaffold containing N- and S-atoms, with versatile pharmacological properties and found ubiquitously in a large number of clinically used drugs. The simplest approach for producing chiral amines involves creating C=N bonds through the reaction of carbonyls and amines, followed by reducing the resulting imine. The efficiency of the reducing agent is importance, it must selectively reduce the C=N bond while leaving the carbonyl compound unaltered.

Methods:

A typical reaction setup involved adding the chiral amine substrate to the reaction vessel containing the Baker's yeast suspension. Place the reaction vessel containing the Baker's yeast suspension and the substrate solution in a sonication bath or use a sonication probe directly immersed in the reaction mixture. The reaction mixture was incubated under optimized conditions with continuous monitoring of progress. Aliquots were withdrawn at regular intervals for analysis to determine conversion rates and enantiomeric excess. Upon completion of the biotransformation, the reaction mixture was quenched in ethyl acetate. The desired product was extracted from the reaction mixture using organic solvents. The stereochemistry and purity of the chiral product were analyzed using chiral HPLC, NMR spectroscopy.

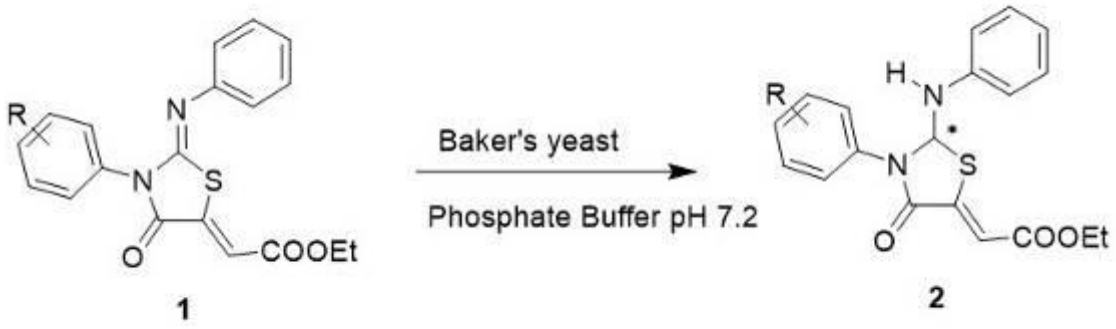
Results:

The biotransformation of sterically challenging chiral amines proceeded with high conversion rates ranging from 80% to 95%. Enantioselectivity was excellent, with enantiomeric excess values consistently exceeding 99% for the desired chiral alcohol products.

Conclusion:

In conclusion, this study demonstrates the efficacy of Baker's yeast as a biocatalyst for the enantioselective reduction of sterically challenging chiral amines, providing a sustainable and efficient route for the synthesis of pharmaceutically relevant chiral building blocks. Through careful optimization of reaction conditions, including pH, temperature, and cofactor regeneration systems, high conversion rates and excellent enantioselectivity were achieved even for substrates with bulky substituents.

Fig. 1


R
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Baker's yeast
Phosphate Buffer pH 7.2
H
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S
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1 **2**

P3-8

A Ketoreductase Utilizes Synthetic Nicotinamide Cofactor Mimetics

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Oxidoreductases are a widely applied enzyme family which is dependent on redox cofactors such as nicotinamide adenine dinucleotide (NADH). These cofactors are relatively expensive and suffer from low stability under process conditions.[1] Recently, so called non-canonical redox cofactors such as 1-substituted nicotinamides gained increasing attention.[2] These cofactors are accessible by fairly straightforward organic synthesis, and allow orthogonal reaction control;[1] however, their implementation into biocatalytic processes can be challenging. The cofactor mimetics typically do not bear the functional moieties interacting with the amino acid residues located in the cofactor binding pocket, leading to a lower enzyme affinity towards these mimetics. Nonetheless, some enzyme classes such as flavoenzymes readily accept cofactor mimetics as an electron source for biocatalytic reductions,[3] whereas other oxidoreductases such as alcohol dehydrogenases and ketoreductases poorly accept them.[4]

In this work, we identified the first ketoreductase accepting a broad variety of cofactor mimetics. We screened libraries of both oxidized and reduced forms of NADH mimetics with varying substituents in the 1- and 3-position of the pyridine moiety (Figure 1). Delightfully, we observed initial reaction rates close to the native cofactor.

Taking into account that the cofactor mimetics display different stabilities at elevated temperatures depending on the pH value, we investigated different reaction parameters, such as reaction time, pH, co-solvent, and temperature to ensure efficient reactions. We further applied the cofactor mimetics for enantioselective ketone reduction to obtain chiral alcohols.

Figure 1. Reduction of carbonyls and oxidation of alcohols catalyzed by a kedoreductase utilizing cofactor mimetics.

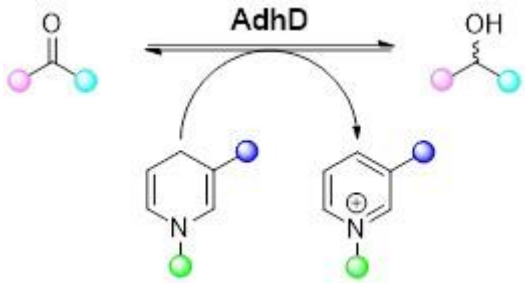
[1] A. Guarneri, W. J. H. van Berkel, C. E. Paul, *Curr. Opion. Biotechnol.* **2019**, *60*, 63-71.

[2] W. B. Black, H. Li, *Methods Mol. Biol.* **2022**, *2433*, 185-198.

[3] A. Guarneri et al., *ChemCatChem* **2020**, *12*, 1368-1375.

[4] L. Josa-Culleré et al., *Catalysts* **2019**, *9*, 207.

Fig. 1



P3-9

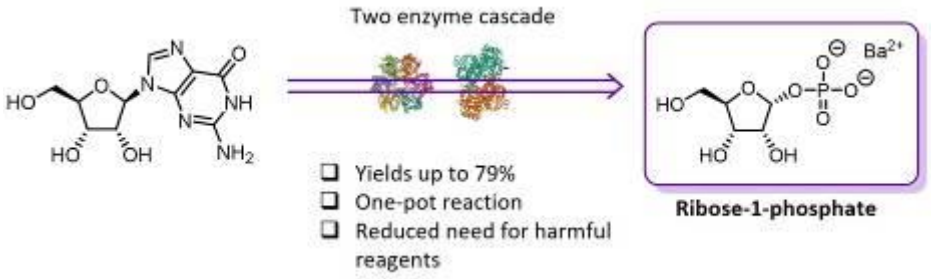
A deamination-driven biocatalytic cascade for the synthesis of ribose-1-phosphate

J. Motter[1], S. Westarp[1,2], J. Barsig[1], C. Betz[1], A. Dagane[1], F. Kaspar[1,3], L. Neumair[1], S. Kemper[4], P. Neubauer[1], A. Kurreck[1,2]
[1]Technical University of Berlin, Chair of Bioprocess Engineering, Berlin, Germany
[2]BioNukleo GmbH, Berlin, Germany
[3]Technische Universität Braunschweig, Institute for Biochemistry, Braunschweig, Germany
[4]Technical University of Berlin, Institute for Chemistry, Berlin, Germany

Pentose-1-phosphates are key metabolites in all living organisms. Recently, they attracted focus in nucleoside analogue synthesis, as they can serve as precursors for the synthesis of difficult-to-access nucleosides. This is illustrated by Merck"s biocatalytic synthesis of the antiviral drug islatravir[1] and molnupiravir[2]. However, due to its lengthy and environmentally taxing synthesis, the availability of the pentose-1-phosphate substrates has been limited. Therefore, we envisioned designing a biocatalytic cascade to shift nucleoside-phosphorylase-mediated phosphorolysis equilibrium positions to near-quantitative conversion. Indeed, coupling the phosphorolysis of guanosine with a thermostable and narrow-spectrum guanine deaminase resulted in increased yields of ribose-1-phosphate. Building on that observation, we developed a one-pot, two-enzyme biocatalytic cascade allowing for high isolated yields of alpha-anomerically pure ribose-1-phosphate (up to 79% at the gram scale). A comparison of route efficiency and waste production showed that our method requires less (toxic) solvents and reagents than other state-of-the-art (chemo)enzymatic methods. Hence, our approach will significantly expand and strengthen the applicability of enzymatic nucleoside syntheses.

1. Huffman, M. A. *et al.* Design of an in vitro biocatalytic cascade for the manufacture of islatravir. *Science* **366**, 1255–1259 (2019).
2. McIntosh, J. A. *et al.* Engineered Ribosyl-1-Kinase Enables Concise Synthesis of Molnupiravir, an Antiviral for COVID-19. *ACS Cent. Sci.* **7**, 1980–1985 (2021).

Fig. 1



P3-10

IN Silico Informed metaGenomic Harvesting Technology - (*INSIGHT) - How a smart platform can accelerate the timelines of genes-to-GMP*

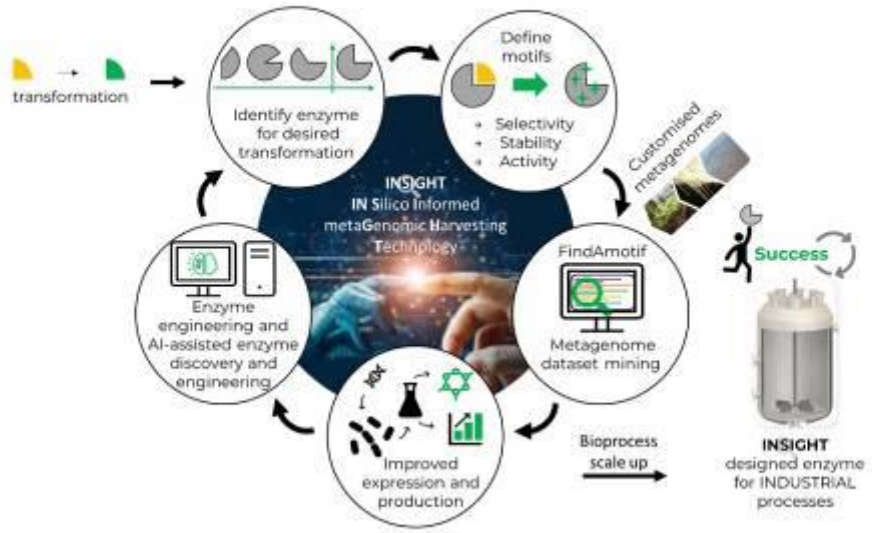
J. Mueller[1], A. Carvalho[1], C. Fleming[2], M. Boyd[2], X. Wang[2], A. Ibrahim[2]
[1]Almac Group, Sciences, Craigavon, United Kingdom
[2]Queen's University Belfast, Belfast, United Kingdom

Biocatalysis is a key enabling technology towards more sustainable and efficient GMP manufacture of active pharmaceutical ingredients. The processes involved in traditional enzyme selection and screening can be resource heavy and limited to publicly available sequenced genomes. Almac"s newly developed INSIGHT platform streamlines enzyme discovery, engineering and development by combining metagenomic and bioinformatic enzyme discovery, high throughput screening, *in silico* enzyme engineering and machine learning. INSIGHT enables Almac to accelerate the development of enzymes to the specific conditions of industrial processes (high substrate loadings and high catalytic efficiency) for the efficient synthesis of chemical intermediates and active pharmaceutical ingredients (APIs). To accelerate development from "genes-to-GMP" Almac integrates enzyme gene library synthesis into INSIGHT"s multi-level enzyme engineering platform which will shorten timelines and make enzyme engineering more affordable.

FIGURE TO BE INSERTED HERE

This presentation will showcase a number of selected examples of the benefits of INSIGHT. It will highlight the stages of INSIGHT from the discovery of novel bulky-bulky Carbonyl Reductases (CREDs) from Almac"s very own metagenomes with superior properties and take the audience through the accelerated engineering of cosubstrate stability in Transaminases that enabled reaction completion. It will also show an example of expression enhancement for a monooxygenase that ensured economic viability of the investigated biotransformation. This accelerated timeline for enzyme discovery and engineering amplifies Biocatalysis as an attractive option for chemical processes even in late stage development, where timelines are short and pressure is high.

Fig. 1



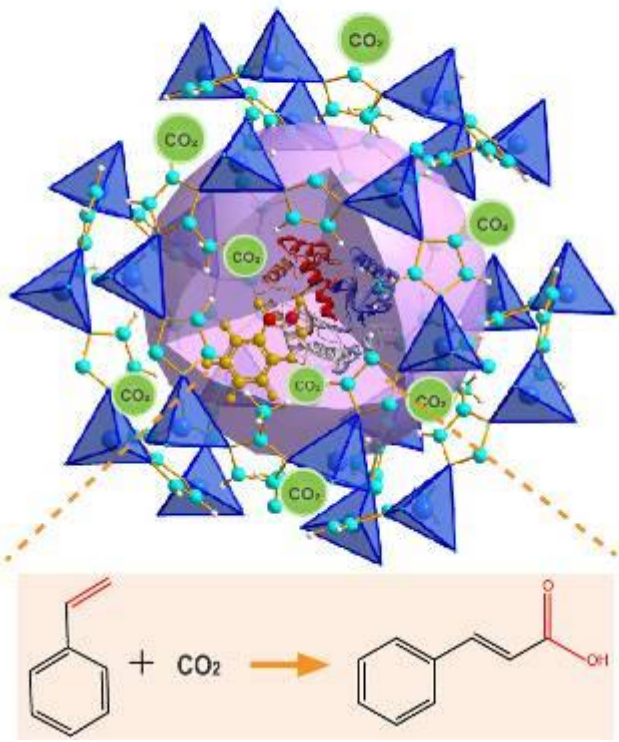
P3-12

CO_2 capture and conversion by dual-functional Enzyme/MOF composites containing Ferulic Acid Decarboxylase

F. Wang[1]
[1]Aarhus University, Aarhus, Denmark

Biocatalytic carboxylation reaction can be applied for CO_2 fixation in an efficient and environmentally friendly way. Recently, decarboxylase-catalyzed reversible (de)carboxylation reactions exhibited high potential for capturing and fixing CO_2 into organic molecules. However, the unfavorable thermodynamic constraints and the inefficient utilization of CO_2 limit biotechnological applications. Hereby, we embedded a ferulic acid decarboxylase (FDC) from *Capronia coronata* into MOF (Metal-organic framework) nanoparticles to synthesize CcFDC/MOF composite. Among various composites tested, CcFDC/ZIF-67 composite exhibited excellent performance for CO2 absorption and subsequent carboxylation reaction, which improved the reaction rate 2-fold compared to the free enzyme. The result of CO_2 absorption suggests that MOF nanoparticles performed as a CO2 concentrator, which can accelerate the catalytic rate and drive the equilibrium toward the formation of carboxylation products by improving the local concentration of CO_2. The ZIF-67 nanoparticles with intact structure can stabilize enzyme molecules when prepared by encapsulation method, leading to high thermal stability (≥90 residual activity after 4 h at 40 ℃) and reusability (≥80 residual activity after 7 cycles) of the composite biocatalyst. Furthermore, CcFDC/ZIF-67 exhibited better carboxylation conversion for different substrates than the free enzyme. Therefore, this CcFDC/ZIF-67 composite can be considered as a dual-functional biocomposite catalyst that can perform synergistic CO_2 capture and conversion.

Fig. 1



P3-13

Application of β-N-Acetylhexosaminidases in Chemo-Biocatalysis

H. Hronska[1], M. Bláhová[1], V. Štefuca[1], M. Rosenberg[1]
[1]Slovak University of Technology in Bratislava, Institute of Biotechnology, Bratislava, Slovakia

Introduction

α-Anomers of aromatic substituted glycosides are often used as important chromogenic/fluorogenic substrates for the rapid and early diagnosis of various diseases or identification and quantification of enzymes. 4NP-α GalNAc and 4MU-α GalNAc are traditional synthetic substrates to determine the activity of α N-acetylgalactosaminidase (α NAGA). It is a crucial enzyme involved in a rare inherited metabolic disorder Schindler disease. One of the possible methods of α-anomers of glycosides preparation is a chemo-enzymatic process starting with anomerically nonselective acid-catalyzed glycosylation and continue with enzymatic hydrolysis of the undesired anomer. In addition, the use of immobilized enzyme can reduce the cost and increase the reusability of the biocatalyst.

Objective

The objective of our work is to apply free or immobilized β-NAGA in the specific hydrolysis of β-anomer from chemically synthetized anomeric mixtures of the chromogenic substrate 4NP-α/β GalNAc and the fluorogenic substrate 4MU-α/β GalNAc. In this way, it is possible to develop a convenient method for preparing α-anomers of mentioned synthetic substrates.

Methods

For the production of the enzyme batch fermentations were performed on 1 L scale using strains of the genus *Penicillium*. The purified enzyme was immobilized by entrapment in PVA-based lenticular hydrogel particles [1,2]. For the quantification of reaction products HPLC methods with UV detection were developed.

Results

In our reaction system, α/β-substituted GalNAc derivatives were chemically synthesized and the β-substituted GalNAc derivatives were enzymatically hydrolyzed, thus allowing for the easy isolation of the α-substituted GalNAc derivatives. Reactions products were simple separated by solvent extraction. The yield of α-anomers glycosides in crystals was 90.5% with the optical purity ≥ 99.6%.

The reusability and long-term stability of the immobilized β-NAGA were determined in the repeated batch mode of operation. Fifty repeated reactions were performed, providing >99% of hydrolysis in each run. The immobilized biocatalyst retained about 90% of its initial activity after 18 months of storage at 4°C [1].

To overcome problem with very low solubility of the aromatic glycosides, enzyme hydrolysis was applied directly on crystal suspensions of α/β-mixtures of aromatic glycosides. The final purity of the crystalline α-anomer was 96% [1].

Conclusion

A simple and convenient procedure was developed to synthesize α-anomers of chromogenic and fluorogenic synthetic enzyme substrates using free or immobilized β-NAGA in chemo-biocatalytic process. The immobilization of β-NAGA in PVA gel provided a stable biocatalyst and enabled its repeated use.

This work was funded by Grant Nr. APVV-20–0208.

[1] Hronska et al., Catalysts **2022**, Vol 12 (5)

[2] Ondrejková et al., Appl. Sci. **2023**, Vol 13 (6)

P3-14

Development of a Thermodynamically Favorable Multi-enzyme Cascade Reaction for Efficient Sustainable Production of ω-Amino Fatty Acids and α,ω-Diamines

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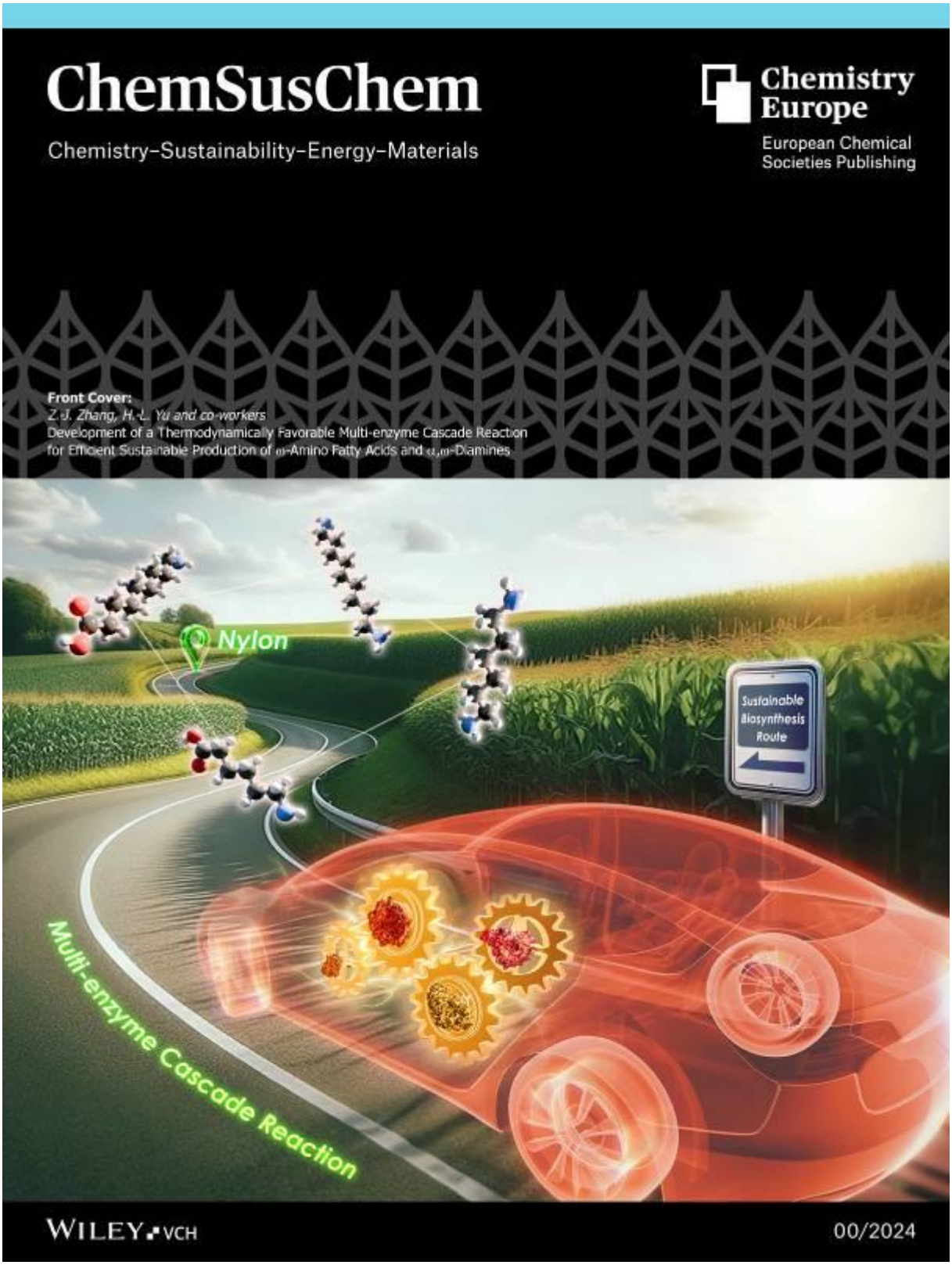
[1]East China University of Science and Technology, School of Bioengineering, Shanghai, China

Aliphatic ω-amino fatty acids (ω-AFAs) and α,ω-diamines (α,ω-DMs) are essential monomers for the production of nylons. Development of a sustainable biosynthesis route for ω-AFAs and α,ω-DMs is crucial in addressing the challenges posed by climate change. Herein, we constructed an unprecedented thermodynamically favorable multi-enzyme cascade (TherFavMEC) for the efficient sustainable biosynthesis of ω-AFAs and α,ω-DMs from cheap α,ω-dicarboxylic acids (α,ω-DAs). This TherFavMEC was developed by incorporating bioretrosynthesis analysis tools, reaction Gibbs free energy calculations, thermodynamic equilibrium shift strategies and cofactor (NADPH&ATP) regeneration systems. The molar yield of 6-aminohexanoic acid (6-ACA) from adipic acid (AA) was 92.3%, while the molar yield from 6-ACA to 1,6-hexanediamine (1,6-HMD) was 96.1%, which were significantly higher than those of previously reported routes. Furthermore, the biosynthesis of ω-AFAs and α,ω-DMs from 20.0 mM α,ω-DAs (C6-C9) was also performed, giving 11.2 mM 1,6-HMD (56.0% yield), 14.8 mM 1,7-heptanediamine (74.0% yield), 17.4 mM 1,8-octanediamine (87.0% yield), and 19.7 mM 1,9-nonanediamine (98.5% yield), respectively. The titers of 1,9-nonanediamine, 1,8-octanediamine, 1,7-heptanediamine and 1,6-HMD were improved by 328-fold, 1740-fold, 87-fold and 3.8-fold compared to previous work. Therefore, this work holds great potential for the bioproduction of ω-AFAs and α,ω-DMs.

https://doi.org/10.1002/cssc.202301477

Cover article of ChemSusChem

Fig. 1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Z.-J. Zhang, H.-L. Yu and co-workers
Development of a Thermodynamically Favorable Multi-enzyme Cascade Reaction for Efficient Sustainable Production of ω-Amino Fatty Acids and α,ω-Diamines
Nylon
Sustainable Biosynthesis Route
Multi-enzyme Cascade Reaction
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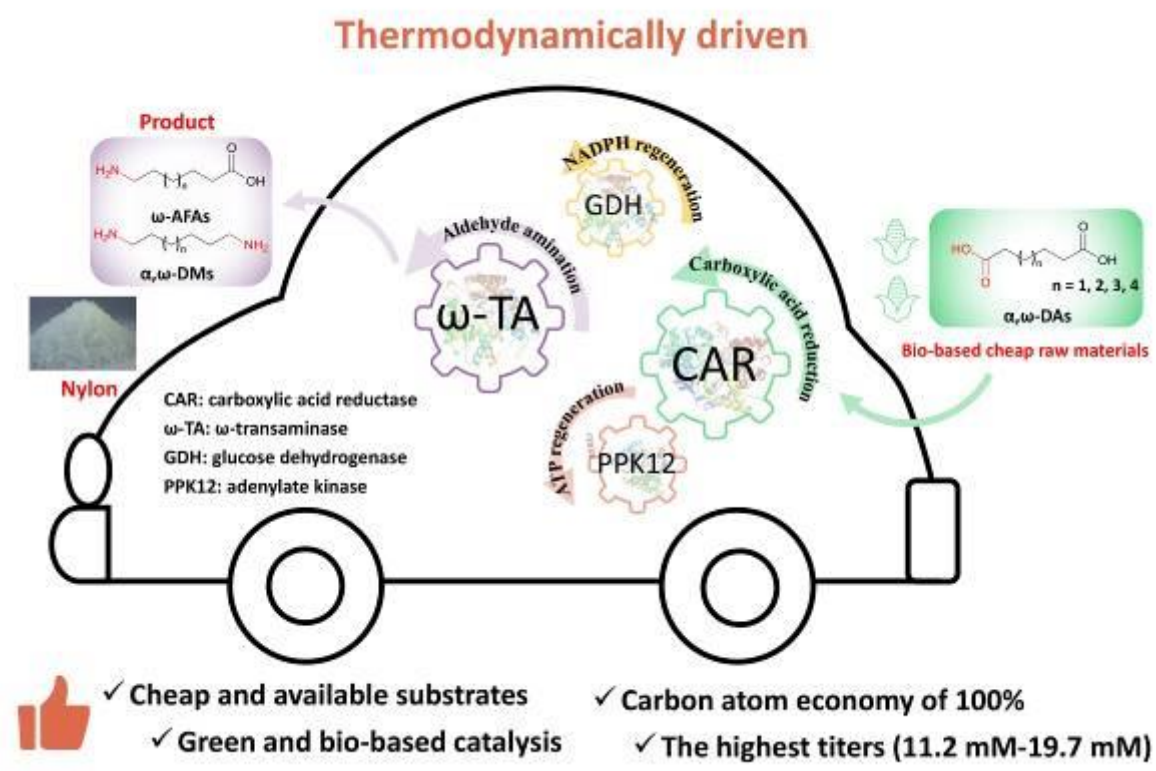
Fig. 2


Thermodynamically driven
Product
ω-AFAs
α,ω-DMs
Nylon
NADPH regeneration
GDH
Aldehyde amination
ω-TA
Carboxylic acid reduction
CAR
ATP regeneration
PPK12
n = 1, 2, 3, 4
α,ω-DAs
Bio-based cheap raw materials
CAR: carboxylic acid reductase
ω-TA: ω-transaminase
GDH: glucose dehydrogenase
PPK12: adenylate kinase
✓ Cheap and available substrates
✓ Green and bio-based catalysis
✓ Carbon atom economy of 100%
✓ The highest titers (11.2 mM-19.7 mM)

P3-15

Enzymatic esterification of dihydroferulic acid with ethylene glycol: maximizing the conversion toward the monoester as a building block for biosourced antioxidant polymer synthesis

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[1]Université de Lorraine, CNRS, LRGP, Nancy, France
[2]Delft University of Technology, Department of Biotechnology, Delft, Netherlands
[3]Université de Lorraine, CNRS, LCPM, Nancy, France

Ferulic acid is a phenolic and anti-oxidant phytochemical that can be found in several vegetal biorefinery wastes. This acid is used in the fields of comestics, pharmaceutics or as a chemical precursor [1]. Among those is the use of ferulic acid for the production of biosourced polymers, although the majority of the work done does not focus on valorising its anti-oxidant abilities. Therefore, generating biosourced monomers from ferulic acid through chemo-enzymatic modifications in order to produce intrinsically anti-oxidant polymers is of significant interest. One reaction of the proposed pathway is the lipase-catalysed esterification of dihydroferulic acid (DHFA), a more reactive derivative of ferulic acid easy to achieve chemicaly, with ethylene glycol (EG), see Figure 1. This reaction can lead to two products: a diester resulting from the grafting of two DHFA units with one EG and a monoester presenting an alcohol function that can be further functionalized to obtain other derivatives such as monomers. Depending on the final target biopolymer, different set of reaction conditions need to be applied to maximize the conversion toward the monoester or the diester derivative. Both ester derivatives can be used as monomer precursor, but the diester requires modifications to the phenolic structure [2], which lead to the loss of the anti-oxidant capacity. Therefore, the conditions to maximize the production of the monoester were studied.

Figure 1: Lipase catalyzed reaction between dihydroferulic acid or its ethyl ester and ethylene glycol.

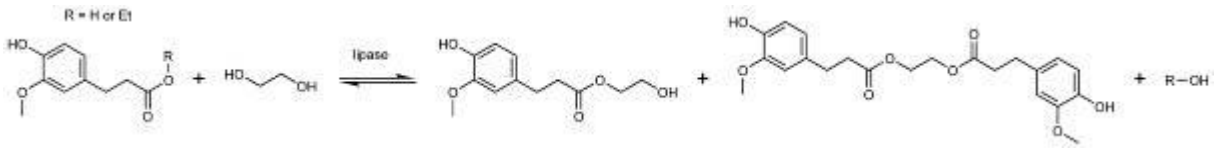
Reactions were performed in dry solvent (2-methyl-2-butanol) and in solvent-free systems, with EG in excess, in the presence of immobilized enzyme Lipozyme 435. The impact of the nature of the substrate on the extent of the reaction was studied, comparing dihydroferulic acid and ethyl hydroferulate as substrates. The impact of the molar ratio between acyl donor and ethylene glycol on the equilibrium was also investigated, as well as the effect of the water activity (aw).

In dry solvent, the dihydroferulic acid and ethyl hydroferulate had conversions as high as 74% and 81%, respectively, when an excess of EG was used (molar ratio = 1:6). The excess also allowed to increase the monoester/diester ratio by a factor of three. Interestingly, no reaction was observed when the reaction was performed in pure EG, but the addition of 10% water led to a conversion of 98% of ethyl hydroferulate. In such conditions, only monoester was produced, with 99% of converted substrate toward it, even at high aw. The maximization of the monoester synthesis allowed its purification and characterization regarding its anti-oxidant capacity. These results represent an important step for a green pathway toward a biosourced and anti-oxidant monomer for subsequent polymerisation.

[1] N. Kumar, V. Pruthi, *Biotechnol Rep (Amst)*. **4**, 86–93 (2014).

[2] F. Pion, A. F. Reano, P.-H. Ducrot, F. Allais, *RSC Adv.* **3**, 8988–8997 (2013).

Fig. 1



P3-16

Substrate flexibility of rutinosidase from *Aspergillus niger*

K. Brodsky[1,2], L. Petrásková[1], M. Kutý[3], P. Bojarová[1], H. Pelantová[1], V. Křen[1]
[1]Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic
[2]University of Chemistry and Technology in Prague, Department of Biochemistry and Microbiology, Prague, Czech Republic
[3]University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic

Introduction: Rutinosidases (6-α-L-rhamnosyl-β-D-glucosidases) are retaining diglycosidases that cleave the glycosidic bond between the (di)saccharide moiety and the respective aglycone. Like many retaining glycosidases, they can also transfer the (di)saccharide moiety to acceptors with a free –OH group and form new glucosides or rutinosides. The crystal structure and molecular modeling of rutinosidase from Aspergillus niger (*An*Rut)[1] show its strong and specific binding to the aromatic aglycone (flavonoid) and a high tolerance at the glycone binding site, especially at position C-6' of the glucose moiety. Revealing the substrate specificity of *An*Rut will help us to synthesize new glucosides and create a library of standards.

Objectives: The substitution of the C-6' position in glucopyranosidic substrates can influence the activity of the enzyme and provide more information about its hydrolytic and synthetic potential with complex substrates. Such as mycotoxins in food, which cannot be properly detected due to lack of standards. In this study, we tested different 6'-*O*-acyl derivatives of isoquercitrin as substrates for *An*Rut[2]. The compounds 6'-*O*-acetyl, 6'-*O*-benzoyl, and 6'-*O*-cinnamoyl derivatives of isoquercitrin (IQ) were also tested as transglycosylation substrates (Fig. 1)

Results: *An*Rut cleaves β-glucopyranosides acylated at C-6' of the glucose moiety (6'-*O*-acylisoquercitrin) with acetyl, benzoyl, phenylacetyl, phenylpropanoyl, cinnamoyl, vanillyl, galloyl, 4-hydroxybenzoyl and 3-(4-hydroxy-3-methoxyphenyl)propanoyl. Molecular modeling based on the crystal structure of *An*Rut showed that large aromatic substituents at C-6' of IQ can block the side tunnel leading to the active site of the enzyme and thus restrict the access of the acceptor for transglycosylation.

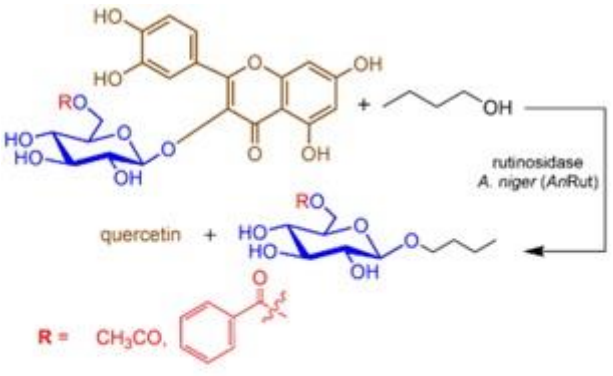
Conclusion: This study shows the great substrate flexibility of *An*Rut at the glycone site. Even large substituents such as gallic acid were accepted by *An*Rut, which is not common for glycosidases. Furthermore, our study supports the previously formulated hypothesis that the side tunnel is used as an access route for transglycosylation acceptors. When this tunnel is blocked, transglycosylation cannot occur. This unique feature of *An*Rut will be the subject of our further research.

Supported by Czech Science Foundation project 24-10000S and travel grant LUC23061 from MEYS

1 Brodsky K, Kutý M, Pelantová H, Křen V, Bojarová P. *Int. J. Mol. Sci*. 2020, 21, 5671.

2 Brodsky K, Petrásková L, Kutý M, Bojarová P, Pelantová H, Křen V. *ChemCatChem*. 2024, e202400028.

Fig. 1



Rutinosidase-catalyzed transglycosylation reactions of *n*-butanol with 6'-*O*-acyl isoquercitrin.

P3-17

Acrylic acid hydrating enzyme from *Fusarium* sp. No. 17 strain

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[1]Osaka Metropolitan University, Graduate School of Agriculture, Osaka, Japan

Introduction

Acrylic acid is one of the short-chain unsaturated fatty acids, and widely used as a basic compound of flexibility polymers. Although research on the microbial conversion of compounds carrying C=C bond has been advanced, there are few reports about conversion of acrylic acid (AA). We have been conducting research on the metabolic pathway of acrylic acid by microorganisms. Among them, we successfully isolated *Fusarium* sp. No.17 strain that hydrates AA to 3-hydroxypropionic acid (3HPA). Furthermore, since the addition of acetyl-CoA (Ac-CoA) was essential for the reaction with the cell-free extract (CFE), this conversion reaction was suggested to be a three-step reaction via acrylyl-CoA and 3-hydroxypropionyl-CoA. In this study, we purified the enzyme from *Fusarium* sp. No. 17 strain and investigated the reaction mechanisms and enzymatic functions.

Results

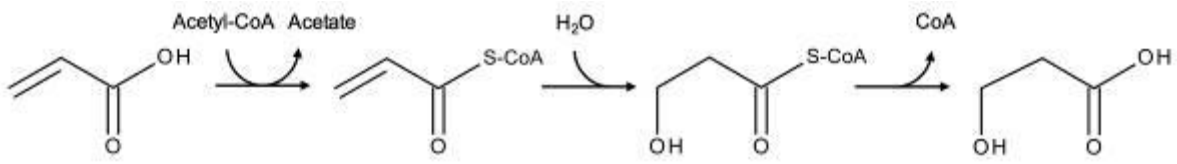
Enzyme purification was performed from CFE prepared from the cells of *Fusarium* sp. No. 17 strain cultured in a medium supplemented with AA. After ammonium sulfate fractionation was performed, enzyme purification was performed by chromatography using various columns such as anion exchange, hydrophobic, and gel filtration columns. After that, the purified fraction was analyzed by SDS-PAGE, and an almost single band was observed as approximately 57 kDa. Furthermore, by means of molecular weight measurement using a gel filtration column, the molecular weight of the enzyme was calculated to be approximately 61 kDa, suggesting that the enzyme is a monomeric protein with a molecular weight of approximately 60 kDa.

Furthermore, to investigate the various properties of this enzyme, activity measurements were performed under various conditions using the purified enzyme. As a result, it was confirmed that the addition of Ac-CoA is essential for the enzyme activity. It was also confirmed that the highest activity was shown at a reaction temperature of 35°C and a pH of 7.0. Furthermore, N-terminal sequence analysis of the purified enzyme using a protein sequencer revealed high homology with acyl-CoA transferases/carnitine dehydratase from the genus *Fusarium* and CaiB from *Escherichia coli*.

Conclusion

Ac-CoA-dependent hydration of AA to 3HPA might proceed as the suggested pathway shown in the figure. Until now, the enzymes catalyzing such reactions have not been reported, so it suggests that the enzyme might be a unique enzyme.

Fig. 1



P3-18

Stereoselective isomerization-reduction one-pot cascade catalyzed by Old Yellow Enzymes

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[1]University of Graz, Institiute of Chemistry, Graz, Austria

PURPOSE OF THE ABSTRACT

Stereoselective methods to access chiral synthons in enantiopure form with high efficiency and low environmental impact are highly demanded by the chemical and pharmaceutical industry. The α-angelica lactone and γ-valerolactones, derived from glucose, are versatile bio-based building blocks with broad industrial applications, *e.g*. as bio-based polymer precursors, natural product and drug components, or fragrance/flavor agents.[1]

We recently reported the promiscuous and stereocomplementary behavior of flavin-dependent Old Yellow Enzymes (OYEs) in the asymmetric isomerization of α-angelica lactone in the absence of nicotinamide.[2] By coupling this reaction to the natural nicotinamide-dependent reductive activity of OYEs, an innovative one-pot cascade to access both enantiomers of γ-valerolactone with high ee values was achieved.[3]

The stereoselective isomerization of substituted β,γ-unsaturated butenolides into the corresponding chiral α,β-unsaturated butenolides is currently limited to a few organocatalytic protocols.[4] We, therefore, set out to develop a mild enzymatic protocol to access a range of substituted chiral lactones via an isomerization-reduction cascade applicable to β and γ-unsaturated butenolides (Figure 1). We demonstrated the ability of OYEs to accept substrates bearing various substitutions at the α- and γ-centers.

Starting from racemic β,γ-unsaturated butenolides, various γ-lactones could be accessed with high stereoselectivity. For instance, 5-butyldihydrofuran-2(3*H*)-one was obtained with 95% *ee* in 80% conversion over two steps using OYE from *Zymomonas mobilis* (NCR), while both enantiomers of 5-benzyldihydrofuran-2(3*H*)-one were obtained in >90% overall conversion and *ee* values between 95% and 94% using NCR and *Bf*OYE4, respectively.

Finally, we are considering the incorporation of an additional third chiral center to enrich molecular complexity and diversify our synthetic platform.

ACKNOWLEDGEMENTS

Acknowledgment is made to the FWF (Austrian Science Fund) P36538-N for supporting this research.

REFERENCES

[1] V. Farina, J. T. Reeves, C. H. Senanayake, J. J. Song, *Chem. Rev.* **2006**, *106*, 2734-2793.

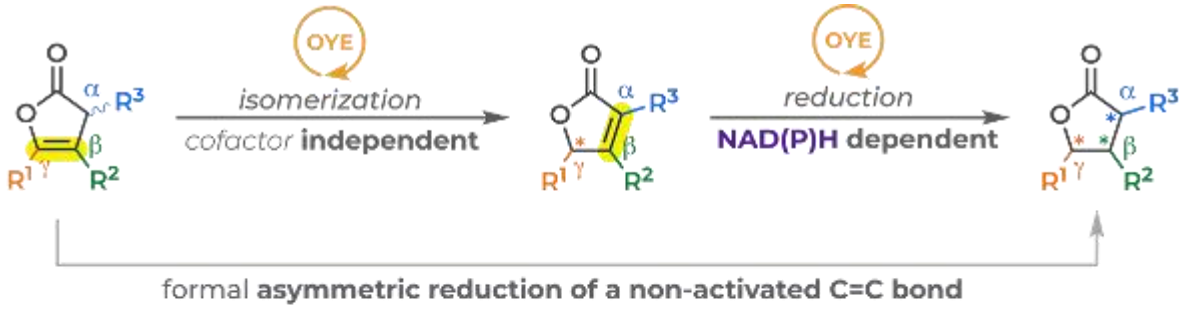
[2] M. S. Robescu, L. Cendron, A. Bacchin, K. Wagner, T. Reiter, I. Janicki, K. Merusic, M. Illek, M. Aleotti, E. Bergantino, M. Hall, *ACS Catal.* **2022**, *12*, 7396-7405.

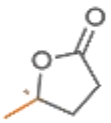
[3] A. Tonoli, K. Wagner, A. Bacchin, T. Reiter, E. Bergantino, M. S. Robescu, M. Hall, *ChemBioChem* **2023**, *24*, e202300146.

[4] Y. Wu, R. P. Singh, L. Deng, *J. Am. Chem. Soc.* **2011**, *133*, 12458-12461.

Figure 1: Biocatalytic access to chiral lactones in a simultaneous one-pot, two-step cascade.

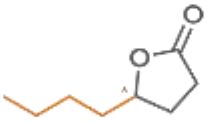
Fig. 1





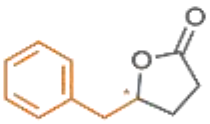
with **NCR**
90% *ee* (*R*)
93% conversion

with ***Bf*OYE4**
92% *ee* (*S*)
77% conversion



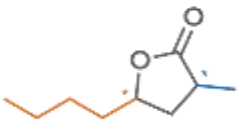
with ***Ps*OYE**
86% *ee* (*R*/*S*)
80% conversion

with **NCR**
95% *ee* (*S*/*R*)
80% conversion



with **NCR**
95 % *ee* (*R*/*S*)
97% conversion

with ***Bf*OYE4**
94% *ee* (*S*/*R*)
95% conversion



tbd *de*
tbd conversion

P3-19

The impact of the BLUETOOLS project on the biocatalytic activity of Servier

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[2]Eötvös Loránd University, Institute of Chemistry, Budapest, Hungary
[3]Budapest University of Technology and Economics, Department of Applied Biotechnology, Budapest, Hungary

1. Introduction

BLUETOOLS is an EU-funded Horizon Europe RIA program aiming at the sustainable exploration and exploitation of marine environments. 14 academic and industrial partners are researching the biological products of marine living systems, bring them to the laboratory and apply them on various fields, such as plastic degradation, medicine, and chemical synthesis.

One of the major contributions to the environmental footprint of a drug is the production of raw materials. Therefore, more environmentally friendly solutions, which reduce waste and energy consumption, could contribute to the more sustainable production of drugs. In the long run, this helps to preserve our environment which is a crucial component of CSR programs of many large enterprises.

2. Objectives

Enzymes can enable more sustainable chemical synthesis methods by lowering energy consumption, producing less waste and resulting in safer processes.[1] At Servier, we started to test and apply enzymes for chemistry six years ago. By screening novel enzyme panels provided by BLUETOOLS partners, we aim to find enzymes that can be the starting points of further optimization and scale-up.

3. Results

We could systematically apply enzymes throughout research and development complementing existing synthetic capabilities. Various enzymes from different classes, such as transaminases, ketoreductases, imine reductases, have been successfully applied in several projects to substitute challenging chemical steps up to 100-gram batches.

4. Conclusions

Access to the vast diversity offered by nature can have a large impact on the type and number of reactions we can target with biocatalysis.[2] BLUETOOLS is a great opportunity to further deepen our expertise in enzyme chemistry, enrich our enzyme portfolio and deliver better solutions for our process chemists.

References

[1] S. Simić et al. Chem. Rev. 2022, 122, 1052-1126.

[2] R. Buller et al. Science 2023, 382, eadh8615 (DOI:10.1126/science.adh8615).

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P3-20

Oxyfunctionalization of Terpenoids by Unspecific Peroxygenases

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Terpenoids, derived from the same C_5 isoprene skeleton, are known as the largest class of natural products with over 80,000 representatives. Their diversity arises from a variety of linkages, subsequent cyclizations and/or rearrangements of two simple C_5 building blocks – isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[2] This is associated with a broad range of applications in the pharmaceutical, flavor, and fragrance industries (see Figure 1).[3] Since modified terpenoids often exhibit more advantageous olfactory properties than their hydrocarbon backbone,[4] their oxyfunctionalization is of utmost importance for the discovery of new flavors and fragrances.

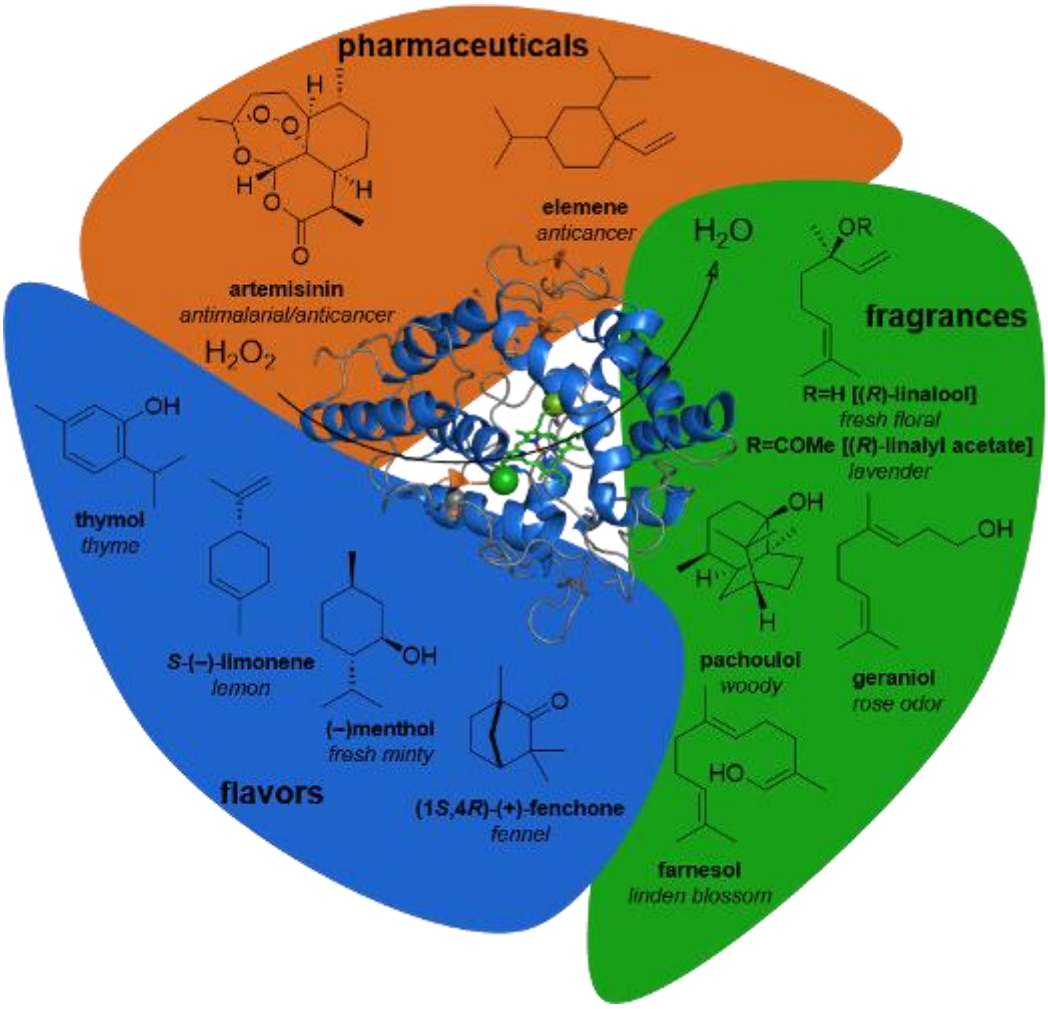
Fig. 1 The application fields of terpenoids and their potential oxyfunctionalization by UPOs.

Here, the two most prominent enzymes involved in the catalysis of oxyfunctionalization reactions are P450 monooxygenases (P450s) and fungal unspecific peroxygenases (UPOs). P450s are well discovered resulting in a vast number of sequences, accepted substrates, and significant variants.[5] However, they have many drawbacks such as (i) instability in organic solvents,[6] (ii) catalytic performance (10,000 turnover numbers vs. over 900,000 for UPOs),[7] (iii) cofactor dependency, and (iv) oxygen dilemma.[8,9] To circumvent these limitations, we turned our attention to UPOs and selected 78 different candidates to screen their activity against terpenoids that had previously been chemo-enzymatically synthesized.[10] To our delight, 34 UPOs accepted the in-house synthesized substrates resulting in mainly three different oxyfunctionalization patterns. For the three most promising UPOs, semi-preparative scale-ups were performed, the oxyfunctionalized products were isolated, and their structure was solved by 1D and 2D-NMR spectrometry. The regioselective oxyfunctionalization of terpenoids highlights the relevance of UPOs, as this reaction cannot be performed in any other chemical means, paving the way for unique flavors and fragrances.

References

[1] Y. Gao, R. B. Honzatko, R. J. Peters, *Nat. Prod. Rep.* **2012**, *29*, 1153–1175.
[2] I. I. Abdallah, W. J. Quax, *KnE Life Sci.* **2017**, *3*, 81–98.
[3] C. S. Sell, *Chemistry and the Sense of Smell*, John Wiley & Sons, **2014**.
[4] V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2019**, *37*, 882–897.
[5] T. Hilberath, A. van Troost, M. Alcalde, F. Hollmann, *Front. Catal.* **2022**, *2*.
[6] M. Hobisch, P. De Santis, S. Serban, A. Basso, E. Byström, S. Kara, *Org. Process Res. Dev.* **2022**, *26*, 2761–2765.
[7] D. Holtmann, F. Hollmann, *ChemBioChem* **2016**, *17*, 1391–1398.
[8] M. Hobisch, D. Holtmann, P. Gomez de Santos, M. Alcalde, F. Hollmann, S. Kara, *Biotechnol. Adv.* **2021**, *51*, 107615.
[9] C. Oberhauser, V. Harms, K. Seidel, B. Schröder, K. Ekramzadeh, S. Beutel, S. Winkler, L. Lauterbach, J. S. Dickschat, A. Kirschning, *Angew. Chem. - Int. Ed.* **2018**, *57*, 11802–11806.

Fig. 1


pharmaceuticals
artemisinin
antimalarial/anticancer
elemene
anticancer
H_2O_2
H_2O
fragrances
R=H [(R)-linalool]
fresh floral
R=COMe [(R)-linalyl acetate]
lavender
pachoulol
woody
geraniol
rose odor
farnesol
linden blossom
thymol
thyme
S-(−)-limonene
lemon
(−)menthol
fresh minty
(1S,4R)-(+)-fenchone
fennel
flavors

P3-21

Synthetic Reagents for Enzyme-Catalyzed Methylation

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The methyl group is one of the most commonly occurring carbon fragments in small-molecule drugs. During the exercise of designing a drug candidate, methyl groups are commonly installed in an effort to improve a molecule"s biological activity and physical properties.[1] Thus, methylation is a key technology in the development of pharmaceutical compounds. Methyltransferase (MT)-biocatalysis may provide powerful options to insert methyl groups into complex molecules with high regio- and chemoselectivity.[2] The major impediment to large-scale application of MTs is the high cost of using stoichiometric cosubstrate *S*-adenosyl-L-methionine (SAM) in addition to its inherent instability and poor atom economy as a methyl donor.[3] As a solution to this problem, we have developed a methodology that uses off-the-shelf reagents, such as methyl iodide (MeI) or methyl tosylate (MeOTs) as methylating reagents for the *in situ* regeneration of SAM.[4,5] In this presentation we will discuss our most recent efforts to optimize methylation biocatalysis for synthetic applications.

[1] H. Schönherr, T. Cernak, T. *Angew. Chem. Int. Ed.* **2013**, *52*, 12256–12267.

[2] E. Abdelraheem, B. Thair, R. F. Varela, E. Jockmann, D. Popadić, H. C. Hailes, J. M. Ward, A. M. Iribarren, E. S. Lewkowicz, J. N. Andexer, P. L. Hagedoorn, U. Hanefeld, *ChemBioChem* **2022**, *23*, e202200212.

[3] I. J. W. McKean, P. A. Hoskisson, G. A. Burley, *ChemBioChem* **2020**, *21*, 2890–2897.

[4] C. Liao, F. P. Seebeck, *Nat. Catal.* **2019**, *2*, 696–701.

[5] X. Wen, F. Leisinger, V. Leopold, F. P. Seebeck, *Angew. Chem. Int. Ed.* **2022**, *61*, e202208746.

P3-22

The potential of a new microbial diamine oxidase for the degradation of histamine in simulated intestinal fluid

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Histamine intolerance is due to an insufficiency of the endogenous enzyme diamine oxidase (DAO) (EC 1.4.3.22). DAO catalyzes the oxidative deamination of various biogenic amines, generating the corresponding aldehydes (imidazole-4-acetaldehyde from histamine), ammonia and hydrogen peroxide. Due to the DAO insufficiency in histamine intolerant individuals, dietary histamine is not sufficiently degraded in the small intestine and surpasses through the intestinal wall into the circulation, causing a disorder of histamine"s endocrine function. Currently, this intolerance is neither objectively diagnosable nor directly treatable by medication.

As the condition "histamine intolerance" is not yet fully understood, the objective of our research was to contribute to the expansion of knowledge about current and prospective treatment approaches by providing an active DAO preparation for *in vitro* histamine degradation experiments. As comparison, a commercially available dietary supplement for histamine intolerance was investigated for its histamine degrading capability under *in vitro* conditions and showed no DAO activity.

In order to provide an alternative, a new DAO (DAO-1) was discovered in the yeast *Yarrowia lipolytica* in an *in silico*screening and was biotechnologically produced. The DAO-1 was investigated specifically regarding its capability to degrade histamine in a simulated intestinal fluid (*in vitro*). It was found that distinctively higher DAO activities are required for a promising supplementation in histamine intolerant individuals than initially assumed. This was especially due to the proteolytic activity of pancreatin peptidases which caused a quick degradation of the DAO-1. However, it was shown that the presence of food compounds like different proteins and sugar distinctively improved the stability of the DAO-1, especially by delaying its proteolytic digestion. The DAO-1 was formulated as a prototype-tablet and degraded a high amount of histamine (22 mg) in the simulated intestinal fluid within 90 minutes.

Hence, it was shown that sufficient DAO activities can be supplied for the preparation of DAO tablets using the efficient biotechnological approach of a microbial DAO production. The histamine degradation achieved with the tablets could already be enough to contribute to the reduction of histamine intolerance symptoms. Furthermore, insights were gained into the stability and general histamine degrading capacity of DAO in simulated intestinal fluid.

P3-23

Amine-tolerant *E. coli* Strains Generated via Adaptive Evolution for Sustainable Synthesis of Chiral Amines

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Chiral amines are building blocks for various pharmaceuticals and fine chemicals. The biocatalytic synthesis of these compounds from prochiral ketones and ammonia is a major advance in sustainable synthetic chemistry.[1–2] Using whole-cells for bioamination reactions is advantageous given their low preparation cost and direct applicability; however, amine toxicity limits the reaction when living cells are used.[3] In fact, to match the toxicity profile of the amine products, whole-cell-based amination reactions are usually performed at low or moderate substrate loadings (3–25 mM), which ultimately results in low amine titers.[3–6] Therefore, we adapted *E. coli* BL21(DE3) cells to growth in the presence of high amine concentrations (100 mM) via adaptive directed evolution in continuous culture. The final adapted strain showed superior tolerance for various structurally different amines, displaying up to six times higher tolerance compared to the wild-type strain. Co-expression of genes encoding for amine dehydrogenase (AmDH) and formate dehydrogenase (FDH) activities in the adapted *E. coli* cells, enabled the stereoselective bioamination (ee >99%) of different ketones with up to 80% conversion at high substrate loading (up to 200 mM) without exogenous cofactor addition. This resulted in up to more than three times higher amine yields (up to 15 g L^{-1}) compared to that obtained using the non-adapted cells. The biocatalytic performance of the adapted cells was correlated to the longer survival and higher population density of the adapted production cells during the reactions. We believe that our *E. coli* system contributes to the development of more robust biocatalysis for amine production.

1. L. Ducrot, M. Bennett, G. Grogan, C. Vergne-Vaxelaire, *Adv. Synth. Catal*. 2021, 363, 328–351.
2. F. G. Mutti, T. Knaus in *Biocatalysis for Practitioners*, Eds. G. D. Gonzalo, I. Lavandera, Wiley-VCH 2021, 143–180.
3. J. A. Houwman, T. Knaus, M. Costa, F. G. Mutti, *Green Chem*. 2019, 21, 3846–3857.
4. L. J. Hepworth, S. P. France, S. Hussain, P. Both, N. J. Turner, S. L. Flitsch, *ACS Catal*. 2017, 7, 2920–2925.
5. J. E. Farnberger, E. Lorenz, N. Richter, V. F. Wendisch, W. Kroutil, *Microb. Cell Fact*. 2017, 16, 132.
6. D. I. Kim, T. U. Chae, H. U. Kim, W. D. Jang, S. Y. Lee, *Nat. Commun*. 2021, 12, 173.

P3-24

Enzymatic functionalization of citroflavonoids: Naringin acylation

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Naringin, a flavonoid recognized for its antioxidant properties [1], poses a challenge in its application to final formulations due to its low solubility in both water and organic solvents. In addressing this issue, we have employed enzymatic acylation to functionalize naringin, catalyzed by immobilized lipase *Candida antarctica* lipase B, and utilized acyl donors of varying chain lengths.

The acylation process involved three different molar ratios (1:3, 1:5, 1:10) in acetonitrile, selected as the preferred reaction solvent. Specifically chosen for this study were three activated acyl donors, acetate (C2), propionate (C3), and laurate (C12) vinyl esters. These vinyl esters were chosen with the explicit goal of evaluating their effects within the gastrointestinal environment. The resulting reaction products and solubility were assessed using HPLC. Conversion rates exceeding 90%, and in some instances, reaching 100%, were achieved within 48 hours. In the subsequent HPLC analysis, only the main monoacylated product was detected, with no observed isomers. This result was further supported by FTIR and MS/MS analyses.

Compared to naringin solubility, all acylated compounds increased their solubility either in water or in solvents, up to 14, 67, and 100-fold for naringin acetate, naringin propionate, and naringin laurate, respectively, as it"s shown in **table 1**.

Table 1. Solubility enhancement factor of naringin derivatives.

Solvent	**Naringin acetate**	**Naringin propionate**	**Naringin laurate**
Water	4	1	1
Ethanol	2	10	11
Methanol	14	58	100
Acetonitrile	11	67	22
Acetone	8	48	82

We reach a faster and efficient process to obtain naringin monoacylates, compared to others reported in previous studies with similar conditions like Yadav [2] and Sun [3]. In addition, less enzyme was used, and no need for further purification. Furthermore, the solubility was improved in different polar solvents by the modification of the naringin structure with an acyl group. These advancements have the potential to optimize the application of naringin within the food and cosmetic sectors, aligning seamlessly with our objective of gut modulation.

[1] D. Barreca, G. Mandalari, A. Calderaro, A. Smeriglio, D. Trombetta, M. R. Felice and G. Gattuso, Plants (Basel), **2020**, 9, 1-23.

[2] H.-M. Li, T.-T. Xu, Q.-X. Peng, Y.-S. Chen, H. Zhou, Y.-Y. Lu and R.-A. Yan, Journal of Food Science, **2021**, 86, 1714-1725.

[3] C. Q. Sun, K. D. Johnson, H. Wong and L. Y. Foo, Frontiers in pharmacology, **2017**, 8, 759.

P3-25

Immobilization of the condensing amidohydrolase MxcM and biocatalytic flow synthesis of imidazoline heterocycles

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Heterocycles are essential structural elements of many pharmaceuticals and agrochemicals. The chemical synthesis of heterocycles typically requires harsh reaction conditions and the use of hazardous agents. Due to these shortcomings, the synthetic utility of enzymes in heterocyclic chemistry is increasingly investigated. In recent years, several members of the amidohydrolase superfamily were described that catalyze intramolecular condensation reactions to form heterocycles in natural product biosynthesis pathways. Most of these condensing amidohydrolases were found to be involved in the formation of benzoxazoles, e.g., in the biosynthesis of the anticancer agent nataxazole.[1] In focus of this study is the condensing amidohydrolase MxcM, which was discovered in the biosynthetic pathway of the siderophore pseudochelin A, where it generates an imidazoline moiety via intramolecular condensation of a β-aminoethyl amide group.[2] Biochemical and kinetic characterization demonstrated that this enzyme features desirable properties for a potential use in chemical processes, as exemplified by its good storage stability and activity in organic solvents.[3] In addition, it does not require cosubstrates for its catalytic activity.

To further evaluate the synthetic utility of this enzyme, we developed a concept for the immobilization of MxcM.[4] Here, a hexahistidine tag was used for fixation on a solid, porous carrier. Our immobilization protocol leads to immobilization yields of ~75% and enzyme loadings between 7 and 8 wt%. In buffer, the remaining activity of the immobilized MxcM amounted to 30–40% compared to the free enzyme. We observed that the immobilization further improved the solvent tolerance of MxcM. Subsequently, we evaluated the performance of immobilized MxcM for biocatalysis in flow (Figure 1). The packed bed-immobilized enzyme reactors featured a good operational stability, indicating that no significant leaching events occurred and that the enzyme remains stable under operation in flow. Notably, the composition of the mobile phase greatly influenced the conversion, while the residence time and the temperature had only minor impact. In future, the presented HPLC-coupled flow system can be used to screen the substrate scope of the amidohydrolase MxcM for synthesis of imidazoline-containing, heterocyclic compounds.

Figure 1. Schematic representation of immobilized enzyme reactor (MxcM-IMER) used for biocatalytic flow synthesis of imidazoline heterocycles.

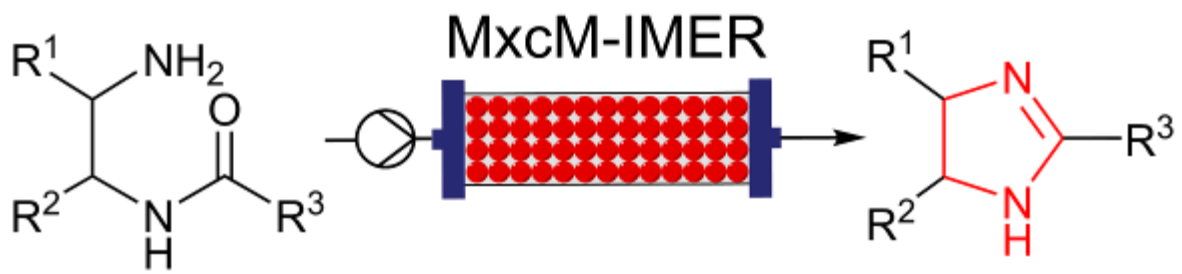
[1] Song H, Rao C, Deng Z, Yu Y, Naismith JH. *Angew. Chem. Int. Ed. Engl.* **2020**, *59*(15), 6054–6061.

[2] Korp J, Winand L, Sester A, Nett M. *Appl. Environ. Microbiol.* **2018**, *84*(22), e01789-18.

[3] Winand L, Vollmann DJ, Hentschel J, Nett M. *Catalysts* **2021**, *11*(8), 892.

[4] Winand L, Theisen S, Lütz S, Rosenthal K, Nett M. *Catalysts* **2023**, *13*(2), 229.

Fig. 1



P3-26

Protein engineering of glucosylglycerol phosphorylase facilitating regio- and stereoselective glycosylation of polyols

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O-Glycosylation of polyols is attracting great interest in manufacturing chiral polyol glucosides available in cosmetics and detergent industries. Achieving synchronously controlled regio- and stereoselectivities in glycosylation of polyols remains challenging. Here, we discovered that the glucosylglycerol phosphorylase from *Marinobacter adhaerens* (MaGGP) presented broad catalytic promiscuity in glycosylation of various polyols, particularly for high carbon 1,2-diols, with excellent regioselectivity and stereoselectivity. Then, we overcame the well-known activity–stability trade-off in enzyme engineering, and simultaneously improved thermostability and catalytic efficiency of MaGGP by 1200- and 13.7-folds, respectively. Crystal structure analysis and molecular dynamics simulation revealed the origin of enhanced thermostability and catalytic efficiency. It can guide the structure-based rational design for developing new catalytic properties.

By virtue of the beneficial mutant, we presented a thermodynamically favorable and ATP-free reaction system for glycosylation of polyols with low-cost starch as glycosyl donor. Several polyol glucosides with high conversion rate were successfully obtained. Furthermore, polypeptide SpyTag-SpyCatcher was employed to construct self-assembled multienzyme complex, and different combinations between enzymes and peptides were constructed and tested. The best self-assembled multienzyme complex exhibited 3-fold higher productivity than that of free enzyme. This synthetic approach overcomes the low atom economies of conventional biocatalytic methods and provides the promise in manufacturing other diversified valuable chiral 1,2-cis-glycosides available in pharmaceutical areas.

Reference

(1) Sun, X; Zhang, T; Liu, Y; Chen, P; Qin, H; Yang, J; Sun,Y. Self-assembled multienzyme complex facilitates synthesis of glucosylglycerol from maltodextrin and glycerol. J. Sci. Food Agric, 2023, 15, 104(1):266-272.

(2) Zhang, T; Liu, P; Wei, H; Sun, X; Zeng, Y; Zhang, X; Cai, Y; Cui, M; Ma, H; Liu, W; Sun, Y; Yang, J. Protein engineering of glucosylglycerol phosphorylase facilitating efficient and highly regio- and stereoselective glycosylation of polyols in a synthetic system. ACS Catal, 2022, 12, 24, 15715–15727.

P3-27

_m_-Nitrobenzoate production by utilizing _p_-aminobenzoate _N_-oxygenase

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1. Introduction

Nitroaromatic compounds have a wide range of industrial applications including pharmaceuticals, pesticides, dyes, and explosives. They are commonly synthesized through aromatic ring nitration using sulfuric and nitric acid. However, this reaction undergoes rigorous condition and needs precise control to prevent it from explosive outcomes. Enzymatic synthesis is an alternative way to produce nitroaromatic compounds as it is environmentally friendly and shows high selectivity. While the enzymatic synthesis of nitroaromatic compounds attracts attention, there are few varieties of enzymes that involved in nitroaromatic compounds synthesis, which limits the range of its application.

2. Objectives

Our objective is to produce *m*-nitrobenzoate, a non-natural nitroaromatic compound, using glucose as a substrate.

3. Results

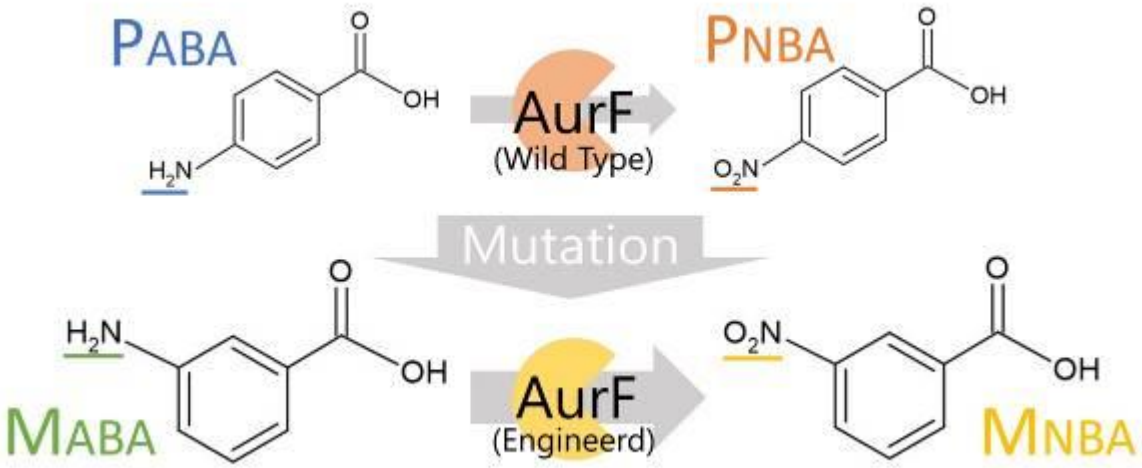
No enzyme that involved in synthesis *m*-nitrobenzoate has been found in nature so far. We engineered AurF: *p*-aminobenzoate *N*-oxygenase from *Streptomyces thioluteus*, which synthesizes *p*-nitrobenzoate from *p*-aminobenzoate by oxidizing the amino group, to modify its substrate specificity. Residues around the active site of AurF were replaced with other amino acids, and the mutants that have high activity towards *m*-aminobenzoate were screened. As a result, a mutant AurFY93F, T167I showed highest conversion rate (80 %) of all the constructed mutants, whereas that of wild-type AurF was 6 %. In addition, AurFY93I and AurFY93L showed relatively high conversion rates, indicating that replacement of Y93 with hydrophobic amino acids improves the activity towards *m*-aminobenzoate. Then, we achieved *m*-nitrobenzoate production from glucose by *Escherichia coli* introduced with PctV from *Streptomyces pactum* in addition to AurFY93F, T167I. PctV catalyzes the reaction forming *m*-aminobenzoate from 3-dehydroshikimate. As the conversion by PctV was found to be a bottleneck in this pathway, further improvement of the expression or activity of PctV is required to increase the production titer of *m*-nitrobenzoate.

4. Conclusion

In this study, we obtained the enzyme that is capable to efficiently synthesize *m*-nitrobenzoate, a non-natural nitroaromatic compound, by introducing mutations to AurF. Furthermore, *E. coli* with AurFY93F, T167I and PctV produced *m*-nitrobenzoate from glucose.

Figure 1. Schematic illustration of engineering AurF's substrate speficity.

Fig. 1


PABA
AurF
(Wild Type)
PNBA
Mutation
MABA
AurF
(Engineerd)
MNBA

P3-28

Heterologous Naringenin Production in the Filamentous Fungus *Penicillium rubens*

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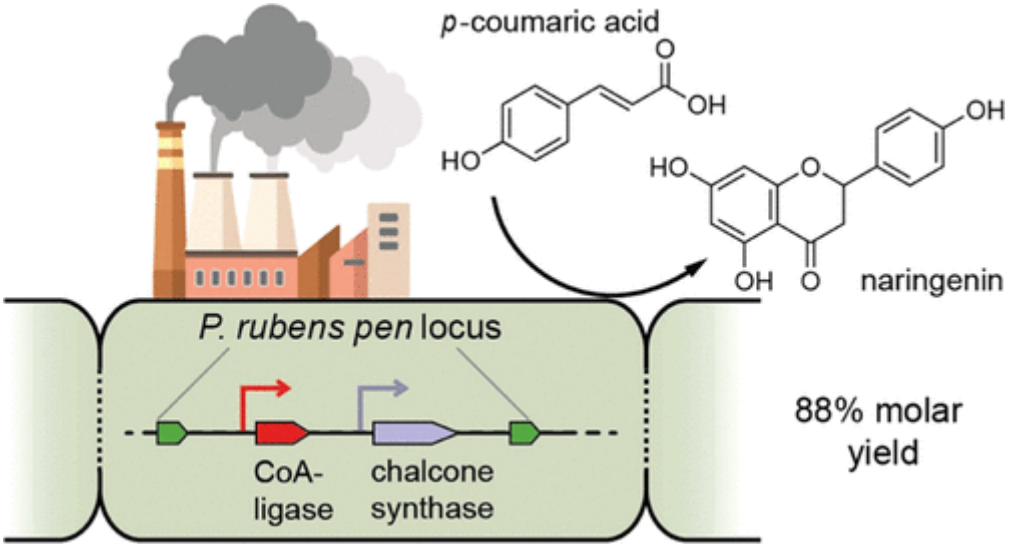
ABSTRACT: Naringenin is a natural product with several reported bioactivities and is the key intermediate for the entire class of plant flavonoids. The translation of flavonoids into modern medicine as pure compounds is often hampered by their low abundance in nature and their difficult and unsustainable chemical synthesis. Here, we investigated the possibility to use the filamentous fungus *Penicillium rubens* as a host for flavonoid production. *P. rubens* is a well-characterized, highly engineered, traditional "workhorse" for the production of β-lactam antibiotics. We integrated two plant genes encoding enzymes in the naringenin biosynthesis pathway into the genome of the secondary metabolite-deficient *P. rubens* 4xKO strain. After optimization of the fermentation conditions, we obtained an excellent molar yield of naringenin from fed *p*-coumaric acid (88%) with a titer of 0.88 mM. Along with product accumulation over 36 h, however, we also observed rapid degradation of naringenin. Based on high-resolution mass spectrometry analysis, we propose a naringenin degradation pathway in *P. rubens* 4xKO, which is distinct from other flavonoid-converting pathways reported in fungi. Our work demonstrates that *P. rubens* is a promising host for recombinant flavonoid production, and it represents an interesting starting point for further investigation into the utilization of plant biomass by filamentous fungi.

Fig. 1



P3-29

An Immobilized Silicon-Carbon Bond-Forming Enzyme for Anaerobic Flow Biocatalysis

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Introduction
The key to establish successful biocatalytic and sustainable applications lies in a holistic design of the reaction processes in suitable reactor systems, which includes the immobilization of enzymes and thus enables simplified product separation and reusability. The recent development of tailored cytochrome enzymes via directed evolution has enabled "new-to-nature" reactivities, such as the biocatalytic formation of carbon-silicon bonds using the cytochrome c from *Rhodothermus marinus*.[1]

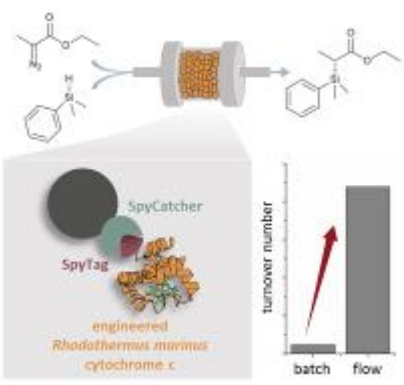
Objective
To maximize the potential of this remarkable biocatalyst by increasing its turnover numbers (TON) and to enable its application in continuous biocatalysis, we recently reported the use of the SpyTag/SpyCatcher (ST/SC)[2] bioconjugation system to immobilise this enzyme.[3]

Results
In our work, we successfully attached the ST to the enzyme without significantly affecting its catalytic activity. Even after immobilization on agarose microparticles, the enzyme retained 60% of its activity, as determined using a newly developed heme-specific analytical methods to quantify the amount of bound enzyme. When optimizing the conditions of the enzyme reaction, we found that using 25% acetonitrile as a co-solvent resulted in increased product formation, but the yield of the reaction was restricted by the reduced lifetime of the diazoester substrate and the solubility of the silane substrate in aqueous solutions. To overcome these limitations, we utilized a continuously operated packed-bed reactor with inline mixing under anaerobic conditions, in which the substrates are supplied as a solution in pure acetonitrile. This minimized preincubation time with buffer and ensured optimal cosolvent concentration for the production of the organosilicon resulting in up to 6-fold TON in continuous flow reactions over a total period of 10 days compared to the free enzyme reaction previously reported[1], and thus in much higher space-time-yields. However, we observed a drop in stereoselectivity under these conditions.

Conclusion
We demonstrated that the silicon-carbon bond-forming cytochrome c can be immobilized efficiently via the ST/SC system and employed in sequential batch reactions as well as in continuous flow reaction processes leading to improved TONs. Further optimization of the enzyme, the reaction conditions as well as the flow setup might enable improvements towards a more economically feasible application of this and similar heme-containing enzymes.

[1] S. B. Kan et al., F. H. Arnold (2016) "Directed evolution of cytochrome c for carbon-silicon bond formation: Bringing silicon to life", *Science*, *354*.
[2] B. Zakeri et al., M. Howarth (2012) "Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin", *P. Natl. Acad. Sci. USA*, *109*.
[3] S. Gallus et al., K. S. Rabe (2023) "An Immobilised Silicon-Carbon Bond-Forming Enzyme for Anaerobic Flow Biocatalysis", *ChemCatChem*, *15*.

Fig. 1



P3-30

A luminescence-based screening platform for lanthanide binding to proteins and peptides

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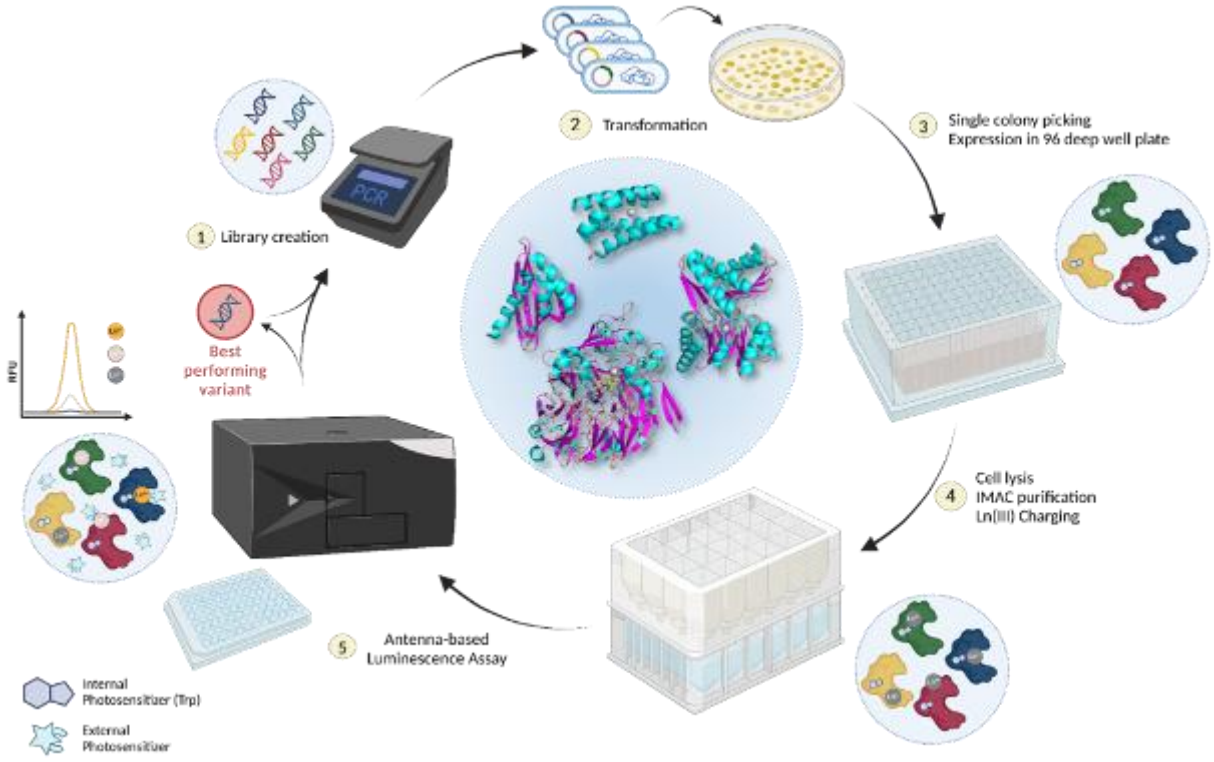
Rare-earth metals are powerful catalysts in photoredox reactions that facilitate radical formation under mild conditions. [1] However, stereocontrol of radical reactions is an innate challenge and often requires sophisticated ligands to tune the enantioselectivity. The design and directed evolution of artificial metalloenzymes (ArMs) is a strategy to efficiently improve the selectivity of such reactions,[2] but the number of available natural or designed lanthanide-binding proteins is limited. This is not just because lanthanides require a complex cellular import system,[3] but also as their strong oxophilicity evokes the tendency for unspecific binding to carboxylic groups and due to the formation of insoluble phosphate salts. [4] We thus aimed to develop an efficient 96-well plate screening platform to identify novel lanthanide binders. To that end, we utilize a dual system of internal and external photosensitizers that enhance lanthanide luminescence. The stringency of this protocol enables the screening of metal-binding peptides fused to a large expression tag, which were used for proof of concept. Furthermore, we could select lanthanide-binding *de novo* protein scaffolds with primitive substrate binding sites from designed smart libraries. The isolated hits have been characterized for metal binding affinity, selectivity, thermostability, and initial enzymatic activities. In conclusion, we could establish a procedure to screen for lanthanide-binding peptides and proteins with prospective applications in developing these scaffolds towards new-to-nature enzymatic reactions.

References

1. Prieto, A. & Jaroschik, F. Recent Applications of Rare Earth Complexes in Photoredox Catalysis for Organic Synthesis. *Curr. Org. Chem.* **26**, 6–41 (2021).
2. Bloomer, B. J., Clark, D. S. & Hartwig, J. F. Progress, Challenges, and Opportunities with Artificial Metalloenzymes in Biosynthesis. *Biochemistry* **62**, 221-228 (2022).
3. Featherston, E. R. & Cotruvo, J. A. The biochemistry of lanthanide acquisition, trafficking, and utilization. *Biochim. Biophys. Acta - Mol. Cell Res.* **1868**, 118864 (2021).
4. Daumann, L. J. Essential and Ubiquitous: The Emergence of Lanthanide Metallobiochemistry. *Angew. Chem. Int. Ed.* **58**, 12795–12802 (2019).

Fig. 1



P3-31

Exploration of key enzymes involved in the xenobiotic biotransformation of the fungus *Cunninghamella echinulata*

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Some fungi are models of mammalian metabolism, and thus a potential alternative to animal testing in drug development. The filamentous fungus *Cunninghamella echinulata* transforms a broad spectrum of xenobiotic compounds to mammalian-equivalent metabolites. Cytochromes P450 (CYPs) are the enzymes mostly responsible for production of phase I drug metabolites. However, the function of these enzymes is not thoroughly studied *in vitro*.

In silico analysis of the *C. echinulata* genome, using the previously identified CYPome of *Cunninghamella elegans*, revealed 37 genes putatively coding for CYPs. Of this, two *C. echinulata* CYPs were selected for analysis based on their similarity to two CYPs in *C. elegans* (5313D1 and 5313E1), which in turn have a high identity (approx. 31%) to the main mammalian CYP involved in drug detoxification, CYP3A4. Aditionally, CYP activity is typically dependent upon reducing power delivered by cytochrome reductases (CPRs), three of which are present in both *C. echinulata* and *C. elegans*.

Expression of CYP and CPR genes in a suitable host will enable investigation of their substrate specificity. A method for heterologous expression in *Pichia pastoris* was previously established in the PI"s group. Using this system, the aforementioned *C. echinulata* CYPs were heterologously expressed in *Pichia pastoris*. While here the activity is relying on the yeast"s own reductases, biotransformation of ibuprofen to 4"-hydroxyibuprofen was detected. Further investigation of these CYPs co-expressed with each reductase will lead to a better understanding of xenobiotic biotransformation in *Cunninghamella echinulata*.

P3-32

Nucleoside transferases of metagenome origin

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1. Introduction

Chemical exchange of nucleobases on nucleosides by classic chemical means is a difficult task. The manipulation of protecting groups consumes solvents, increases step count and generates more waste. To overcome these difficulties enzymes could impose a potential solution. Nucleoside 2'-deoxyribosyltransferases (NDTs) [1] or possibly nucleoside phosphorylases (NPs) [2] could be exploited on modified sugar moieties and non-natural nucleobases to construct a chemoenzymatic way for advanced pharmaceutical compounds as a green and sustainable alternative.

2. Objective

This study aims to find, develop and characterize enzymes within the framework of the Horizon Europe project BLUETOOLS, which can catalyze the transfer reaction between a nucleoside-like and a nucleobase-like molecule at an industrially acceptable way (Figure 1).

3. Results

We have identified a few enzymes of metagenome origin – provided by Bluetools partners - that can exchange nucleobases on 2'-fluoro nucleosides beside natural ribonucleosides. The nucleobase specificity of NDTs is more relaxed which has been demonstrated in the case of these enzymes, too.

Reaction condition optimization enabled higher transfer rate and reduced hydrolysis but the extent of modification on the substrate structure is still limited. These preliminary results indicate that the enzymes need to be modified to improve their activity towards more complex target molecules.

4. Conclusion

We have found novel enzymes which can catalyze nucleobase exchange on non-natural nucleosides but further development is needed. The full reaction space will be broadened by enzyme engineering which is supposed to help solve problems related to industrial applicability, too.

References

[1] J. Del Arco et al. *Biotechnol. Adv.* **2021**, *51*, 107701.

[2] S. Kamel et al. in Enzymatic and chemical synthesis of nucleic acid derivatives. (Eds J. Fernández-Lucas, M.-J. Camarasa Rius), p. 1-20, **2019**.

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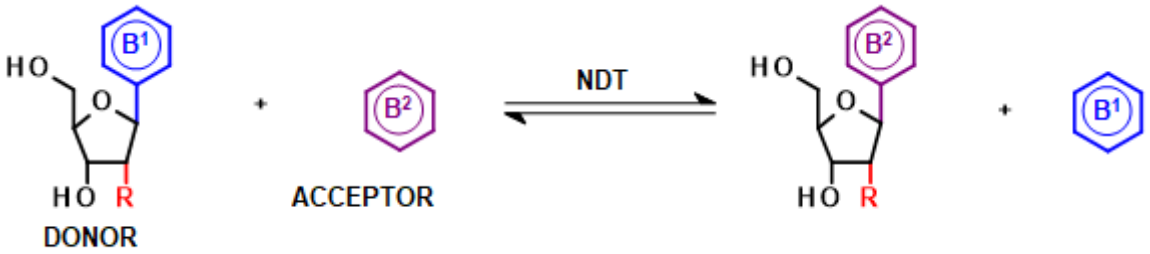
Project no. C2265084 has been supported by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund.

Fig. 1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P3-33

Application of thermostable phenolic acid decarboxylase in deep eutectic solvents

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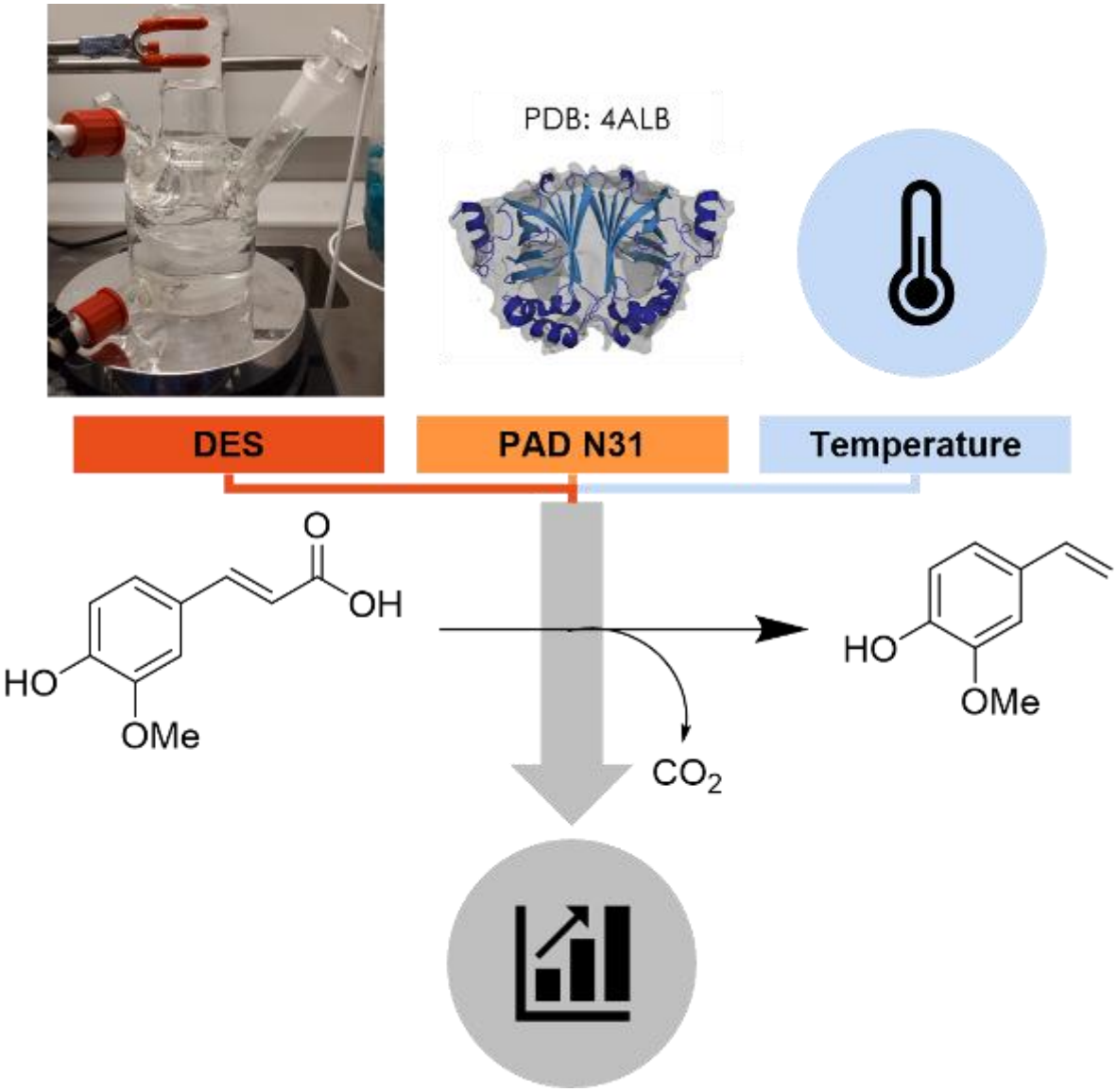
The synthesis of hydroxy styrenes by enzymatic decarboxylation of diverse hydroxy phenolic acids (e.g., ferulic acid, *p*-coumaric acid, caffeic acid, and sinapic acid) is a relatively new development. This environmentally friendly conversion also benefits from the extraction of renewable lignin-based substrates. However, enzymes usually need aqueous reaction media to work optimally. Whereas, most organic compounds are poorly soluble in water. A way to overcome this challenge is the use of non-conventional media, particularly deep eutectic solvents (DESs).[1,2] DESs are tunable in their physicochemical properties and are better compatible with enzymes than alternative organic solvents.[3] Despite the numerous advantages, the main challenge is the viscosity of DESs (e.g., 235±18 mPa·s for choline chloride:glycerol while water with 0.89 mPas·s, both at room temperature). Lowering impractical viscosities can be done by using higher temperatures3 and thus the need for an enzyme that tolerates higher temperatures and DESs is necessary.

Figure 1: Schematic illustration of the intensification of the decarboxylation of ferulic acid with PAD N31 in DESs at elevated temperatures. Abbreviations: PAD – phenolic acid decarboxylase, DES – deep eutectic solvent.

This study focuses on the thermally stable mutant phenolic acid decarboxylase (PAD) N31[4] and its characterization in two DESs, namely choline chloride:glycerol (ChCl:Gly, 1:2 mol/mol) and choline chloride:ethylene glycol (ChCl:EG, 1:2 mol/mol) with maximum 20 vol.% of buffer. By pushing the boundaries of the solubility by using DESs and higher temperatures (up to 70 °C) an intensification of the process can be achieved. We found the solubility of ferulic acid is around 9 times higher in ChCl:Gly (1:2) with 20 vol.% buffer and 13 times higher in ChCl:EG (1:2). For the buffer a K_M value of 4.6±1.2 mM and a V_{max} of 87±14 U/mg was achieved. For ChCl:Gly (1:2) and ChCl:EG (1:2) a V_{max}/K_M was determined with 0.137±0.07 U/mg and 0.193±0.02 U/mg, respectively. Both cases revealed first-order kinetics over a broad ferulic acid range, indicating very high K_M values.

1. Schweiger, A.K.; *et al., ACS Sustain. Chem. Eng.* **2019**,*19*, 16364–16370.
2. a) Petermeier, P. *et al.*, *Green Chem.* **2022**, *24*, 6889–6899.; b) Petermeier, P. *et al.*, *Commun. Chem.* **2024**, *7*, 57.
3. Zhang, N. *et al.*, *ACS Sustain. Chem. Eng.* **2021**, *9*, 8347–8353.
4. Myrtollari K. *et al. ACS Sustain. Chem. Eng.* **2024**, *12*, 3575–3584.

Fig. 1


PDB: 4ALB
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CO_2

P3-34

Aerobic 1,2-propanediol production from glucose by decoupling metabolic pathways in *Escherichia coli*

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[2]RIKEN, Center for Sustainable Resource Science, Yokohama, Japan

1. Introduction

Microbial production of chemicals gets attention due to increase environmental concerns and its studies using various microorganisms have been worked. 1,2-propanediol (1,2-PDO) is one of commodity chemicals and its sustainable production is promising because the high pressure and high temperature are needed in conventional chemical method. In general, the microbial 1,2-PDO production, particularly when using *Escherichia coli*, has been studied under anaerobic condition. However, this anaerobic cultivation process faces several challenges such as low cell growth and the limit of the supply of precursors of 1,2-PDO.

2. Objectives

We aim to achieve high 1,2-PDO production with high yield using engineered *E. coli*. For gaining sufficient cell growth, we focused on aerobic 1,2-PDO production.

3. Results

To avoid above hurdles involved in conventional microbial 1,2-PDO production, we divided metabolic pathways into two roles; production of target chemical using glucose and cell growth using xylose. We achieved aerobic 1,2-PDO production by deletions five genes (*eno*, *eda*, *sdaA*, *sdaB* and *tdcG*) involved in central metabolism in MG1655Δ*gloA* strain to isolate the 1,2-PDO synthetic pathway from central metabolism and by introducing the xylose assimilation pathway, Weimberg pathway from *Caulobacter crescentus*. The enhancement of D-xylonate assimilation and the deletion of byproduct synthetic pathway competing with 1,2-PDO production improved 1,2-PDO production. We finally achieved 2.48 ± 0.15 g/L 1,2-PDO with a 0.27 ± 0.02 g/g-glucose yield after 72 h cultivation in engineered strain.

4. Conclusion

We demonstrated aerobic 1,2-PDO production in engineered *E. coli* with utilizing substrates effectively. Our results could be applied to other chemical production suffering from production constraints like 1,2-PDO.

P3-35

Engineering of *Corynebacterium glutamicum* for *trans*-cinnamic acid and styrene production from glucose

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[2]Kobe University, Graduate School of Science, Technology and Innovation, Kobe, Japan

1. Introduction

Styrene stands as an exceptionally versatile chemical compound, predominantly utilized in the synthesis of polystyrene. The versatility of trans-cinnamic acid (tCA) extends across a wide spectrum of applications, ranging from aromatic constituents in perfumery to essential components in food, cosmetics, pharmaceuticals, and agrochemicals. Nevertheless, the quest for tCA production from more economical carbon sources such as glucose remains unattained. In parallel, the realization of styrene production from C. glutamicum has yet to be reported.

2. Objectives

Our objective is the development of an efficient styrene and tCA producing *Corynebacterium glutamicum* by enzyme engineering and metabolic engineering.

3. Results

Through screening for phenylalanine ammonia lyase (PAL), we identified BdPAL6 derived from *Brachypodium distachyon* as exhibiting the highest catalytic activity for tCA synthesis. By enhancing the flux to phenylalanine production and optimizing the gene expression patterns, we produced 3.59 g/L of tCA from glucose in test-tube cultures. Furthermore, 12.3g/L of tCA was successfully produced from 80g/L of glucose using jar fermenter. Subsequently, we unveiled the indispensability of co-expressing prenyltransferases encoded by UbiX for the enzymatic activity of ferulic acid decarboxylase (FDC) in *C. glutamicum*. After selection of the appropriate promoter and re-optimizing the expression pattern, 2.73 g/L of styrene was produced in test-tube cultures, furthermore, 5.0 g/L of styrene was successfully produced in jar fermenter.

4. Conclusion

We developed the metabolically engineered C. glutamicum to produce tCA and styrene from glucose. The selection of PAL for tCA synthesis, optimization of gene expression for increased production, and the necessity of co-expressing prenyltransferase UbiX for styrene production were demonstrated. We achieved significant tCA and styrene yields in both test-tube cultures and jar fermenters, suggesting C. glutamicum as a promising host for the production of these compounds.

P3-36

Whole-cell photocatalysis with a surface-displayed *de novo* cerium enzyme

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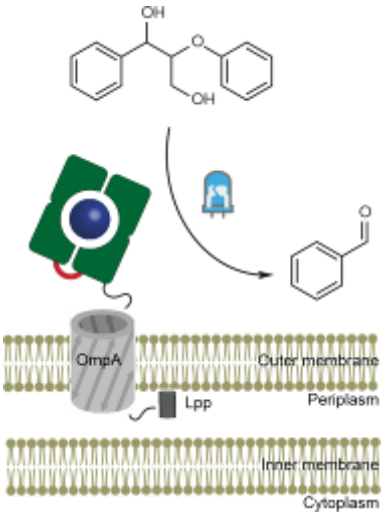
Photobiocatalysis has emerged as a powerful tool for enabling new-to-nature reactions with enzymes.1 Artificial photoenzymes can be generated by incorporating organic or metal-based photocatalysts into a protein scaffold. In organic synthesis, the use of earth-abundant and inexpensive lanthanide complexes for photoredox catalysis has gained popularity.2 Especially cerium complexes are well suited to catalyze a broad spectrum of photoreactions under relatively mild conditions.3 Inspired by this, we generated a cerium-dependent photoenzyme by equipping a *de novo* protein scaffold with a dative cerium binding site.4,5 The resulting photoenzyme catalyzes the cleavage of 1,2-diols, such as hydrobenzoin, to the respective carbonyl products. Moreover, expressing the enzyme on the surface of *E. coli* and subsequent charging with cerium(III) allowed the use of the system in a whole-cell catalysis approach. We show that the whole-cell photobiocatalyst can be applied for the degradation of lignin surrogates into synthetically useful building blocks. In the future, this cell surface display strategy will facilitate more efficient directed evolution of the protein by reducing the screening effort, as purification of the enzyme is no longer necessary. Furthermore, it opens the possibility for using the enzyme in *in vivo* selection systems.

Figure 1:E. coli surface display of the de novo cerium enzyme and light dependent degradation of a lignin surrogate.5

References

1. Emmanuel, M. A. *et al.* Photobiocatalytic Strategies for Organic Synthesis. *Chem. Rev.* **123,** 5459–5520 (2023).
2. Prieto, A. & Jaroschik, F. Recent Applications of Rare Earth Complexes in Photoredox Catalysis for Organic Synthesis. *Curr. Org. Chem.* **26,** 6–41 (2022).
3. Qiao, Y. & Schelter, E. J. Lanthanide Photocatalysis. *Acc. Chem. Res.* **51,** 2926–2936 (2018).
4. Caldwell, S. J. *et al.* Tight and specific lanthanide binding in a de novo TIM barrel with a large internal cavity designed by symmetric domain fusion. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 30362–30369 (2020).
5. Klein, A. S. *et al.* A de novo metalloenzyme for cerium photoredox catalysis. *ChemRxiv* 10.26434/chemrxiv-2024-6g4px (2024)

Fig. 1



P3-37

Substrate scope of two alkene cleaving lignostilbene-α,β-dioxygenases

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[3]BASF SE, Ludwigshafen am Rhein, Germany

Oxidative alkene cleavage is an important reaction in organic chemistry. While synthetic organic methodologies either use transition metal oxides or small organic molecules as stoichiometric oxidant, there are several enzyme families that can perform this reaction, using mostly iron as a cofactor.[1] Lignostilbene cleavage dioxygenases (LSDs), which are a subfamily of carotenoid cleavage dioxygenases, are non-heme dependent enzymes able to cleave *p*-hydroxy-substituted stilbenes employing molecular oxygen as oxidant.[2] Suggestions on the mechanism have been made based on EPR spectroscopy as well as MM/QM calculations, which both indicate that the cleavage reaction starts with a radical attack of an iron-oxo complex in the β-position of the substrate and then continues with the addition of the second oxygen to the α-position of the substrate.[2b, 3] Recently, LSDs have been used for a chemoenzymatic cascade towards flavor aldehydes.[4]

In this work, the substrate scope of the two LSD variants NOV1 S283F, from *Novosphingobium aromaticivorans* DSM 12444, and CO-03 C26N, from *Sphingomonadales bacterium,* has been investigated covering a panel of stilbene and styrene derivatives as well as bicyclic compounds. Additionally, investigations on the position of radical attack were performed using two radical clocks bearing a cyclopropyl ring either in α- or β-position of the substrate.

The substrate scope investigations showed that only *p*-hydroxy-substituted compounds without electron-withdrawing group in the β-position were converted. Additionally, only NOV1 S283F was active on resveratrol. The isolated reaction products were characterized by NMR. The radical clock (*E*)-4-(2-cyclopropylvinyl)phenol was not accepted by the enzymes. The compound 4-(1-cyclopropylvinyl)phenol was converted by as well as in absence of enzyme. The isolated reaction products of this substrate have been analysed by NMR, where the cyclopropyl ring was not found, indicating a radical attack on the β-position followed by ring opening.

In conclusion, the substrate scope of the two lignostilbene cleaving dioxygenases was shown to be limited to *p*-hydroxystyrene derivatives and resveratrol.

References:

[1] F. G. Mutti, *Bioinorg. Chem. Appl.* **2012**, *2012*, 626909.

[2] a) E. Kuatsjah, A. C. K. Chan, R. Katahira, S. J. Haugen, G. T. Beckham, M. E. P. Murphy, L. D. Eltis, *J. Biol. Chem.* **2021**, *296*, 100758; b) R. P. McAndrew, N. Sathitsuksanoh, M. M. Mbughuni, R. A. Heins, J. H. Pereira, A. George, K. L. Sale, B. G. Fox, B. A. Simmons, P. D. Adams, *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 14324-14329.

[3] J. Lu, W. Lai, *ChemistryOpen* **2019**, *8*, 228-235.

[4] S. Giparakis, M. Winkler, F. Rudroff, *Green Chem* **2024**, *26*, 1338-1344.

Acknowledgments: The COMET center: acib: Next Generation Bioproduction is funded by BMK, BMAW, SFG, Standortagentur Tirol, Government of Lower Austria und Vienna Business Agency in the framework of COMET - Competence Centers for Excellent Technologies. The COMET-Funding Program is managed by the Austrian Research Promotion Agency FFG.

P3-38

Biocatalytic depolymerization of lignin into mono-aromatic molecules

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Introduction

Lignin, an amorphous aromatic heteropolymer and major component in lignocellulose, can serve as a sustainable source for aromatic platform [1]. In nature, different biocatalytic approaches exist to depolymerize lignin, of which reductive pathways have been found to involve glutathione-dependent etherases and lyases, combined with NAD-dependent Cα-alcohol dehydrogenases, that specifically cleave the β-O-4 linkages in lignin model compounds [2]. To utilize this pathway for lignin depolymerization as an *in vitro* cascade, an NADH-dependent GAR is additionally introduced to enable *in situ* cofactor regeneration [3]. Depending on the lignin source, this cascade allows for the production of up to three different types of phenylpropanoid products, namely guaiacyl (G)-, syringyl (S)- and phenyl (H)-hydroxypropanone (Figure 1).

Objectives

To increase the yield of desired monoaromatic products, lignins from different sources and extracted via different methods were tested in this cascade. Furthermore, important reaction parameters were varied and their impact on product yield improvement was studied.

Results

Lignins derived from different biomass sources resulted in varying ratios of released phenylpropanoids, in agreement with the lignin"s composition as determined by 2D NMR analysis. Furthermore, the depolymerization of lignins with a high proportion of unmodified β-O-4 aryl ether linkages resulted also in higher monomeric product yield. Additional investigations into the impact of individual reaction parameters revealed that lignin concentration as well as cosolvent concentration had a major impact on the total monoaromatic product yield achieved by the cascade.

Conclusion

Lignin can be depolymerized effectively using this enzyme cascade with specific formation of three different phenylpropanoid products. Furthermore, product yields up to 200 mg/L could be achieved by utilizing lignins with high proportion of β-O-4 linkages as well as adjusting lignin and cosolvent concentrations.

References

[1] R. Rinaldi et. al., *Angew Chem Int Ed Engl.* **2016**, 55, 8164-8215.

[2] J. Husarcíková et al., *Appl Microbiol Biotechnol.* **2018**, 102, 5391-5401.

[3] J. Reiter et al., *Green Chem*. **2013**, 15, 1373-1381.

Fig. 1

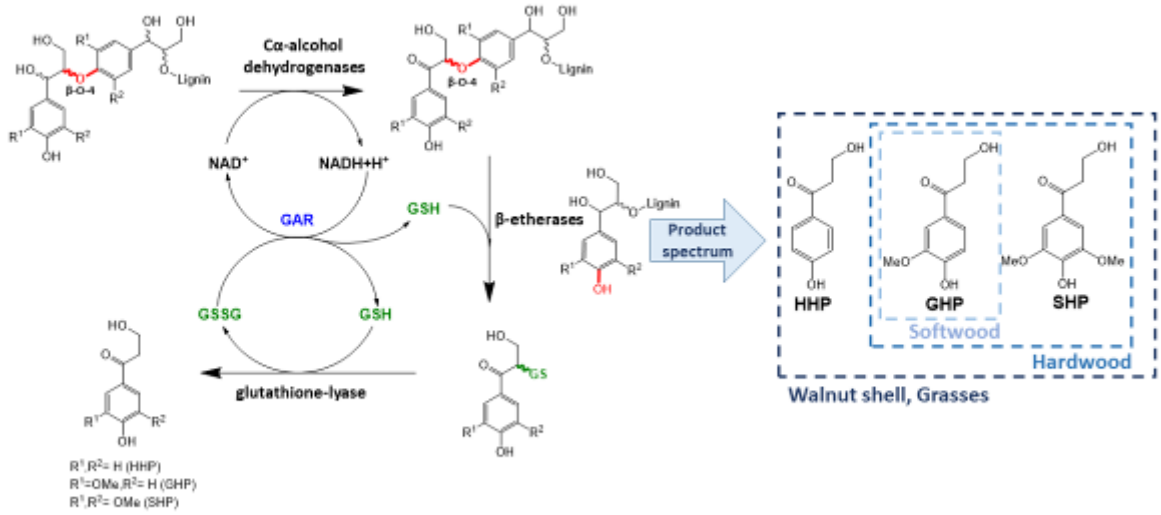

Figure 1: Lignin depolymerization via reductive β-O-4 aryl ether cleavage using an enzymatic cascade as well as resulting major monoaromatic products.

P3-39

Sulfation of phenolic compounds by novel aryl sulfotransferases

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Introduction: Polyphenols are widespread substances in nature and represent an important group of secondary plant metabolites. Studies have shown that these substances have various therapeutic, antioxidant and anti-inflammatory effects. Polyphenols are metabolized in the body during biotransformation Phase II, when they can be sulfated, glucuronylated or methylated. Defined standards of sulfated flavonoids are required for metabolic studies and to determine their bioactivity. The synthesis of sulfated metabolites could help to understand the biotransformation of polyphenols and their effects on the human body. Isolation of these metabolites from biological material is impractical, but they can be synthesized in vitro using chemical and chemo-enzymatic approaches[1,2].

Objectives: So far, only a few aryl sulfotransferases (ASTs) have been described. The best known are the ASTs from *Desulfitobacterium hafniense*[3] and *E. coli* CFT073[4]. Our aim was therefore to produce and characterize new ASTs for the sulfation of phenolic compounds. Based on the sequences of the known enzymes, four new potential ASTs were selected. The new ASTs were produced in *E. coli* and purified to homogeneity. The enzymes were characterized and their substrate specificity was determined with various flavonoids and phenolic acids.

Results: All produced enzymes were capable to sulfate the selected substrates using *para*-nitrophenyl sulfate as a sulfate donor. The presence of the sulfated products was confirmed by HPLC-MS analysis. Based on the tests of the individual ASTs, one of the new ASTs was selected for the preparative production of kaempferol sulfate. The product of the synthesis was isolated and structurally characterized.

Conclusion: Some of the new ASTs, such as the ASTs from *Campylobacter fetus* and *Desulfofalx alkaliphila*, were more effective in sulfating phenols than previously known ASTs. These enzymes can also be used for the preparative sulfation of flavonoids, such as kaempferol, which was enzymatically produced for the first time in this study. This research underlines the importance of finding new opportunities for synthetic applications and subsequently for deconjugation.

Supported by Czech Science Foundation, No. 23-04654S.

[1]Kolaříková, V., Brodsky, K., Petrásková, L., Pelantová, H., Cvačka, J., Havlíček, L., Křen, V., Valentová, K., *Int. J. Mol. Sci*. **23**, 15171 (2022)

[2]Brodsky, K., Káňová, K., Konvalinková, D., Slámová, K., Pelantová, H., Valentová, K., Bojarová, P., Křen, V., Petrásková, L. *ChemSusChem* **15** (2022)

[3]van der Horst, M.A., van Lieshout, J.F.T., Bury, A., Hartog, A.F., Wever, R. *Adv. Synth. Catal*. **354**, 3501–8 (2012)

[4]Malojčić, G., Owen, R.L., Grimshaw, J.P.A., Brozzo, M.S., Dreher-Teo, H., Glockshuber, R. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 19217–22 (2008)

P3-40

Conversion of yeast *Saccharomyces cerevisiae* β-1,3/1,6-glucans to functional disaccharides, laminaribiose and gentiobiose, using endo-glucanases PsLam81A and PsGly30A

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The use of biomass-derived polysaccharides for food and nutrition is one of the measures to achieve a carbon-neutral bioeconomy, according to the European Polysaccharide Network of Excellence (EPNOE) Research Roadmap 2040. Yeast cell walls (CWs) are inexpensive, non-toxic and compositionally valuable industrial by-products consisting mainly of β-1,3/1,6-glucan polysaccharides (up to 70% of the total mass) and can be obtained from spent brewer"s yeast or during the production of yeast extract. Currently, yeast CWs are treated by physical, chemical and enzymatic methods to obtain β-glucans with immunomodulatory properties. A common drawback of these extraction methods is the low specificity, resulting in β-glucan products with inconsistent biological activity. The enzymatic conversion of yeast CWs-related by-products into water-soluble oligosaccharides, e.g. the disaccharides laminaribiose and gentiobiose, suggests an alternative approach for the application of β-glucans. Laminaribiose is a potent prebiotic and seed germination enhancer, whilst gentiobiose is a flavour enhancer with a refreshing bitter taste resembling chocolate, cocoa and coffee.

The aim of our study was to identify bacterial enzymes capable of selectively releasing laminaribiose and gentiobiose from β-1,3/1,6-glucans present in the CWs of the yeast *Saccharomyces*. The crude yeast CWs preparation was used as a carbon source to screen environmental samples (e.g. soil, water) for the presence of microorganisms that efficiently hydrolyse yeast CWs. The secretome of the *Paenibacillus* sp. GKG was analysed using an LC-MS/MS-based proteomics approach to identify potential carbohydrate-active enzymes (CAZymes). The genes encoding potential CAZymes, PsLam81A and PsGly30A, were successfully cloned and heterologously expressed in *Escherichia coli*.

The hydrolysis selectivity pattern for different β-glucan standards revealed that PsLam81A is an endo-β-1,3-glucanase and PsGly30A is an endo-β-1,6-glucanase. PsLam81A and PsGly30A hydrolysed yeast CWs to laminaribiose and gentiobiose as the main hydrolysis products, respectively. PsLam81A showed the highest activity on laminarin (108.8 ± 1.7 U/mg), the truncated form PsLam81AΔCBM56 lacking the carbohydrate-binding module showed decreased hydrolysis efficiency on water-insoluble polysaccharides compared to the full-length enzyme PsLam81A. PsGly30A showed distinctive properties compared to other GH30 family β-1,6-glycosidases – high catalytic efficiency for pustulan (1262±82 U/mg) and low hydrolytic activity towards gentiobiose. In addition, a novel strategy for the chemoenzymatic synthesis of *p*NP-β-gentiobioside (*p*NPG2) was demonstrated. *p*NPG2 was used to monitor the dynamics of the residual activity of PsGly30A in yeast CWs hydrolysate**.**

Thus, we have demonstrated that the selected hydrolases are suitable for the valorisation of yeast CWs by enzymatic conversion.

P3-41

Biodiesel production through lipase-based reactions and innovative enzymatic approaches

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The global demand for energy has led to both environmental degradation and the depletion of energy reserves. To address this challenge, there is a pressing need to explore alternative fuel sources, such as biodiesel. Biodiesel, a renewable energy option synthesized through the transesterification reactions of vegetable or animal oils with short-chain alcohols like methanol, offers a promising solution [1]. First-generation biodiesel utilizes food crops like soybean or palm oil, while second-generation biodiesel employs non-food sources such as waste cooking oil or acidic oil [2]. Lipasesstand out as key catalysts for biodiesel production for their stability, biodegradability, efficiency, and catalytic activity [2]. Additionally, cutinases exhibit potential in biodiesel production by catalyzing hydrolysis, esterification, and transesterification reactions, proving valuable in detergent and biodiesel industry [2, 3]. Acyltransferases also emerge as intriguing biocatalysts with promising applications in the green oleochemical industry [2, 4].

In our study, we explore the potential of seven biocatalysts for second-generation biodiesel production. These include *Rhizopus oryzae* lipase (Biolipasa-R, Biocon®-Spain), immobilized *Candida antarctica* lipase B (Novozym® 435), LIP2 from *Yarrowia lipolytica*, whole cells of *Yarrowia lipolytica* with surface-displayed LIP2 [5], cutinases from *Humicola insolens* (HiC) and *Fusarium oxysporum* (FoC), and an acyltransferase from *Pyrobaculum calidifontis* VA1 (PestE) [6]. Although our research with cutinases and acyltransferases is in its preliminary stages, promising results have been achieved in pNPB hydrolysis reactions, with HiC exhibiting a specific activity of 50.1 U/mg, FoC 327.1 U/mg, and PestE 26.7 U/mg. These biocatalysts will undergo protein engineering and further evaluation for biodiesel production using oils sourced from the biodiesel industry.

As for lipases, we evaluate their performance based on biodiesel conversion, reaction time, catalytic efficiency, and immobilization effectiveness. To enhance productivity, lipases are immobilized on diatomaceous earth, a cost-effective and environmentally friendly silicon dioxide-based material, as well as on commercial supports like Purolite® methacrylate resins. Initial findings reveal the highest conversion rates with free Biolipasa-R, achieving an 89% conversion rate with sunflower oil, followed by immobilized Biolipasa-R on macroporous divinylbenzene, achieving 81%. Additionally, for acidic oil, favorable results were obtained with secreted LIP2, yielding a conversion rate of 76%.

References:

[1] Moschona et al., *Appl. Biochem. Biotechnol.,* **2024**, *accepted*.

[2] Spanou et al., *Frontiers Catal.,* **2024**, *4*, 1360702.

[3] Bermúdez-García et al., *Appl. Biochem. Biotechnol.,* **2016**, *182*, 1014-1036.

[4] Mandal et al., *Biores. Technol.*, **2020**, *297*, 122439.

[5] Georgiadis et al., *Microorganisnms*, **2023**, *11*, 1152.

[6] Terholsen et al., *ChemBioChem*, **2022**, *23*.

P3-42

Biocatalytic CH-oxidation reactions with white-rot fungi

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The transformation of aliphatic hydrocarbons to oxygen-containing products is of vital practical importance both for laboratorial and industrial chemistry. The development of an effective approach for the oxidation of aliphatic CH-bonds by using enzymatic strategies attracts a lot of attention in order to replace hazardous reagents such as strong electrophiles or oxidizing agents like nitric or sulphuric acid with eco-friendly natural biocatalysts. White-rot fungi (WRF) have the unique ability to degrade the robust natural polymer lignin [1] by developing an enormously rich enzymatic profile [2]. This makes WRF, which are among the most "aggressive" natural species, quite promising for effective aliphatic CH-oxidation reactions. In comparison to numerous biocatalytic systems, such as bacteria or yeasts, WRF-based systems stand out due to their exceptional enzyme stability, non-toxic nature, and simplicity in use.

The oxidative activity of growing cultures of WRF with already sequenced genomes toward adamantane, diamantane and their derivatives such as ketones and alcohols has been studied. It was found that *Trametes versicolor* and *Cerrena zonata* demonstrate efficient oxidation of CH-bonds in diamondoid ketones, concurrently reducing the carbonyl group. The biotransformation to the corresponding dihydroxy-derivatives occurred within only three days in growing cultures, which is unexpectedly fast for such atypical bulky substrates that notably differ from the fundamental lignin skeleton. These products were isolated with high preparative yields and fully characterized.

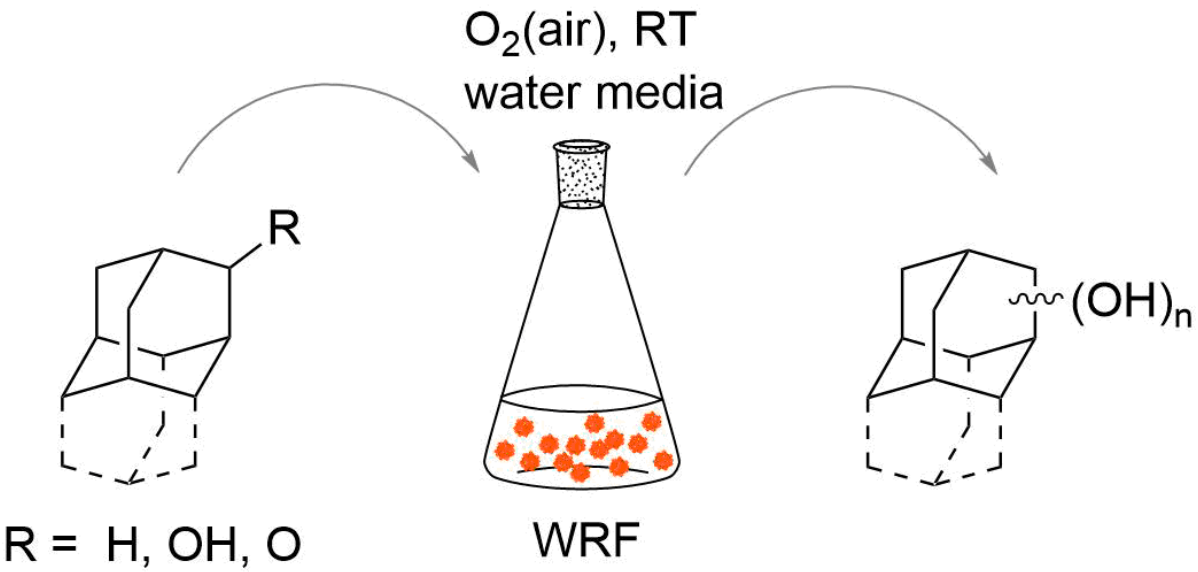
Figure 1. Biotransformations catalyzed by WRF

In case of *C. zonata*, the membrane-bound nature of the responsible enzymes, which belong to the class of oxidoreductases, was disclosed. These enzymes were only expressed in the cultures after their supplementation with a small amount of cage hydrocarbon ketones such as adamantanone or diamantanone; No activity has been detected in isolated membrane fractions of non-supplemented cultures of *C. zonata*. The isolation and purification of the key enzymes utilizing traditional biochemical techniques are currently underway. In parallel, transcriptome analyses of non-supplemented and supplemented cultures were also performed. The results indicate that the target proteins represent P450-type monooxygenases. Currently, these bioinformatic data are used for *in silico* analysis in order to identify the enzymes responsible for the biotransformation.

This research could provide crucial insights for both, chemistry and biology, as fungi generate complex mixtures of various enzymes and produce a diverse range of products. At the same time, isolated and characterized enzymes can improve the understanding of the mechanisms of WRF action, since information about these processes is currently insufficient.

1. M. Madadi, A. Abbas, *J. plant pathol. microbiol.*, 2017. **8**(2): 1000398.
2. T.K. Lundell, M.R. Mäkelä K. Hildén, *J. Basic Microbiol.*, 2010. **50**(1): 5-20.

Fig. 1


O_2(air), RT
water media
R
$(OH)_n$
R = H, OH, O
WRF

P3-43

Multi-enzyme/whole cell catalytic production of short-medium chain terminal alkane diols and diacids

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The rising issue of plastic pollution and the limited motivation for mechanical recycling urges the academia and industry to develop the technologies that enable close- or open- loop recycling and upcycling. To address this, the ACTPAC project seeks to develop a practical method to transform chemically inert C-C backboned plastic waste, specifically polyethylene (PE), into high-value monomers and biochemicals. This method combines chemical and biological processes to convert polyethylene through a series of steps: from PE to alkanes, then to terminally functionalized monomers, and finally back into polymers, establishing a complete production cycle (**Figure 1**).

Figure 1: Processing path from waste polyethylene plastics to oxy-functionalized alkanes and new polyester plastics.

One of the pivotal challenges in this transformation is the biotransformation of alkanes into α,ω-alkandiols and diacids, particularly due to the difficulty in directing C-H oxy-functionalization at the least reactive terminal positions. The ACTPAC project aims to utilize the unique capabilities of Cytochrome P450 (CYP450) enzymes to overcome this challenge. Specifically, Cytochrome P153A (CYP153A) enzymes have garnered attention lately due to their ability to catalyze the selective terminal α,ω-hydroxylation of medium to long-chain alkanes and fatty acids [1]. Furthermore, some CYP153A orthologs have been proven to catalyze the full terminal oxidation of alkanes into their corresponding alkanedioc acids [2]. However, utilization of CYP153A enzymes is still challenged by low conversions, requirement for cofactor regeneration and overoxidation of products. That"s why this work aims to develop a biotransformation platform for the efficient terminal regioselective oxidation of medium to long-chain alkanes into their corresponding α,ω-alkandiols and diacids. Our strategy involves screening and characterization of promising CYP153A orthologs capable of hydroxylating and oxidizing medium length alkanes into α, ω-diols and diacids. Engineering of selected CYP153A enzymes to fine-tune their specificity and activity towards targeted substrates through computational methods and machine learning algorithms. Lastly, scale-up is conducted in large-scale bioreactors, with a focus on enhancing efficiency, cofactor regeneration, and fine-tuning reaction conditions to maximize yields. Through collaboration with industrial partners, we seek to bring this technology to a level where it can make a significant impact on PE plastic waste management and recycling practices, contributing to a more sustainable circular economy.

This project is part of the ACTPAC consortium founded by Horizon Europe ZEROPOLLUTION, grant nr. 101135289.

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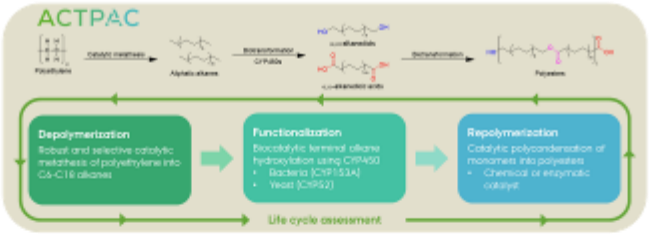
[1] Wang, L.; ...; Li, Y. Mol Biol Rep 2023, 50 (8), 6955–6961.

[2] Funhoff, E. G.; …; van Beilen, J. B., J Bacteriol 2006, 188 (14), 5220–5227.

Fig. 1


ACTPAC
Depolymerization
Robust and selective catalytic metathesis of polyethylene into C6-C18 alkanes
Functionalization
Biocatalytic terminal alkane hydroxylation using CYP450
Bacteria (CYP153A)
Yeast (CYP52)
Repolymerization
Catalytic polycondensation of monomers into polyesters
Chemical or enzymatic catalyst
Life cycle assessment

P3-44

Specific Terminal Oxidation of Xylene Derivatives by Unspecific Peroxygenase

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Xylene and its derivatives play pivotal roles in various industries, yet their use comes with inherent health and environmental risks. The imperative to develop sustainable methods for their transformation cannot be overstated. In this study, an unspecific peroxygenase from *Thielavia terrestris* (*Tte*UPO) was screened. This enzyme exhibited remarkable 100% chemoselectivity in producing a monoacidified oxidation product from xylene, representing a noteworthy advancement in enzymatic oxidation processes. A fusion protein combining *Tte*UPO and *Ao*FOx was engineered and a continuous stream of H2O2 was introduced to counter the sensitivity of *Tte*UPO to H2O2. These strategic interventions yielded significant improvements in the oxidation efficacy against p-xylene and its derivatives. Notably, the study achieved a 60% yield of a 2.4 mM monoacidified product from p-xylene.This research not only presents a cutting-edge strategy for transforming xylene compounds but also underscores its importance in promoting environmental protection and advancing the principles of green chemistry.

P3-45

Intensified Production of Ethyl Butyrate Using Different Lipases and Ethanol Fermentation by *Zymomonas mobilis* in a Biphasic System

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The short-chain fatty acid ethyl ester, ethyl butyrate, holds promise as a sustainable aroma compound as well as a biofuel. Compared to diesel, the ester has an heating value (25.39 MJL^{-1}) which is lower than the heating value of diesel with a value of 35.77 MJL^{-1}. Since the density of both is comparable with 0.886 g cm^{-3} and approximately 0.84 g cm^{-3} for ethyl butyrate and diesel respectively, the ester could potentially be used as diesel additive. The production route for the ester involves multiple steps, such as separate substrate fermentations, esterification step, and downstream processing. Therefore, a process intensification that combines the fermentation and esterification steps could be promising for reducing the time required as well as preventing product or substrate inhibitions and enabling an in-situ product removal by adding a second phase to the system.

Lipases are among the most used enzyme families for esterification reactions in industrial applications. These enzymes can catalyze the esterification reaction and the hydrolysis of the ester, but require an interface layer between organic and water phase for any catalytic efficiency. The production of ethyl butyrate from ethanol and butyrate has already been achieved using different lipase, such as lipase A and B from *Candida antarctica,* lipase from *Candida rugosa, Mucor miehei.* Moreover, many immobilized versions are used. Often, these esterification reactions are carried out in neat organic environments or with a low amount of water. However, since we want to combine the esterification and fermentation the amount of water phase, especially media, should be at least 50 %.

Producing ethanol from microorganisms is a significant field in addressing the current climate crisis. Therefore, it is well established that ethanol can be produced using either *Saccharomyces cerevisiae* or *Zymomonas mobilis.* Regarding the goal for production of ethyl butyrate, the ethanol fermentation should be carried out in a biphasic system using *Z. mobilis* as ethanol producer while butyrate will be supplemented for the esterification.

To investigate the most suitable lipase, a screening of different lipases in multiple systems with a varying organic to aqueous phase values is conducted. The characteristic values km¸vmax, and ttn are determined for each system and each substrate concentration. The biphasic fermentation is conducted with different organic solvents like hexadecane, dodecane, and hexane, in varying organic phase to media ratios, comparable to the enzymatic experiments. The growth rate and the ethanol yield in the systems used are compared to evaluate the most suitable system

Changing the ratio of the organic solvent to media, influences the esterification efficiency as well as the ethanol yield in the fermentation process, respectively. This work is used as preliminary experiment to combine these in the future for an intensification of the process and the production of ethyl butyrate.

P3-46

Engineering *G. oxydans* for the production of functionalized sugar acids and the development of a screening assay for new strains and mutated enzymes

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Gluconobacter oxydans has great biotechnological potential due to its ability to incompletely oxidize a large variety of sugars, polyols, and related compounds. It is able to grow in solutions with highly concentrated substances and can tolerate a low pH. These oxidations are catalyzed by membrane-bound dehydrogenases (mDHs) with the active site facing toward the periplasm, therefore avoiding the transport of substrates and products in and out of the cytoplasm. We developed a platform for the functional expression of heterologous mDHs in *G. oxydans* BP9.1, devoid of its native mDHs, thereby increasing the specific activity of the heterologous mDHs and avoiding unwanted side reactions.

This study aims to use this platform to produce cellobionic acid (CBA), galactaric acid (GA), and potentially other relevant sugar acids with space-time yields relevant for industrial usage. CBA is an alternative to the animal-derived lactobionic acid, while GA could be a replacement for tartaric acid. Both can be used in pharmaceutics, cosmetics, and other applications. The membrane-bound glucose dehydrogenase (mGDH) from *Pseudomonas taetrolens* DSM 21104 was expressed in *G. oxydans* BP9.1 to construct a strain that produces CBA and GA with a high space-time yield. For further optimization, different promotors have been tested in a plasmid-based expression so the best one could be integrated into the genome of our chassis strain *G. oxydans* BP9.1. In parallel, random in vitro mutagenesis was used to create variants of the mGDH from *P. taetrolens*. An assay was established to screen for mutants with a high oxidation rate against cellobiose. In this plate assay, carbonate in the medium serves as an indicator for acidification. By oxidizing the cellobiose in the medium, the colonies form halos on the plates, whose size correlates with the amount of CBA produced. This assay can also be used to identify new strains isolated from various high-sugar fruits with mGDHs that have the ability to oxidize the substance of choice.

In conclusion, we created a strain based on the already industry-established *G. oxydans* 621H strain that is able to oxidize cellobiose into CBA and galacturonic acid into GA in industrial-relevant amounts. With the carbonate plate assay, new strains and mutated enzymes can be screened easily for oxidative activity against cellobiose and other sugars on a large scale in a short time.

P3-47

C-C Bond Formation via Biocatalytic Formylation of Resorcinol

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Formylation, the addition of a formyl (-CHO) group, stands as a pivotal transformation in organic synthesis, playing a crucial role in the synthesis of valuable intermediates and end products. Despite the availability of various formylation techniques,[1] the utilization of biocatalysts remains limited. This scarcity is attributed to the prevalent reliance on harsh synthetic reagents and conditions in most existing methods.[2]

This research aims to establish a highly efficient biocatalytic method for C-C bond formation, with a specific focus on the formylation of resorcinol (**Figure 1**). The study centers on the application of *Pp*ATase, an acyltransferase originating from *Pseudomonas protegens*, known for its capability to catalyze the Friedel–Crafts C-acylation of phenolic substrates in buffer.[3] The research seeks to examine enzyme homologues and to identify the most effective biocatalyst. Additionally, the investigation involves exploring the substrate scope, encompassing the evaluation of various formyl sources and improving the reaction conditions to enhance overall efficiency.

Figure 1. Formylation of resorcinol catalyzed by acyltransferase

Among the enzyme homologues tested, three showed activity towards resorcinol. Consequently, *Cs*ATase, an acyltransferase originating from *Chromobacterium sphagni*, was chosen for further analysis and its activity was compared to that of *Pp*ATase in the formylation of resorcinol. Upon evaluating various aliphatic and aromatic formyl donors, phenyl-formate emerged as the best formylating agent, achieving 99% and 84% conversion in the formylation reaction catalyzed by *Cs*ATase and *Pp*ATase, respectively.

In conclusion, this research endeavors to bridge the gap in biocatalytic formylation techniques and by focusing on the formylation of resorcinol and harnessing the catalytic potential of enzymes such as *Pp*ATase and *Cs*ATase, we aim to pioneer a novel approach to C-C bond formation.

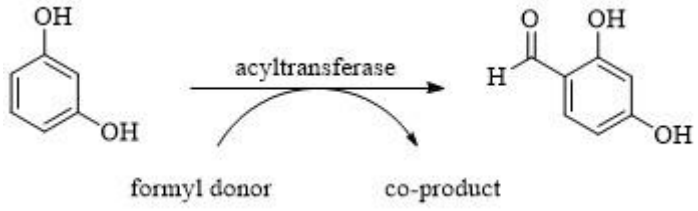
References

[1] W. Kantlehner, *Eur. J. Org. Chem*., **2003**, *14*, 2530-2546.

[2] Ø. W. Akselsen, L. Skattebøl, T. V. Hansen, *Tetrahedron Lett.*, **2009**, *50*, 6339-6341

[3] N. G. Schmidt, T. P. Keller, N. Richter, B. Wiltschi, K. Gruber, W. Kroutil, *Angew. Chem. Int. Ed*., **2017**, *56*, 7615 –7619

Fig. 1



P3-48

Repurposing of *Cv*FAP

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The fatty acid photodecarboxylase from *Chlorella variabilis* (*Cv*FAP) is an enzyme with a great potential for the production of biofuels, since it can perform the decarboxylation of fatty acids (FAs) *via* radicals, to produce alkanes in a clean and mild way using only visible blue light.[1] However, most studies up to date have used FAP mainly within its natural limits. The radicals that this enzyme forms are useful reactive intermediates in organic synthesis,[2] and therefore we hypothesize that can also catalyze non-natural radical reactions, which would not only increase the scope of applicability of this promising enzyme, but also provide alternative green and sustainable routes for the synthesis of high-value chemical synthons and pharmaceutical precursors.

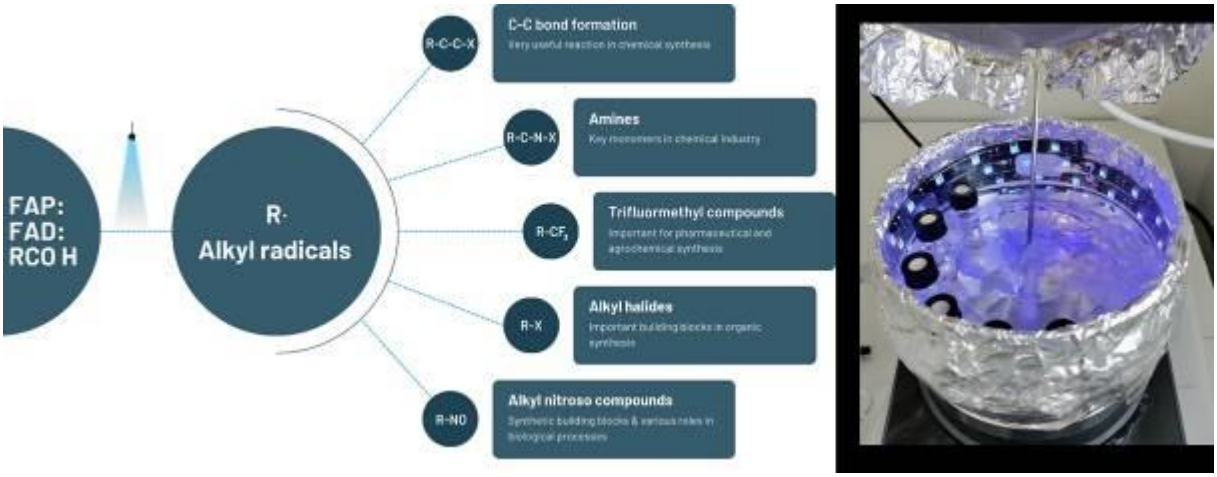
Bearing this in mind, in this work we expressed *wild type* FAP, and selected mutants that generate short, medium and long chain alkyl radicals. Next, we screened different radical traps together with the fatty acid substrates, aiming to form C-C, C-N and C-X bonds. We performed a thorough study of the reaction conditions, and detected the products using chromatographic techniques coupled with MS.

Figure 1. *(Left)* Scheme of the target products. *(Right)* Photoreactor used for the reaction under illumination with blue light (LED).

[1] aD. Sorigué, B. Légeret, S. Cuiné, S. Blangy, S. Moulin, E. Billon, P. Richaud, S. Brugière, Y. Couté, D. Nurizzo, *Science* **2017**, *357*, 903-907; bL.-E. Meyer, B. E. Eser, S. Kara, *Current Opinion in Green and Sustainable Chemistry* **2021**, *31*, 100496; cP. Santner, L. K. Szabó, S. N. Chanquia, A. H. Merrild, F. Hollmann, S. Kara, B. E. Eser, *ChemCatChem* **2021**, *13*, 4038-4046.

[2] M. Yan, J. C. Lo, J. T. Edwards, P. S. Baran, *Journal of the American Chemical Society* **2016**, *138*, 12692-12714.

Fig. 1



P3-49

Exploring Late-Stage Scaffold Construction in Chemoenzymatic Natural Product Synthesis

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Introduction

Late-stage scaffold building is a challenging aspect of natural product synthesis, particularly when it involves the formation of heterocycles. A structurally diverse class of aromatic prenyltransferases has been identified that catalyse both, the cyclisation of tryptophans to a pyrroloindole scaffold and the chemically demanding C-C coupling of prenyl moieties of different chain lengths to the C3-position.[1]

Objectives

Bacterial and fungal aromatic prenyltransferases perform regio- and stereoselective prenylation of tryptophan-containing diketopiperazines. The elucidation of their substrate and prenyl-donor scopes reveals a catalyst-toolbox providing access to diverse prenylated compounds.

Results

Structurally diverse aromatic prenyltransferases were expressed and purified, allowing their biochemical characterisation. A screening method was established to efficiently and reliably test for activity as well as substrate and prenyl-donor scopes.[2] The prenyltransferases were successfully used in the biocatalytic prenylation of tryptophan-containing diketopiperazines. The C3-prenylated compounds were structurally analysed in order to fully characterise the prenyltransferases.

Conclusion

Aromatic prenyltransferases are promising biocatalysts for chemoenzymatic total synthesis approaches, as they provide access to valuable natural products and their analogues.[3]

References

[1] C. Sun, W. Tian, Z. Lin, X. Qu, *Nat. Prod. Rep.* **2022**, *39*, 1721-1765; 'Biosynthesis of pyrroloindoline-containing natural products'.

[2] D. A. Amariei, M. Haase, M. K. T. Klischan, M. Wäscher, J. Pietruszka, *ChemCatChem* **2024**, e202400052; 'High-Throughput Colorimetric Detection and Quantification of Indoles and Pyrroloindoles for Enzymatic Activity Determination'.

[3] M. Haase, B. David, B. Paschold, T. Classen, P. Schneider, N. Pozhydaieva, H. Gohlke, J. Pietruszka, *ACS Catal.* **2024**, *14*, 227-236; 'Application of the C3-Methyltransferase StspM1 for the Synthesis of the Natural Pyrroloindole Motif'.

P3-50

Leveraging Nature's Catalysts: Methyltransferases in Late-Stage Modifications

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Introduction

In the realm of drug discovery, sometimes the smallest tweak can yield the most astonishing results. One such phenomenon, known as the "magic methyl effect," describes the strong influence that the addition of a methyl group can have on the physicochemical properties and biological activity of a compound.[1, 2] In pharmaceutical research, the strategic introduction of methyl groups can modulate various characteristics of drugs, such as influencing solubility and metabolic stability, altering conformation, and impacting the binding affinity.[1, 3]

Objectives

Utilizing nature"s toolbox, the goal is to employ methyltransferases, enzymes that catalyse the transfer of a methyl group to a wide range of substrates, for late-stage decorations. Focusing on *O*- and *C*-methyltransferases interesting building blocks as well as natural products and interesting bioactive compounds will be targeted for methylation exploiting the potential of the "magic methyl effect".

Results

Using *in silico* molecular docking experiments, databanks were screened for promising enzymes for the methylation of the target compounds. Subsequently the enzymes were tested for the methylation of various compounds.

Conclusion

The exploration of the "magic methyl effect" in drug discovery underscores the potential for precision enhancement of pharmaceuticals. Through the targeted utilization of methyltransferases, our research aims to capitalize on nature's catalytic potential for late-stage modifications. Initial screening and testing have identified promising enzymes for substrate methylation, setting the stage for further investigation.

References

[1] P. W. Ondachi *et al.*, *J. Med. Chem.* **2018**, *61*, 7525-7545; 'Potent and Selective Tetrahydroisoquinoline Kappa Opioid Receptor Antagonists of Lead Compound (3R)-7-Hydroxy-N-[(1S)-2-Methyl-1-(Piperidin-1-Ylmethyl) Propyl]-1, 2, 3, 4-Tetrahydroisoquinoline-3-Carboxamide (PDTic)'.

[2] P. d. S. M. Pinheiro, *et al.* , *Pharmaceuticals* **2023**, *16*, 1157; 'The Magic Methyl and Its Tricks in Drug Discovery and Development'.

[3] D. Aynetdinova, *et al.*, *Chem. Soc. Rev.* **2021**, *50*, 5517-5563; 'Installing the "magic methyl"–C–H methylation in synthesis'.

P3-51

Development of immobilized lipases for the biosynthesis of eicosapentaenoic acid lysophospholipid

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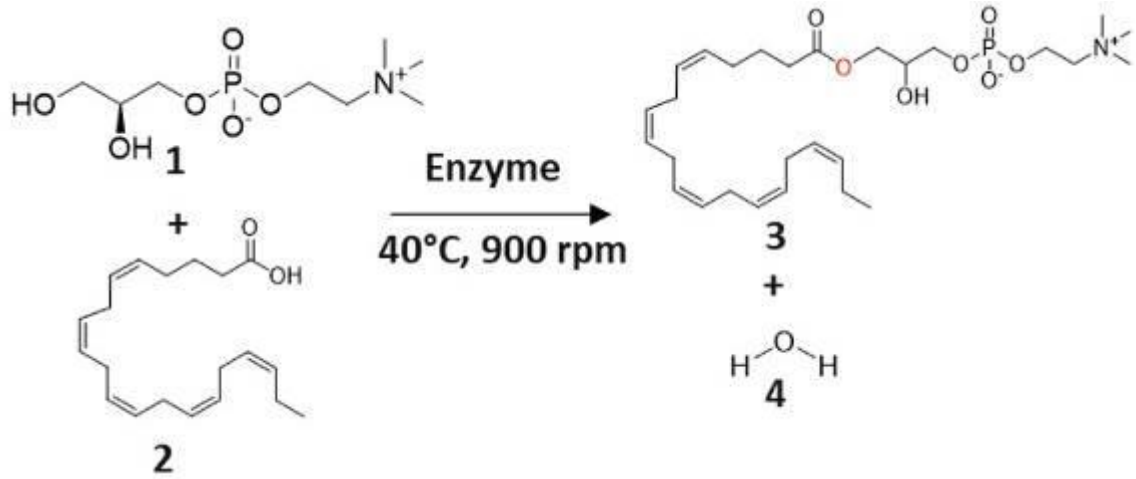
Introduction: Eicosapentaenoic acid (EPA) is an omega-3 fatty acid with human health benefits[1]. However, administered in the phospholipid form the bioavailability of EPA is the highest, hence opening range of possibilities for its administration. Aim: We performed a screening immobilization of 5 commonly used lipases[2] to fabricate a heterogeneous biocatalyst capable to synthesize EPA lysophospholipid (EPALIP) in a sustainable manner. Results: To this aim, we performed an immobilization screening by chemically modifying Macro-Prep support, an epoxy methacrylate resin distributed by Bio-Rad, generating 9 supports with different functional groups. The immobilization parameters were determined following the equations used elsewhere[1]. After performing the immobilization screening (1 mg protein g-1 support) we select the immobilization chemistry which offers the highest yield (Y,%) and relative recovery activity (rRA,%) for each enzyme. In this sense MANAE was choose for *Rhizopus oryzae* lipase (Y: 53; rRA: 64), *Rhizomucor miehei* lipase (Y: 98; rRA: 90) and *Thermomyces lanuginosus* lipase (TLL; Y: 99; rRA: 84) using ionic interactions, while heterofunctional support Cu2+/E was selected for *Candida rugosa* lipase (Y: 98; rRA: 6.5) and *Candida antarctica* B (CALB; Y: 99; rRA: 28) using metal coordination and covalent bonding. To further discriminate among biocatalysts, we evaluated the thermal stability of the selected biocatalyst by determining the temperature at which the enzyme retains 50% of its activity after 1 h of incubation (T50). In all cases we observed an increase of 5 to 20 °C in the T50 after immobilization, demonstrating the stabilization of lipases through proper immobilization chemistry. Finally, we perform the solvent-free esterification (Fig 1) of EPA and glycerophosphocholine (GPC) to synthesize EPALIP using immobilized biocatalyst at 40 ºC and 900 rpm. We detected the synthesis of EPALIP by MALDI-TOF only in the reactions catalysed by CALB and TLL. After testing immobilization loads of 1, 10, 30, 68 mg g-1 with CALB and TLL, the most promising enzymes, we found that CALB and TLL biocatalysts at 10 mg g-1 synthesized EPALIP, TLL being up to 10-fold more efficient than immobilized CALB.

Conclusion: From these results we found that tailored heterogeneous biocatalysts with improved stability are able to synthesize EPALIP in a sustainable manner (solvent-free and using an alcohol as solvent reaction, respectively).

[1] Ahmmed 2019, 10.1111/1541-4337.12510. [2] Castejón 2020, 10.1016/j.nbt.2020.02.006. [3] Santiago 2023, 10.1021/acs.biomac.2c01364.

Fig 1. Esterification reaction for synthesis of EPALIP. 1, GPC. 2, EPA.3, EPALIP. 4, Water.

Fig. 1



P3-52

Biotransformation and antimicrobial studies of flavonoids with bromine and chlorine atoms

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Flavonoids are known for their anti-inflammatory properties, antioxidant, and antimicrobial ones. However, due to their low water solubility, orally administered flavonoids are poorly absorbed, and therefore, their therapeutic potential is limited. It can be improved using glycosylation via biotransformation, which has been proven to be an effective way to increase water solubility and bioavailability of flavonoids. In our study, we carried out the biotransformation of chalcone, flavanone, and flavone, which contained bromine and chlorine atoms, using the strains *Beauveria bassiana* KCH J1.5, *Isaria fumosorosea* KCH J2 and *Isaria farinosa* KCH J2.6. Then, we checked the antimicrobial activities of aglycones and one glycoside 8-bromo-6-chloroflavone 4''-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside on four pathogenic and four probiotic bacteria. We measured the effect of flavonoids on bacteria growth over a 72-hour period at hourly intervals using a microplate reader. The chalcone exhibited the highest antibacterial properties, followed by flavones and then flavanones. We also noticed that the glycoside derivative had enhanced antimicrobial effect against pathogenic bacteria, while being less harmful to probiotic bacteria than the corresponding aglycone. Our research suggests that bromine and chlorine atoms in flavonoids increase their antimicrobial properties, and that attachment of a glycoside unit to a flavone selectively enhances its bactericidal properties and bioavailability of the compound. This study adds to the knowledge about compounds not previously described in the literature with their antibacterial properties and further potential for their use in industry.

P3-53

Precise Genome Editing via CRISPR/Cas9 for *in vivo* Production of Sialyllactose

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[2]Hamburg University of Technology, Institute of Technical Biocatalysis, Hamburg, Germany

Sialyllactose (SL) is one of the human milk oligosaccharides (HMOs), the third most abundant component of breast milk after lactose and fat. HMOs are crucial for human health and have a positive impact on the growth of naturally occurring human gut bacteria, such as bifidobacteria[1]. In addition, HMOs prevent the adsorption of pathogens by acting as acceptor mimics for pathogen binding. SL, present in human breast milk as 3'-SL and 6'-SL, are essential components in neutralizing toxins and preventing bacteria and viruses from adhering to the epithelial surface of breast-fed infants[2].

The production of HMOs is receiving increasing attention in biotechnology due to a high demand for HMOs e.g. as food additive for breast milk substitutes.

One biotechnological approach to produce HMOs involves using metabolically engineered strains to overcome challenges such as the need for multiple enzyme cascades, cofactor regeneration, substrate uptake and product release, as well as the prevention of intermediate degradation.

To address these challenges, an *Escherichia coli* K12 strain is modified using CRISPR/Cas9 for the synthesis of SL, as illustrated in Figure 1. During strain construction, genome editing will be used to synthesize and regenerate necessary substrates and cofactors in the cell, while preventing unwanted degradation of substrates and products.

The method of choice is CRISPR/Cas9, as it allows multiple genes to be inserted and knocked out simultaneously.

The gene knock-out was designed in a way that a deletion occurs in the genome so that the gene sequence is removed except for the start codon and the last 7 amino acids. For a gene knock-in, the gene sequence of the GOI is introduced in combination with a T7RNA polymerase promoter. Gene-Overexpression is achieved by placing the expression under the control of the selected promoter. For this purpose, the T7RNA polymerase is introduced into the genome. The T7RNA polymerase knock-in is achieved with a simultaneous *lacZ* knock-out.

Figure 2 shows exemplarily the alignment of the ATP-dependent 6-phosphofructokinase isozyme 1 (*pfkA*) knock-out region.

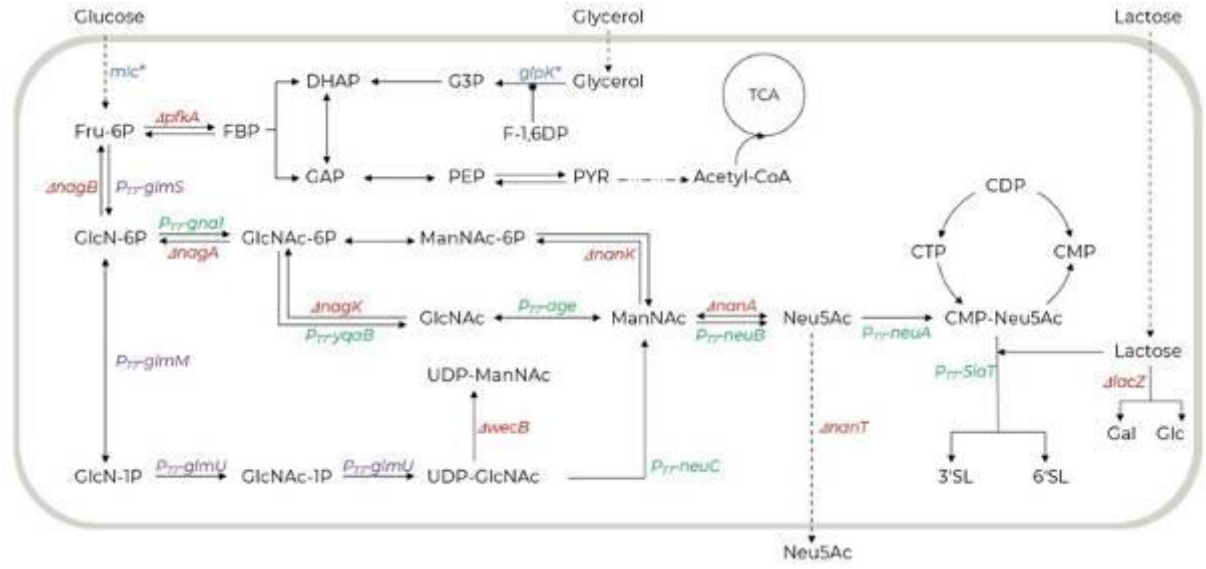
The sequencing (Figure 2) confirms that the knock-out of the gene *pfkA* using CRISPR/Cas9 was successful and further genes have already been knocked out.

The newly synthesized strain will enable the production of pure SL, helping to overcome limitations, e.g. for mothers in need of milk substitution and the inaccessibility by chemical synthesis.

[1] Guo, L. et al. "Enzymatic Synthesis of 6'-Sialyllactose, a Dominant Sialylated Human Milk Oligosaccharide, by a Novel *exo*-α-Sialidase from Bacteroides fragilis NCTC9343." *Applied and environmental microbiology*, vol. 84,13 e00071-18. 2018. https://doi.org/10.1128/AEM.00071-18

[2] Li, Z. et al. "Multi-Enzymatic Cascade One-Pot Biosynthesis of 3’-Sialyllactose Using Engineered *Escherichia coli*." *Molecules* vol. 25,16 3567. 2020. https://doi.org/10.3390/molecules25163567

Fig. 1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GAP
PEP
PYR
Acetyl-CoA
CDP
PTT-gnal
GlcN-6P
ΔnagA
GlcNAc-6P
ManNAc-6P
ΔnanK
CTP
CMP
ΔnagX
PTT-yqaB
GlcNAc
PTT-age
ManNAc
ΔnanA
PTT-neuB
Neu5Ac
PTT-neuA
CMP-Neu5Ac
Lactose
PTT-glmM
UDP-ManNAc
PTT-SiaT
ΔlacZ
ΔwecB
ΔnanT
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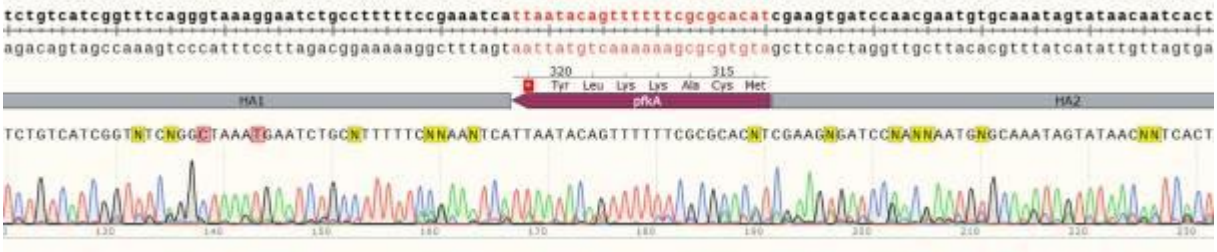
Fig. 2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Tyr Leu Lys Lys Ala Cys Met
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P3-54

Investigation of regio- and stereoselectivity of oxidation of *O*-alkenyl substituted pyridines utilizing non-heme iron PmlABCDEF monooxygenase

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1. Introduction

A variety of useful products, from medicine to agriculture, can be obtained from oxidation reactions. In industry, such reactions are carried out by chemical methods, which are often polluting and do not always show the desired regio- and stereoselectivity. An alternative to them are more environmentally friendly biocatalytic methods, which allow the insertion of an oxygen atom with the desired regio- and stereoselectivity.

Important building blocks and intermediate compounds in organic chemistry – epoxides and *N*-oxides – can be obtained with the help of various monooxygenases (Dong et al., 2018). Nevertheless, there is a lack of research on enzymes that possess the ability to oxidize the terminal unconjugated double bond found in aryl alkenes and *N*-heteroaromatic compounds to produce suitable epoxides.

2. Objectives

This work aims to (1) use organic chemistry methods to synthetize *O*-substituted pyridinols having alkenyl chains of different length; (2) to use these compounds as substrates with non-heme iron monooxygenase PmlABCDEF (Petkevicius et al., 2022) investigating regio- and stereoselectivity of final reaction products.

3. Results

We have synthesized six different *O*-alkenylated pyridine-3-ols. Obtained compounds were used as substrates to whole cells of *Pseudomonas putida* KT2440 bacteria producing recombinant PmlABCDEF monooxygenase. Biocatalysis end products were purified and identified as epoxides, *N*-oxides, and compounds having both epoxide and *N*-oxide moieties. To detect and determine the obtained product, we used methods such as chromogenic identification reaction with Preußmann reagent, thin layer chromatography (TLC), high-performance liquid chromatography-mass spectrometry (HPLC-MS), nuclear magnetic resonance (NMR), and high-resolution mass spectrometry (HRMS). We also investigated PmlABCDEF monooxygenase's ability to convert aryl alkenes to epoxides stereo-selectively.

4. Conclusion

We have shown that PmlABCDEF monooxygenase effectively converts various *O*-alkenyl-substituted pyridines. The ratio of obtained products (epoxides vs. *N*-oxides) depends on the chain length. This enzyme also shows a potential in stereoselective oxidation as 3-(oxiran-2-ylmethoxy)pyridine was obtained with 92.8 % ee.

References

Dong, J., Fernández-Fueyo, E., Hollmann, F., Paul, C. E., Pesic, M., Schmidt, S., Wang, Y., Younes, S., & Zhang, W. (2018). Biocatalytic Oxidation Reactions: A Chemist"s Perspective. *Angewandte Chemie International Edition*, *57*(30), 9238–9261. https://doi.org/10.1002/anie.201800343

Petkevicius, V., Vaitekunas, J., Sadauskas, M., Schultes, F. P. J., Tischler, D., & Meškys, R. (2022). The versatility of non-heme diiron monooxygenase PmlABCDEF: a single biocatalyst for a plethora of oxygenation reactions. *Catalysis Science and Technology*, *12*(24), 7293–7307. https://doi.org/10.1039/d2cy01167k

P3-55

The CYPome of *Cunninghamella elegans*: A Model for Xenobiotic Biotransformation

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Cunninghamella spp. are recognized as mammalian models of drug and xenobiotic metabolism. These fungi can catalyse phase I and phase II drug metabolism. Cytochromes P450 (CYPs) are enzymes mostly responsible for phase I (oxidative) metabolism; although, the function of these enzymes is not thoroughly studied in vitro. In silico analysis of the genome sequence of *C. elegans* identified 32 genes putatively coding for CYPs. However, the low sequence homology of CYPs restrains a prediction of function based on solely on protein sequence. Furthermore, CYP activity is typically dependent upon reducing power delivered by cytochrome reductases (CPRs), three of which are present in *C. elegans*. Heterologous expression in a suitable host is the best option to study their substrate specificity. Cyp5313D1 expressed in *Pichia pastoris*converted flurbiprofen to 4′-hydroxyflurbiprofen, the same metabolite was produced by *C. elegans* cultures. Furthermore, co-expression of CYPs cyp5208A3 and cyp5313D1[PN1] with one of the three CPRs led to improved biotransformation of various xenobiotic substrates (transfluthrin, β-cyfluthrin and λ-cyhalothrin, ibuprofen and diclofenac). Recently, significant progress has been made in optimizing the cloning and expression of a new enzyme from *C. elegans* in *P. pastoris*: the bifunctional cyp5205A8, a self-sufficient enzyme capable of functioning without the aid of CPRs. Biotransformation experiments have revealed cyp5205A8 as a fatty acid hydroxylase. It catalyses the subterminal (ω-1 to ω-3) hydroxylation of C7-C14 fatty acids. Additionally, in silico analysis aims to identify key amino acid residues for potential site-directed mutagenesis investigations, aligning with the overarching goal of enhancing CYP functionality. The identification of substrate activities in these enzymes holds the potential to reduce animal use and offer a more sustainable route to drug metabolite production.

P3-56

Unspecific peroxygenases for the oxidation of HMF

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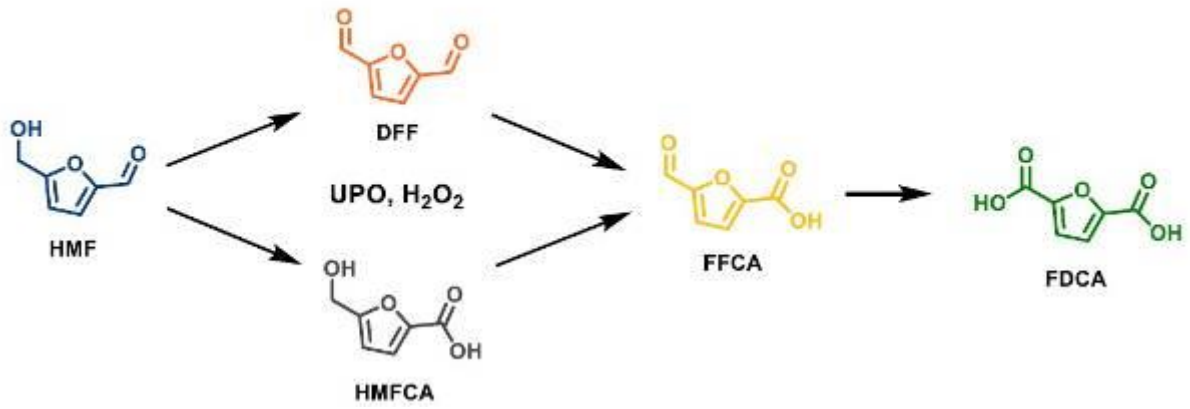
2,5-Furandicarboxylic acid (FDCA) is a valuable chemical that finds application in the polymer industry [1]. This building block can be accessed via renewable starting materials, i.e. via the catalytic oxidation of biomass-derived 5-hydroxymethylfurfural (HMF), and thus attracts great interest [2]. The inclusion of FDCA in the list of the top 12 biobased chemicals by the U.S. Department of Energy (DOE) back in 2004[3] encouraged an upsurge of research resulting in a wide toolbox of catalytic oxidation methods towards FDCA. The main advantages of biocatalysts in this regard are their selectivity and mild reaction conditions, which are essential for a sustainable process. One challenge of enzymatic processes is to obtain high product titers. From this point of view, unspecific peroxygenases (UPOs) have shown great potential as oxidation catalysts [4]. Since so far, only AaeUPO has been used in cascades with oxidases for the oxidation of HMF [5] in this work we screened 23 different UPOs and found that HspUPO [6] and UPOx8 are the first UPOs capable of performing three consecutive oxidation steps from HMF to FDCA (Figure 1). Moreover, we observed that the chemoselectivity in the first oxidation step can be increased by adding cosolvents, although accompanied by lower conversions. The combination of HspUPO with HMFO [7] and variants thereof [8] in a self-sufficient cascade led to increased efficiency of the system.
Our results highlight the potential of UPOs for the synthesis of FDCA and contribute to the development of sustainable and efficient methods for the production of value-added chemicals from biomass-derived feedstocks.

Figure 1. Oxidation of HMF to FDCA by UPO.

[1] A. F. Sousa et al., Polym. Chem. 2015, 6, 5961.
[2] H. Cong et al., Catalysts 2021, 11, 1113.
[3] T. Werpy et al., Top Value Added Chemicals from Biomass: Volume I - Results of Screening for Potential Candidates from Sugars and Synthesis Gas, 2004.
[4] F. Tonin et al., Org. Process Res. Dev. 2021, 25, 1414.
[5] a) J. Carro et al., Biotechnology for biofuels 2018, 11, 86; b) A. Karich et al., Microorganisms 2018, 6, 5.
[6] L. Rotilio et al., ACS Catal. 2021, 11511.
[7] W. P. Dijkman et al., Angew. Chem. Int. Ed. 2014, 126, 6633.
[8] M. Pickl et al., Angew. Chem. Int. Ed. 2018, 57, 2864.

The COMET center: acib: Next Generation Bioproduction is funded by BMK, BMDW, SFG, Standortagentur Tirol, Government
of Lower Austria und Vienna Business Agency in the framework of COMET - Competence Centers for Excellent Technologies. The COMET-Funding Program is managed by the Austrian Research Promotion Agency FFG.

Fig. 1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FDCA

P3-57

Exploring a Novel Rossmann Fold-Type Enzyme Participating in the Catabolism of Modified Nucleosides

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More than 100 modified nucleosides with different structures and functions are known to be components of DNA and RNA. Living organisms use these modified nucleosides to ensure the regulation of gene expression, function of various RNA molecules, use them as secondary metabolites and other essential tasks. While much is known regarding their synthesis, knowledge about their catabolism is lacking.

In this work, we set out to identify catabolism pathways for 2'-modified uridine compounds. We identified a plasmid from a metagenomic library containing a gene encoding a putative Rossmann fold-type enzyme that was annotated as *S*-adenosyl-L-homocysteine hydrolase (SAHase). Unlike canonical SAHases, our enzyme showed no activity with *S*-adenosyl-L-homocysteine (SAH). Formation of NADH was also detected, meaning that our noncanonical SAH hydrolase (ncSAHase) reaction mechanism differs from canonical SAHases as no NAD+ regeneration occurs. These findings show that the identified ncSAHase and other homologous enzymes compose a new subgroup of Rossmann fold-type enzymes. Our ncSAHase was active with modified uridine and guanosine compounds, especially, 2'-O-methyluridine and other 2'-modified uridines. The ncSAHase reaction with nucleosides produces NADH, free base, and modified 2-(hydroxymethyl)furan-3-one (Fig. 1). The activity of ncSAHases *in vitro* with various modified nucleosides might propose the new catabolic pathways for modified uridine, guanosine and, with the help of cytidine deaminase, cytidine nucleosides. At the same time, the formation of reactive furan-3-ones opens possibilities for the synthesis of modified saccharides, noncanonical nucleosides, or other compounds.

In summary, we identified a novel Rossmann fold-type enzyme that catalyzes base removal and modified furan-3-one formation from nucleosides.

Fig. 1

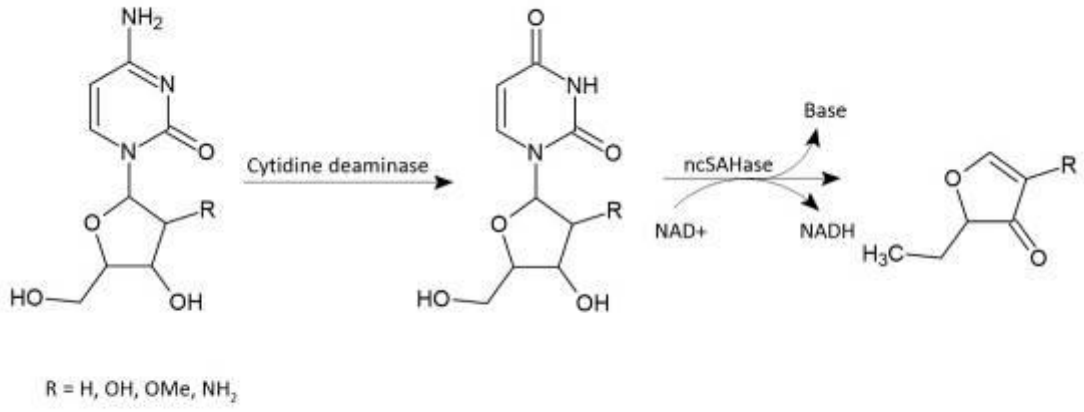

Figure 1. Reaction catalyzed by ncSAHases.

P3-58

Enzymatic synthesis of the chiral neurotransmitter noradrenaline

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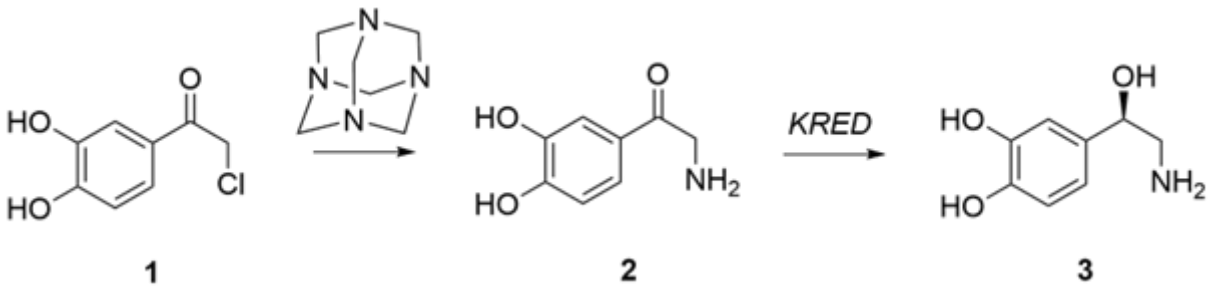
Green chemistry and biocatalysis has become a core field in the industrial biotechnology world, due to the need for sustainable and affordable methods for producing pharmaceuticals, fine chemicals and polymers, but most importantly due to enzymes" excellent catalytic efficiency.

Currently our focus is on the development of ketoreductase (KRED) superfamily. It has been demonstrated that KREDs are incredibly selective in the reduction of a variety of ketones, including bridged ring systems and rings with five, six, and seven members. One very known function common to the KRED superfamily is the transformation of ketones to chiral alcohols. At Sterling, our initial efforts are to synthesise the chiral neurotransmitter noradrenaline (compound **3**, **fig. 1**) which can be accessed from the chloroketone (**1**) after treatment with hexamine.

Fig. 1. Proposed route to noradrenaline, 3, from commercially available chloroketone, 1.

Docking studies and enzyme engineering will be performed on lead candidate enzymes to enhance their substrate specificity and overall yield. Enzyme immobilisation will be applied to enhance stability and overall performance. Finally, lead candidate enzymes that exhibit great potential in the biocatalytic reaction will be used in scale-up productions.

Fig. 1



1 2 3

P3-59

Discovery and characterization of a new 4,6-α-glucanotransferase for the modification of starch and its hydrolysates

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The majority of Lactic acid bacteria are recognized for their ability to produce glucansucrases, enzymes that transform sucrose into various α-glucans. Recently, a subfamily of these enzymes (E.C.2.4.1.-), known as 4,6-α-glucanotransferases(4,6-α-GTase), has been discovered that is able to produce α-glucans with increased α-(1,6) linkages from substrates containing α-(1,4) linkages, such as starch and starch hydrolysates. To expand the catalytic potential of these biocatalysts, a reference set of characterized 4,6-α-GTase enzymes was used for identification of new potential enzymes across various databases via BLAST. The identified sequences were then aligned for construction of a maximum likelihood phylogenetic tree consisting of 172 sequence annotations. GtfB-like 4,6-α-GTase from *Ligilactiobacillus salivarius* GJ-24 was identified and selected for further characterization. The full and N-terminally truncated enzyme was expressed in *Escherichia coli*. The hydrolytic and the transglycosylation activities of full and truncated GtfB-ΔN confirmed their catalytic ability for the bioconversion of amylose as the substrate. The reaction selectivity was characterised by HPAEC and TLC studies. NMR and HPSEC analyses further assist with the characterization of reaction end-products and the determination of the extent of formation of α-(1,6) linkages. Glucans rich in α-(1,6) linkages will escape the digestion in the small intestine and reach the colon where they act as prebiotic or dietary fibers which can then contribute to overall health.

P3-60

Commercial development of enzymatic processes for industrial applications

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Enzymicals develops biocatalysts for a variety of industries such as life science, pharma and fine chemicals, flavor & fragrance, plastics etc. using a proven platform to access unique enzymes from biodiversity as well as engineered enzymes. Our tool box of several established production organisms such as *E. coli*, *Pichia* and *Bacillus* enables the commercial production of the biocatalyst for industrial use. Tailor-made biocatalysts, combined with innovative route design, can improve manufacturing processes of chemical compounds by eliminating steps, increasing yields, and reducing solvent usage, all leading to reduced waste formation and energy consumption. Specialized in enzyme screening and biocatalyst development, from initial catalyst-lead finding to process optimization and pre-scale up, Enzymicals covers extensive expertise and experience in the field of enzyme technology. Here we present examples of our services from selected projects such as enzymatic plastic degradation and enzyme screening.

P3-61

Triple expression system towards controllable production of flavonoid rhamnosylation cascade

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Molecular and synthetic biology tools are increasingly utilised in the design of biocatalysts, including genetically engineered microorganisms, recombinant proteins, and novel enzymatic pathways [1]. These tools facilitate the creation of more efficient, controllable and tailored for specific application catalysts, devoid the undesirable characteristics. Among the key aspects of the biocatalyst design is their production process. In this study, we present the characterisation of a set of bacterial plasmids dedicated to recombinant expression in broadly used *Escherichia coli* or *Pseudomonas putida.*

Figure 1. The framework of the expression cassette design. Abbreviation: TF – transcriptional factor; RS – restriction site; RBS – ribosome binding site; CDS – coding sequence.

The set includes plasmids with four different bacterial expression cassettes (Fig. 1), which can be used alone or freely combined in up to three-gene monocistronic expression systems using Golden Standard Molecular Cloning assembly [2]. Due to the independent induction of each cassette, it enables the autonomous expression of up to three recombinant enzymes from one plasmid and in proportion, which guarantees optimal efficiency of the cascade. The expression of triple-enzyme cascade consisting of sucrose synthase from *Glycine max* (*Gm*SuSy), chimeric UDP-rhamnose synthase (*Vv*RHM-AtNRS_ER) [3] and flavonol-7-*O*-rhamnosyltransferase from *Arabidopsis thaliana* (*At*UGT89C1) was used as an example of designed plasmids implementation. Flavonoid rhamnosides are of particular interest to the pharmaceutical industry due to their improved antiviral and antibacterial activities [4]. Therefore, developing new, efficient methods for their synthesis is highly appealing.

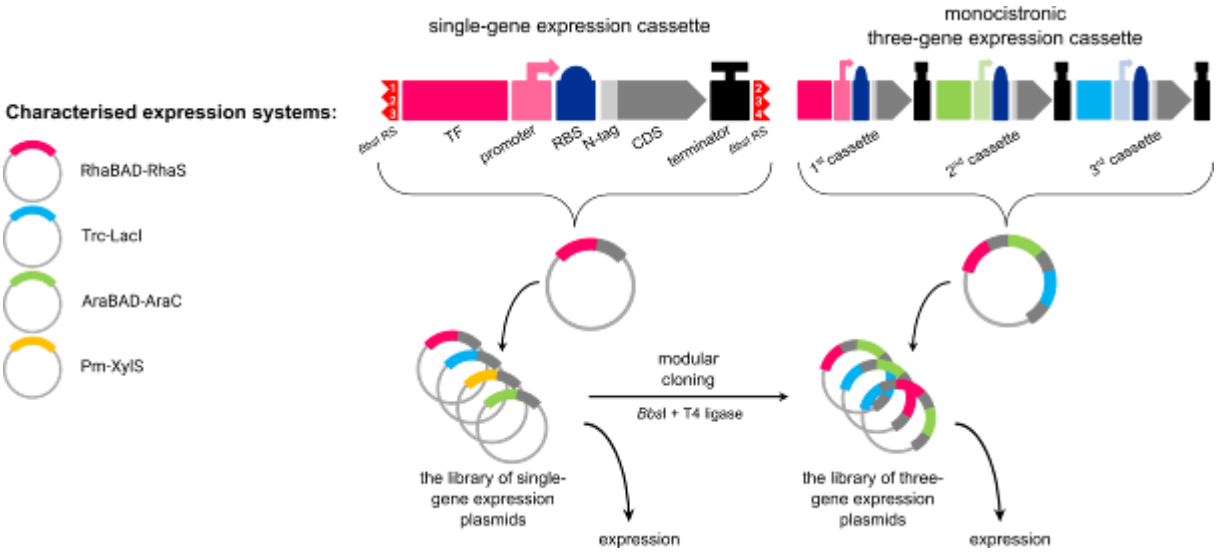
Acknowledgements

This work was supported by the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement no. 814650 (SynBio4Flav) and by the Wrocław University of Environmental and Life Sciences (Poland) as part of the research project no N070/0011/23.

References

1. Kant Bhatia S, Vivek N, Kumar V, Chandel N, Thakur M, Kumar D, et al., Bioresour Technol. 2021;324:124596.
2. Blázquez B, León DS, Torres-Bacete J, Gómez-Luengo Á, Kniewel R, Martínez I, et al. Nucleic Acids Res. 2023;gkad758:1–16.
3. Pei J, Chen A, Sun Q, Zhao L, Cao F, Tang F. Biochem Eng J, 2018;139:33–42.
4. Kim BG, Kim HJ, Ahn JH. J Agric Food Chem. 2012;60:11143–8.

Fig. 1



P3-62

Exploring Diverse Glycone Preferences of *Aspergillus niger* Rutinosidase

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Introduction

Rutinosidases (6-α-L-rhamnosyl-β-D-glucosidases) represent a class of retaining diglycosidases pivotal in cleaving the glycosidic bond between saccharide moieties and their respective aglycones in glucosides and rutinosides. Among these, the most extensively studied is the rutinosidase derived from *Aspergillus niger* (*An*Rut) [1]. Insights from its crystal structure and molecular modeling [2] reveal a robust, specific affinity towards aromatic aglycones (flavonoids) and remarkable adaptability at the glycone binding site. Notably, this enzyme demonstrates a broad acceptance of both β-glucopyranosides and rutinosides, showcasing significant flexibility in binding to the glycone moiety [3].

Objectives

We aimed to investigate the variability of various mono- and diglycosides, mostly as *p*-nitrophenyl glycosides. Our objective was to explore the potential utilization of alternative carbohydrates, focusing on their applicability in transglycosylation reactions with alcoholic and phenolic acceptors.

Results

We have shown that *An*Rut can cleave β-glucopyranosides glycosylated at C-6 of the glucose moiety – natural substrates rutinosides but also isomaltosides (6-α-d-glucopyranosyl-β-d-glucosides). 1-4-linked diglycosides (e.g., maltoside and lactoside) are not hydrolyzed, showing a certain preference for the C-6 substitution of Glc. *An*Rut cleaves unsubstituted β-glucopyranosides well and we have now shown that other β-glycosides, such as β-d-galactopyranoside, β-d-xylopyranoside and α-l-arabinopyranoside (which is a structural analog of β-d-Gal), are also well accepted by the enzyme. Transglycosylation with various acceptors, including phenolic acceptors was also successfully tested.

Conclusion

This study demonstrates the great substrate flexibility of rutinosidase at the glycone site, so that the substrate specificity of *An*Rut can be extended to the C-6-modified glucosides [4] and some other monoglycosides. The high transglycosylation potential of this enzyme, which also enables the glycosylation of phenolic acceptors, extends the application potential of this versatile enzyme.

[1] Šimčíková D, Kotik M, Weignerová L, Halada P, Pelantová H, Adamcová K, Křen V. *Adv. Synth. Catal*. 2015, 357,107–117.

[2] Brodsky K, Kutý M, Pelantová H, Cvačka J, Rebroš M, Kotik M, Kutá Smatanová I, Křen V, Bojarová P. *Int. J. Mol. Sci*. 2020, 21, 5671.

[3] Křen V, Bojarová P. *Biotech. Adv*. 2023, 108217.

[4] Brodsky K, Petrásková L, Kutý M, Bojarová P, Pelantová H, Křen V. *ChemCatChem*. 2024, e202400028.

Supported by Czech Science Foundation project 22-00197K and travel grant LUC23061 from MEYS.

P3-63

Regio- and Stereoselective Oxidative Phenol Coupling by Mushroom Unspecific Peroxygenases

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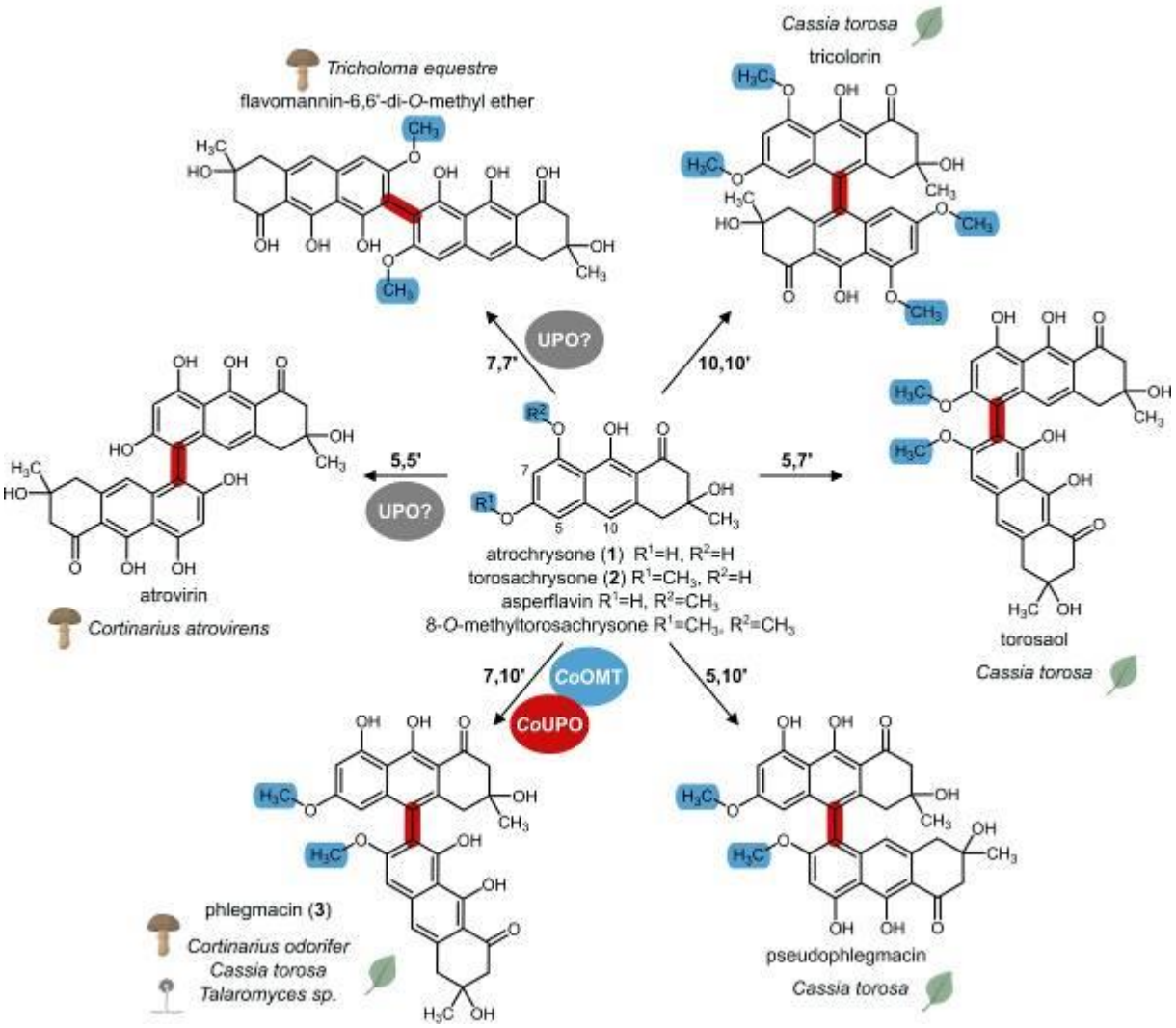
Dimeric pre-anthraquinones are widespread in nature with remarkable diversity among different kingdoms such as fungi or plants and possess various bioactivities.[1] They include compounds of different coupling types, e.g., phlegmacin, atrovirin, and flavomannin groups originating from the universal building block atrochrysone (**1**). However, the regio- and stereoselective control of the coupling reaction remains elusive.[2] The kingdom of fungi, particularly the division of Basidiomycota, is a producer of a large variety of these compounds with various modifications of **1**, such as methylation, oxidation, and dimerization (**Fig. 1**).

Fig. 1 Oxidative phenol coupling of dimeric pre-anthraquinones. The monomers atrochrysone (**1**), its methylated derivatives torosachrysone (**2**), asperflavin, and 8-*O*-methyltorosachrysone are symmetrically or unsymmetrically homo-coupled to natural products of fungal and plant origin.

Expanding on Ascomycota-employed oxidative phenol coupling (OPC) enzymes, *i.e.*, laccases and cytochrome P450 (CYP) enzymes, we identified a different basidiomycete enzymatic setup for the formation of biaryls. An unspecific peroxygenase (UPO) from the mushroom *Cortinarius odorifer* catalyzes the regioselective OPC of torosachrysone (**2**) to form phlegmacin (**3**). The biosynthesis of **3** was reconstituted by heterologous production of *Co*UPO in *Aspergillus niger* together with the non-reducing polyketide synthase (*Co*PKS4)[3] and an *O*-methyltransferase (*Spo*M).[4] The results further show **3** as the only dimeric product from the heterologous host, despite the plausible formation of five other regioisomers. This proves the regioselectivity of *Co*UPO for the 7,10"-coupling of **2**. Additionally, *in vivo* and *in vitro* feeding experiments showed the atropoisomeric formation of **3**.[5] We have shown that the oxidative phenol coupling in basidiomycetes proceeds *via* an unprecedented UPO-catalyzed dimerization and thereby enlarge the biocatalytic portfolio of OPC-type reactions beyond laccases, peroxidases, and CYP enzymes that have previously been reported.[6] This landmark study further opens a new avenue for C–C bond formation with potential application in organic synthesis.

References:
[1] a) W. Hüttel, M. Müller, *Nat. Prod. Rep.* **2020**, *38*, 1011; b) G. Bringmann *et al*., *Biaryls in nature: a multi-facetted class of stereochemically, biosynthetically, and pharmacologically intriguing secondary metabolites*, Springer, Vienna, **2001**; c) M. Gill, *Nat. Prod. Rep.* **2003**, 615.
[2] M. Gill, W. Steglich, *Pigments of fungi (Macromycetes)*, Springer, Vienna, **1987**.
[3] N. A. Löhr *et al*., *Angew. Chem. Int. Ed.* **2022**, *134*, e202116142.
[4] W. Thiele *et al*., *ACS Chem. Biol.* **2020**, *15*, 844.
[5] L. Platz *et al*., *under review* **2024**.
[6] a) S. Obermaier *et al*., *Angew. Chem. Int. Ed.* **2019**, *58*, 9125; b) C. R. Benedict *et al*., *Phytochemistry* **2006**, *67*, 356; c) L. S. Mazzaferro *et al*., *J. Am. Chem. Soc.* **2015**, *137*, 12289.

Fig. 1


Tricholoma equestre
flavomannin-6,6'-di-O-methyl ether
Cassia torosa
tricolorin
UPO?
7,7'
10,10'
5,5'
UPO?
5,7'
atrochrysone (1) R1=H, R2=H
torosachrysone (2) R1=CH3, R2=H
asperflavin R1=H, R2=CH3
8-O-methyltorosachrysone R1=CH3, R2=CH3
atrovirin
Cortinarius atrovirens
torosaol
Cassia torosa
7,10'
CoOMT
CoUPO
5,10'
phlegmacin (3)
Cortinarius odorifer
Cassia torosa
Talaromyces sp.
pseudophlegmacin
Cassia torosa

P3-64

Heterologous production of aurachin C in *Escherichia coli*

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Introduction

Aurachins are a family of prenylated quinoline antibiotics [1], which possess potent antiprotozoal properties [2]. In the natural producer *Stigmatella aurantiaca* Sg a15 a farnesyl moiety is added to 2-methyl-4-quinolone by the farnesyltransferase AuaA to produce aurachin D. The latter is then *N*-hydroxylated to aurachin C by the Rieske type oxygenase AuaF [3]. Recently, a method for the biocatalytic production of aurachin D from the synthetic substrate 2-methyl-4-quinolone using recombinant *E. coli* cells was described [4].

Objective

Due to their biological properties, the aurachins have gained attention as biochemical tool compounds and as drug candidates. Therefore, there is some interest in their cost-efficient production. The aim of this work was to intensify the biosynthesis of aurachin D in the heterologous host *E. coli* and to endow this bacterium with the *auaF* gene so that it can produce aurachin C.

Results

The switch from a complex fermentation medium to a defined minimal medium initially decreased the aurachin D titer in *E. coli* from 63.6 mg L^{-1} to 30.5 mg L^{-1}, but simplified the downstream processing. By increasing the glucose concentration in the minimal medium to 15 g L^{-1} and the NH4Cl concentration to 2 g L^{-1}, aurachin D titers comparable to the complex medium were reached. With the increase of the substrate concentration from 90 mg L^{-1} to 180 mg L^{-1} an aurachin D titer of 104.2 mg L^{-1} could be achieved. Another rise of the product titer to 117.2 mg L^{-1} was possible after extending the cultivation time from 24 h to 48 h.

Plasmid-based co-expression of AuaA and AuaF enabled the production of aurachin C (6.6 mg L^{-1}) besides aurachin D (31.5 mgL^{-1}). Further studies are necessary to clarify the incomplete conversion of aurachin D into aurachin C.

Conclusion

The intensification of aurachin D production was successful and the product titer was almost doubled. Moreover, a system for aurachin C production was established. In the future new aurachin C derivatives can be produced by precursor-directed biosynthesis and screened for their antiprotozoal properties.

References

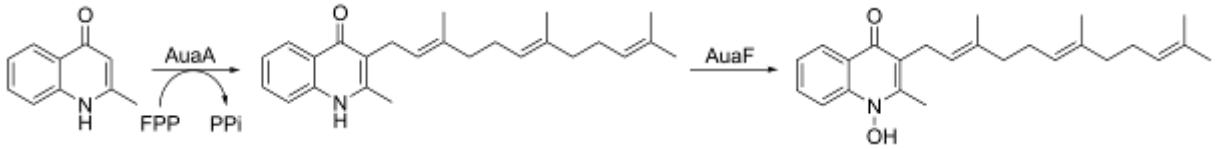
[1] Kunze B, Höfle G, Reichenbach H. The aurachins, new quinoline antibiotics from myxobacteria: production, physico-chemical and biological properties. *J. Antibiot.* **1987**; *40(3)*: 258.

[2] Kruth S, Zimmermann CJ-M, Kuhr K, Hiller W, Lütz S, Pietruszka J, Kaiser M, Nett M. Generation of aurachin derivatives by whole-cell biotransformation and evaluation of their antiprotozoal properties. *Molecules* **2023**; *28(3)*: 1066.

[3] Pistorius D, Li Y, Sandmann A, Müller R. Completing the puzzle of aurachin biosynthesis in *Stigmatella aurantiaca* Sg a15. *Mol. Biosyst.* **2011**; *7(12)*: 3308.

[4] Kruth S, Schibajew L, Nett M. Biocatalytic production of the antibiotic aurachin D in *Escherichia coli*. *AMB Expr.* **2022**; *12(1)*: 138.

Fig. 1


O
N
H
AuaA
FPP
PPi
O
N
H
AuaF
O
N
OH

P3-65

The lipase – initiated chemoenzymatic cascade reaction leading to the C=C double bond cleavage

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Biocatalysis plays an important role in modern organic synthesis. Among others, lipases are the most widely used enzymes as they catalyze "natural" reactions such as hydrolysis, esterification, and transesterification, often in a stereoselective or chemoselective way [1]. However, lipases also catalyze non-natural reactions, what makes these enzymes even more attractive. This ability of enzymes is known as a promiscuity [2].

Recently, we have focused our attention on enzymatic Michael addition reaction. However, when we studied the reaction of cinnamic acid esters with dimedone in the presence of lipase we did not obtain Michael adduct, but discovered a new activity of these enzymes as xanthendione derivatives products were obtained (Figure 1).

The unpreceded lipase activity towards the Michael addition leading to the C=C double bond cleavage was discovered. Various esters of cinnamic acid derivatives were converted to valuable xanthendiones in the reaction with dimedone. It is worth to note, that under special conditions, two various tetraketones were obtained from one ester molecule.

References

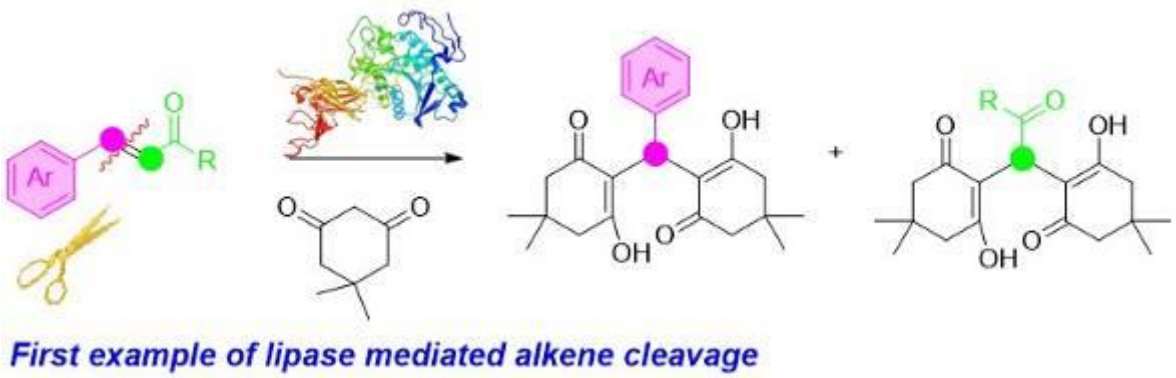
[1] P.-Y. Stergiou, et. al. *Biotechnol. Adv.,31* (*2013*), 1846-1859.

[2] K. Hult, P. Berglund, *Trends Biotechnol*. *25 (*2007), 231-238.

Acknowledgments

The work was supported by the National Science Centre, Poland, under research project Opus no 2023/49/B/NZ7/02469 and **COST Action CA 21162** "**Establishing a Pan-European Network on Computational Redesign of Enzymes (COZYME)**".

Fig. 1



P3-67

A safer and more sustainable by design perspective in biocatalytic amide-bond coupling

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[2]Technical University of Denmark, Copenhagen, Denmark
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The poster will be presenting our work in the preprint you can find in this link https://doi.org/10.26434/chemrxiv-2023-x8f25

Background: Amide bond formation is critical in many areas of chemistry, but traditional methods often rely on toxic chemicals, coupling agentsm organic solvents, and generate substantial waste. This approach is incompatible with the growing focus on green chemistry principles.

Question: How can we develop a more sustainable and environmentally friendly platform for amide bond formation?

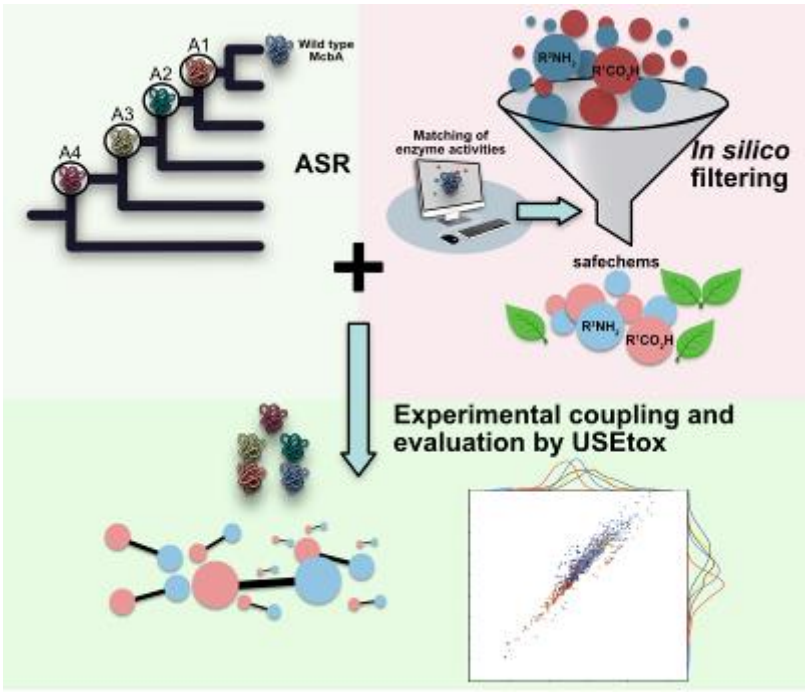
Methods:

In Silico Substrate Selection: Computational filtering of commercially available amines and acids based on their predicted human and environmental toxicity to generate a library of amines and acids with lower toxicity, named "safechems" in our work **Enzyme Engineering:** Matching the pool of safer amines and acids with the substrate scope of characterized amide bond synthesising enzymes. Marinacarboline amide bond synthetase (McbA) served as a template to create robust and versatile ancestral enzymes through computational modelling. **Experimental coupling**: A subset of the safechems were coupled by the wild-type and ancestral enzymes in 384 well plates, analysed by UPLC-MS **Evaluation of human and ecotoxicological impact** by USEtox

Results: From 105 commercially available amines and acids we generated a controlled chemical space with 54 acids and 188 amines. Evaluation with USEtox proved that the filtering resulted in chemicals with lower toxicity impact. The ancestral enzymes exhibited an increased thermostability of 20 C. Of 272 possible amides in the experimental coupling, 38 were detected with substantial DAD peaks, whereas 32 of them were novel structures.

Conclusions: This research demonstrates the effectiveness of the "Safer and more Sustainable by Design" strategy in biocatalytic amide bond formation. Notably, restricting the building blocks to those with minimal predicted toxicity did not hinder the discovery of novel amides. This highlights the potential for this approach to achieve both environmental sustainability and the development of new functionalities. Furthermore, the successful matching of the controlled chemical space (defined by "safechems") with the known substrate scope of characterized enzymes facilitated the identification of a suitable scaffold template. Subsequent ancestral sequence reconstruction yielded stable enzymes with complementary activities, expanding the range of achievable amide couplings.

Fig. 1


A1
A2
A3
A4
Wild type
McbA
ASR
Matching of
enzyme activities
In silico
filtering
safechems
Experimental coupling and
evaluation by USEtox

P3-68

The development of novel bio-catalytic strategies to construct enantiopure sulfoxides

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Abstract

Flavoenzymes have the potency to catalyse a vast number of monooxygenation reactions owing to the chemical versatility of cofactor flavin.[1] In BVMO-catalysed oxidation reactions, one atom of molecular oxygen is introduced to the organic substrate, and the other oxygen atom undergoes reduction to water. Having water as a by-product, BVMO attracts interest to be explored as a synthetic tool to address green chemistry.

This enzyme has not only been used for performing Baeyer-Villiger oxidations[2] but also for other oxidation reactions including sulfoxidations, oxidations of boron and selenium-containing compounds, epoxidations, and N-oxidations.[3] Chemo- and stereo-selectivity are the major focus of previous studies. Only a few works conducted on substrates presented more than one group susceptible to BVMO-catalysed oxidation.[4,5]

This work aims to set up a biocatalytic methodology for constructing enantiopure sulfoxides bearing multiple oxidative sites. A series of sulfides were synthesised using standard synthetic techniques as substrates and screened by a panel of BVMO enzymes. Selected enzymes were investigated and used for the methodology development.

References

1 M. Hall, *Enzymes (Essen)*, 2020, **47**, 37–62.

2 H. Leisch, K. Morley and P. C. K. Lau, *Chem Rev*, 2011, **111**, 4165–4222.

3 G. de Gonzalo, M. D. Mihovilovic and M. W. Fraaije, *ChemBioChem*, 2010, **11**, 2208–2231.

4 M. D. Mihovilovic, B. Müller, M. M. Kayser, J. D. Stewart, J. Fröhlich, P. Stanetty and H. Spreitzer, *J Mol Catal B Enzym*, 2001, **11**, 349–353.

5 F. Zambianchi, S. Raimondi, P. Pasta, G. Carrea, N. Gaggero and J. M. Woodley, *J Mol Catal B Enzym*, 2004, **31**, 165–171.

P3-69

Metal promiscuity in SvS-A2 and SvS-WT

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Bacterial diterpene cyclases are gaining recognition in synthetic biology and biocatalysis for their ability to sustainably produce complex, multicyclic compounds. Previously, through ancestral sequence reconstruction (ASR), our group generated an ancestor to spiroviolene synthase (SvS-WT) from *Streptomyces violens* that catalyzes the cyclization of geranylgeranyl pyrophosphate (GGPP) to spiroviolene via a three Mg^{2+} ion cluster[1].

We have observed differences in the access to the metal binding site and spatial arrangement of the three Mg^{2+} ions between SvS-WT and ancestral variant (SvS-A2) of spiroviolene synthase based on our obtained crystal structure and generated homology model[2] (see attached figure of the SvS-A2 (wheat) and SvS-WT (light blue) metal-binding sites overlayed with three Mg^{2+} ions complexed with GGPP (a) and FPP (b). The SvS-A2 (wheat) and SvS-WT (light blue) metal-binding sites overlayed with four Mg^{2+} ions complexed with GGPP (e) and FPP (f)).

Here, we are experimentally evaluating the effect of dicationic cofactor type and concentration on the activity and promiscuity for SvS-A2 and SvS-WT for the cyclization of GGPP to spiroviolene and the alternative substrate farnesyl pyrophosphate (FPP) to hedycaryol (see attached figure of the cyclization of GGPP and FPP catalyzed by SvS-A2 and SvS-WT with different di-cationic metal ions as cofactors). Different di-cationic metals result in large variations in specificity and relative activity for both SvS-A2 and SvS-WT).

References:

1. Hendrikse, N. M., Charpentier, G., Nordling, E. & Syrén, P.-O. Ancestral diterpene cyclases show increased thermostability and substrate acceptance. *FEBS J.* **285**, 4660–4673 (2018).
2. Schriever, K. *et al.* Engineering of Ancestors as a Tool to Elucidate Structure, Mechanism, and Specificity of Extant Terpene Cyclase. *J. Am. Chem. Soc.* **143**, 3794–3807 (2021).

Fig. 1

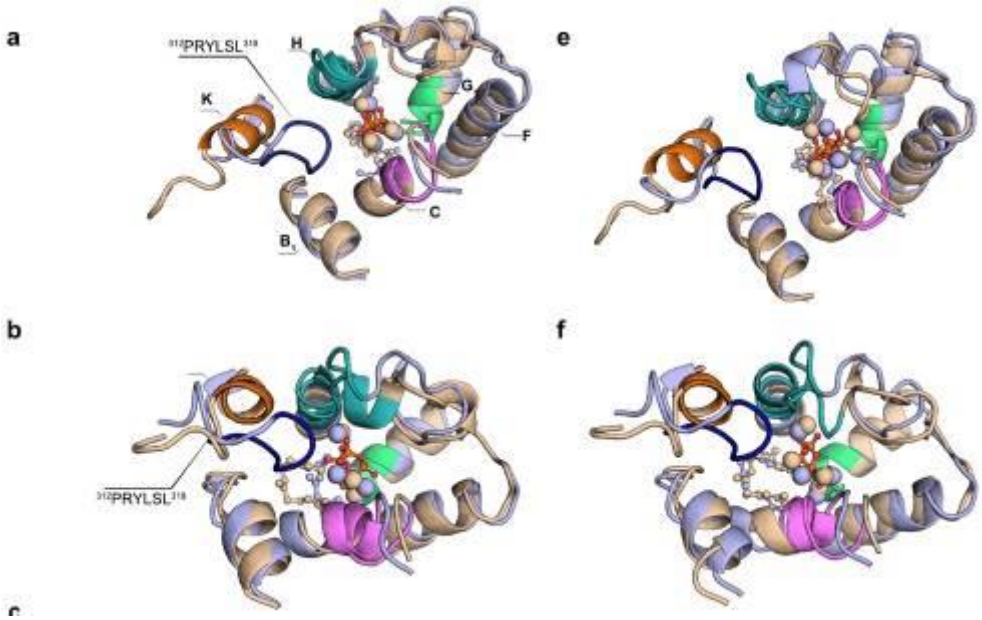
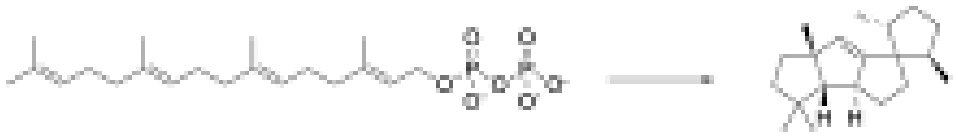


Fig. 2

SvS-A2, SvS-WT

Cofactor: Mg^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}



GGPP

Spirovetiene

FPP

Hedycaryol

P3-70

Robust Ketoreductase with High Enantioselectivity Towards Selected Substrates

T. Petrovičová[1], Z. Hegyi[1], I. Smonou[2], M. Rebroš[1]
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[2]University of Crete, Department of Organic Chemistry, Heraklion, Greece

Ketoreductases (KREDs) catalyse efficiently the reduction of carbonyl groups in a great variety of carbonyl compounds to produce optically pure secondary alcohols [1]. KRED (*Hansenula polymorpha*), which is recombinantly produced and researched in this work, was previously used for the synthesis of (*S*)-2-chloro-1-(3-chloro-4-fluorophenyl)-ethanol, a chiral key intermediate for an anticancer candidate compound, in high yield and excellent enantioselectivity [2].

The aim of the study was produce, characterize and apply this robust recombinant KRED (*Hansenula polymorpha*) in the reduction of the various ketones to achieve high conversion, enantioselectivity and stability. To improve the enantioselectivity with the most interesting substrates we want to apply computational design of point mutations, create the library of mutants, which will be screened in the reaction with acetophenone.

KRED was successfully overexpressed in *Escherichia coli* BL21(DE3) in a high cell density process, with biomass concentration 49.7 g L^{-1} and high specific activity (2220.1 ± 16.1 U g^{-1} DCW) [3]. KRED in combination with glucose dehydrogenase (GDH) created two-enzyme system, which was thoroughly characterized in terms of optimal pH, temperature, and stability in the reduction of the model substrate ethyl-2-methyl acetoacetate. Successful co-immobilization of KRED and GDH into PVA particles was developed [4], to achieve reusability of this two-enzyme system. The purified enzyme showed activity towards 31 substrates, including aliphatic and aromatic ketones, acetophenone and substituted acetophenones, aryl alkyl ketones, diketones, aliphatic ketones and β-keto esters [3].

In most of the bioreductions the *R*-enantiomer was formed, suggesting anti-Prelog preference for the KRED. This result is particularly important since there are very few known ketoreductases in the literature that show anti- Prelog selectivity during ketone reduction, especially for aliphatic ketones such as 2-octanone and 3-octanone. The modification and shift of enzyme enantioselectivity towards wider range of substrate by computational redesign of this KRED is highly demanded and approached.

Acknowledgement

This work was supported by the Slovak Research and Development Agency under the contract no. APVV-18-0254; Cost Action CA21162 (COZYME), and Project 101159534 — WIDEnzymes.

[1] D.J. Pollard, J.M. Woodley. Trends Biotechnol. 25 (2007) 66–73.

[2] R.L. Hanson, S. Goldberg, A. Goswami, T.P. Tully, R.N. Patel. Advanced Synthesis & Catalysis 347 (2005) 1073–1080.

[3] T. Petrovičová, D. Gyuranová, M. Plž, K. Myrtollari, I. Smonou, M. Rebroš. Molecular Catalysis. 502 (2021) 111364.

[4] T. Petrovičová, K. Markošová, Z. Hegyi, I. Smonou, M. Rosenberg, M. Rebroš. Catalysts. 8 (2018) 168.

P3-71

Substrate promiscuity of fungal laccases

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Laccases are versatile biocatalysts that are applied in a variety of industries, such as the food and textile industry[1]. In fungi they have been found to produce dimeric preanthraquinones with high regio- and stereoselectivity[2]. These natural products exhibit bioactive properties interesting for pharmaceutical applications and there is a large diversity due to their inherent regio- and stereo isomerism regarding their biaryl bond[3].

This work determines the substrate promiscuity of three laccases from filamentous fungi to investigate the influence of the chemical structure on regio- and stereoselectivity of the dimerization reaction. A small library of preanthraquinones has been synthesized over four steps with a subsequent dimerization by the Ustilaginoidin laccases UstL, CheL and MytL (Scheme1). Analytical scale reaction indicated full conversion after 24 hours and the reaction could be easily up-scaled to milligram scale.

To conclude, we have expanded the list of laccases capable of performing C-C coupling reactions on non-natural substrates and broadened the opportunities for development of oxidative phenol coupling enzymes as a tool in organic synthesis.

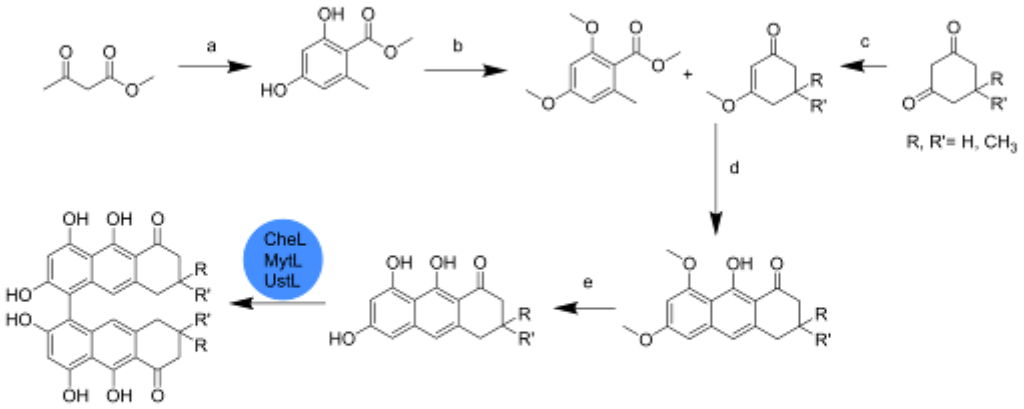
Scheme 1: Synthetic Route towards monomeric preanthraquinone derivatives and subsequent dimerisation by laccases. a) NaH, n-Buli -78°C, 24h, reflux., 24 h, then HCl r.t. 24h; b) K2CO3, MeI, reflux 48 h; c) TMSCl, MeOH, r.t., 24h; d) DIPA, n-BuLi, THF, -78°C → r.t., 3h; e) BBr3, DCM, r.t., 24h.

[1] M. D. Cannatelli, A. J. Ragauskas, *Chem. rec.* **2017**, *17*, 122.

[2] a) L. Fürtges, S. Obermaier, W. Thiele, S. Foegen, M. Müller, *ChemBiochem.* **2019**, *20*, 1928; b) S. Obermaier, W. Thiele, L. Fürtges, M. Müller, *Angew. Chem. Int. ed.* **2019**, *58*, 9125.

[3] a) W. Hüttel, M. Müller, *Nat. prod. rep.* **2021**, *38*, 1011; b) H. Aldemir, R. Richarz, T. A. M. Gulder, *Angew. Chem. Int. ed.* **2014**, *53*, 8286.

Fig. 1


a
b
c
d
e
R, R'= H, CH3
CheL
MytL
UstL

P3-72

Hydrogen-driven synthesis of chemicals in whole cells

D. Siebert[1], A. Al-Shameri[1], V. Sieber[1]
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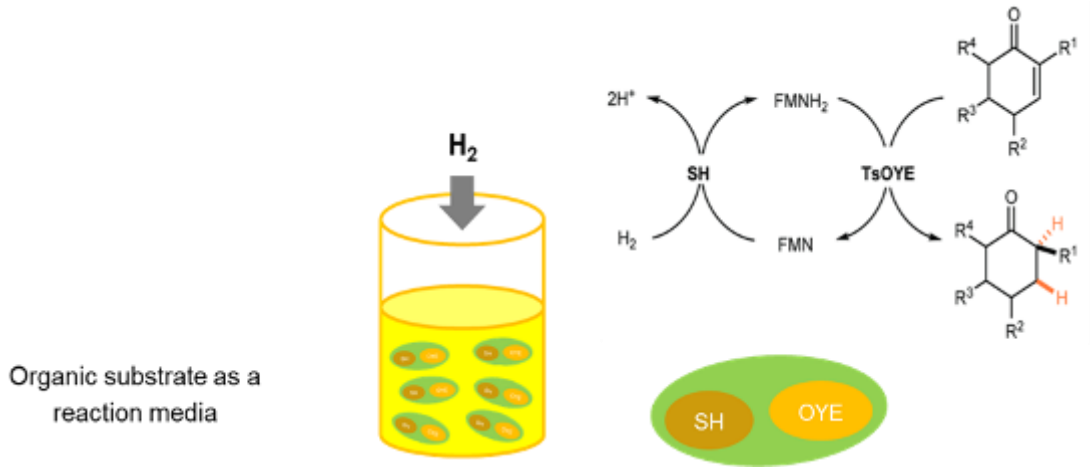
Biocatalysis-based reactions have the potential to be a clean and renewable platform to produce fine chemicals. Previously we described an oxygen-free oxidative biocatalytic system for the conversion of sugars into fine chemicals based on hydrogen evolution. This study introduces a sustainable approach to chemical synthesis using green hydrogen within whole-cell systems. By harnessing the enzymatic properties of the soluble hydrogenase (SH), we propose a method where we couple SH with an ene reductase (OYE) for the chemical reduction of terpenoids. This methode will minimize the reliance on traditional reducing reagents and reduces environmental impact. Moreover, by employing the substrate itself as a solvent, we further enhance the sustainability of the process by reducing the need for conventional aqueous/organic solvents. Through enzymatic catalysis, whole-cell biotransformation, and solvent optimization, we present a promising strategy for a green chemical synthesis applicable to pharmaceuticals, agrochemicals, and specialty chemicals. We discuss recent progress, challenges, and future directions in this field, underscoring the potential of green hydrogen-driven whole-cell catalysis for sustainable chemical synthesis.

[1] Al-Shameri A, Siebert DL, Sutiono S, Lauterbach L, Sieber V. "Hydrogenase-based oxidative biocatalysis without oxygen". Nat Commun. 2023

Fig. 1



P3-73

Optimization of reaction conditions for production of FDCA using whole-cell catalysis

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[1]KTH Royal Institute of Technology, Fibre and Polymer Technology, Stockholm, Sweden

2,5-furandicarboxylic acid (FDCA) is a desirable product as it is a building block for the bio-based polymer polyethylene furanoate (PEF), however; the carboxylation of 2-furoic acid is performed chemically at high temperatures (Banerjee et al, 2016). The enzyme HmfF from *Pelatomaculum thermopropionicum* (*Pt*HmfF) has been characterized and shown to catalyze the reversible (de)carboxylation of 2-furoic acid to FDCA (see Fig. 1) at high CO_2 loadings (Payne et al, 2019). Previous work on the production of FDCA with *Pt*HmfF using whole cells resulted in 41% conversion after 3h (Lopez-Lorenzo et al, 2023).

INSERT FIGURE HERE!

Fig. 1: Reversible reaction scheme of 2,5-Furandicarboxylic acid to 2-Furoic acid catalyzed by *Pt*HmfF

In this work, reaction conditions were optimized to achieve the highest yield while keeping sustainability in mind. Briefly, *E. coli* CD43 cells were cultivated in different media with bioreactors or in Erlenmeyer flasks in a shaking incubator. The carbon dioxide source was tested, using carbon dioxide gas, sodium bicarbonate and potassium bicarbonate.

References:

Banerjee, Aanindeeta, et al. "Carbon dioxide utilization via carbonate-promoted C–H carboxylation." Nature, vol. 531, no. 7593, Mar. 2016, pp. 215–219, https://doi.org/10.1038/nature17185.

Lopez-Lorenzo, Ximena, et al. "Whole-cell mediated carboxylation of 2-furoic acid towards the production of renewable platform chemicals and Biomaterials." ChemCatChem, vol. 15, no. 6, 21 Feb. 2023, https://doi.org/10.1002/cctc.202201483.

Payne, Karl A.P., et al. "Enzymatic carboxylation of 2-furoic acid yields 2,5-furandicarboxylic acid (FDCA)." ACS Catalysis, vol. 9, no. 4, 15 Feb. 2019, pp. 2854–2865, https://doi.org/10.1021/acscatal.8b04862.

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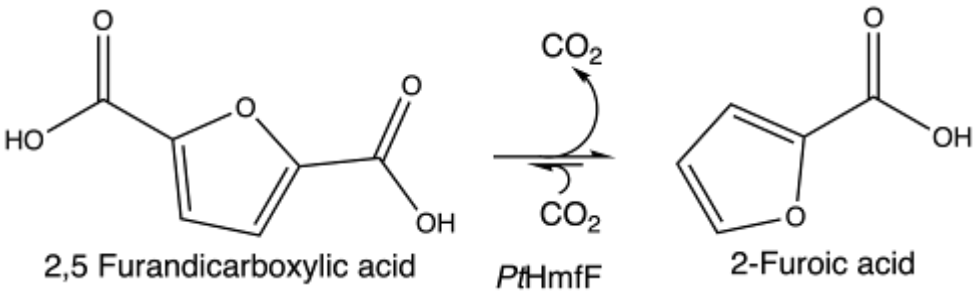

P3-74

Hydrogen-Driven Isobutanol Production

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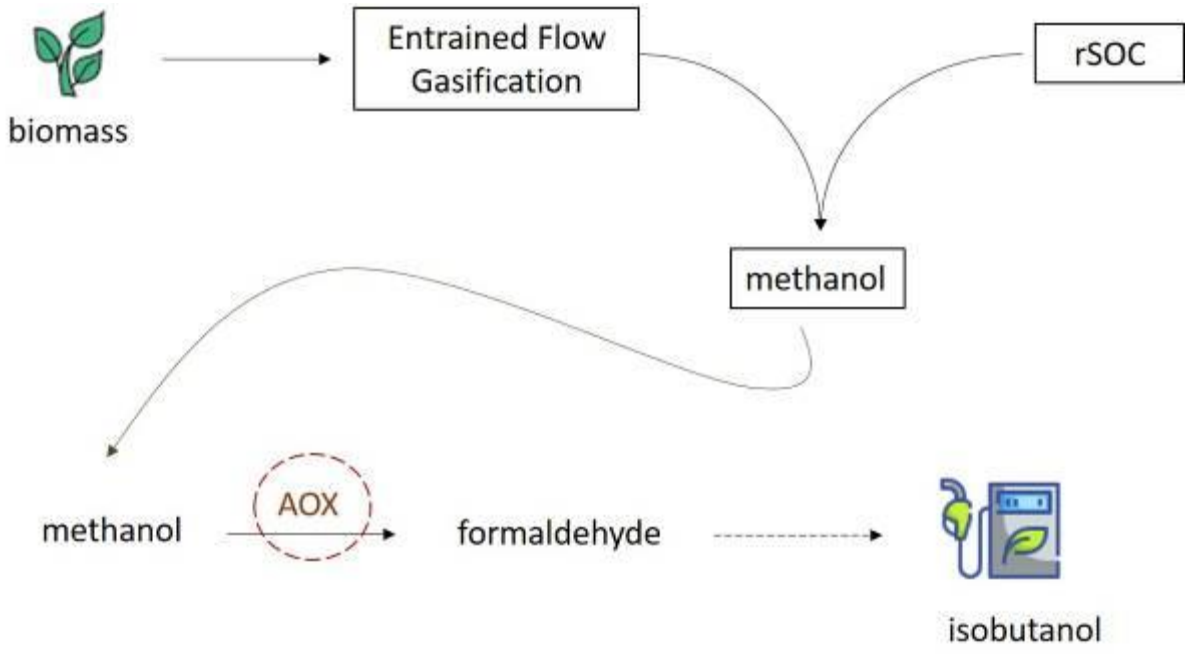
Facing rapid global warming, the need for green technologies, which utilize renewable resources, is rising. This also includes the synthesis of important chemicals like isobutanol, which has been produced with fossil fuels as feedstock [1]. In the context of green chemistry, an enzymatic route is applied for the synthesis of isobutanol, starting with green methanol as substrate, which can be produced by utilizing hydrogen and other syngas components. This enzymatic route starts with an alcohol oxidase (AOX), which catalyzes the reaction of methanol to formaldehyde. The focus of this work lies on finding a suitable AOX, which has a low activity on and affinity for isobutanol and that it can also be expressed in *E. coli* for further engineering. Furthermore, this alcohol oxidase is applied in the enzymatic cascade and isobutanol production is evaluated.

Figure 1: Production of green methanol, followed by the conversion of methanol to formaldehyde with the help of an alcohol oxidase (AOX) and subsequent production of the biofuel isobutanol.

[1] Fu, Chuhan; Li, Zhuoxi; Jia, Chuhua; Zhang, Wanli; Zhang, Yulei; Yi, Conghua; Xie, Shaoqu (2021): Recent advances on bio-based isobutanol separation. In Energy Conversion and Management: X 10, p. 100059. DOI: 10.1016/j.ecmx.2020.100059.

Fig. 1



P3-75

Choosing the Optimal Denitrifying Bacterial Culture for Autotrophic and Mixotrophic Bioremediation Strategies: Insights into Nitrate-Reducing Fe(II)-Oxidizing Bacteria

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The increasing interest in autotrophic and mixotrophic denitrification utilizing nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) highlights their potential for efficient nitrate and heavy metal removal. While this approach promises cost-effective and environmentally friendly solutions, fundamental research on NRFeOx is still developing. Traditionally categorized into autotrophs and mixotrophs, recent studies reveal a third group of bacteria of autotrophic nature, which cannot grow autotrophically using Fe(II) as the sole electron donor, although they do perform nitrate-reducing Fe(II) oxidation.In this contribution, we elucidate the distinctions among NRFeOx to provide a robust foundation for applied scientists in selecting optimal bacterial isolates or enrichment cultures for their systems. We analyzed a range of commonly used isolated bacterial strains, well-studied enrichment cultures, and novel enrichment cultures under denitrifying Fe(II)-oxidizing conditions. Our research assessed their performance in nitrate removal, Fe(II) oxidation, Fe(III) (oxyhydr)oxide mineral formation, and byproduct profiles. In addition, we explored the interplay between abiotic processes and microbial activity, shedding light on their collective impact on denitrification efficiency.Key findings reveal that the selection of NRFeOx for bioremediation purposes significantly affects the production of harmful intermediates such as nitrite and climate-active gases like N2O and NO. NRFeOx does not equal NRFeOx. We like to underscore the necessity of refining our understanding of NRFeOx, offering critical insights to enhance both the effectiveness and environmental impact of nitrate and heavy metal removal technologies.

(1) Pan, Y., Ying-Ying F., Ke Z., Tian T., Yu-Sheng L., and Han-Qing Y. (2023) "Microbial Mixotrophic Denitrification Using Iron(II) as an Assisted Electron Donor". Water Research X 19 (May):100176. (2) Park, S., Ji-Hoon L., Tae Joo S., Hor-Gil H., and Min Gyu K. (2018) "Adsorption and Incorporation of Arsenic to Biogenic Lepidocrocite Formed in the Presence of Ferrous Iron during Denitrification by Paracoccus Denitrificans". Environmental Science & Technology 52 (17): 9983–91. (3) Becker, S., Enright, A., and Kappler, A. (2021) "7 LIVING ON IRON". Metals, Microbes, and Minerals - The Biogeochemical Side of Life. De Gruyter.

P3-76

Enzymatic Hydrolysis of an Industrial Yeast Extract By-product for Enhanced Fermentation Application

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1 Hamburg University of Technology, Institute of Technical Biocatalysis, Hamburg, Germany
2 Ohly GmbH, Hamburg, Germany

IL4-1

Repurposing Enzymes for Non-Natural Reactions

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Enzymes are gaining increasing attention in the context of sustainable synthesis, as they can contribute to the 'greening' of chemical manufacturing. Two selected examples will illustrate how enzyme catalysis can offer attractive approaches to address the sustainability challenge in modern synthesis and highlight the current shortcomings in delivering efficient biocatalysts for non-natural reactions.

i) We recently identified a case of asymmetric hydride-free isomerization of non-activated C=C-bonds by flavin-dependent Old Yellow Enzymes (OYEs).[1] We coupled this redox-neutral step to a bioreduction by designing a fusion bi-molecular protein to generate a one-pot enzymatic cascade for the stereodivergent reduction of a-angelica lactone.[2] Since the stereoselectivity of OYEs is difficult to predict and a universal structure-stereoselectivity relationship for OYEs is still elusive, we leverage natural protein diversity to develop a stereocomplementary platform applicable to prochiral lactones.

ii) Nitro compounds are vital in organic synthesis, yet conventional methods for their production are non-selective and environmentally harmful. Inspired by the occurrence of nitro compounds in nature,[3] we recently achieved the biocatalytic hydronitration of a,b-unsaturated carboxylic acids by a lyase in the presence of nitrite. To broaden substrate options beyond fumaric acid, we aim to explore computational tools like QM/MM methodologies and machine learning to understand, predict, and redesign the protein's structure, so as to enable efficient conversion of various conjugated carboxylic acids. Studying the aspartase/fumarase superfamily provides additional insights to tailor the biocatalyst for non-natural reactions.

Figure 1. Concept of A) enzymatic hydronitration of unsaturated carboxylic acids using nitrite salt; B) asymmetric isomerization-reduction of (pro)chiral butenolides with Old Yellow Enzymes.

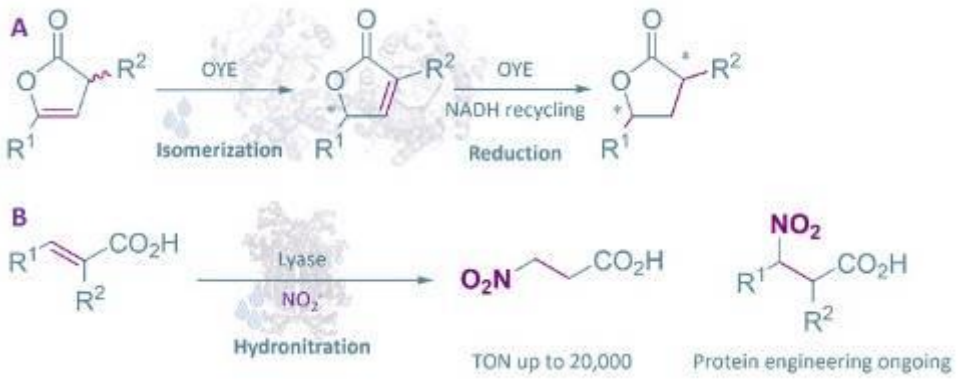
Acknowledgement is made to the ACS GCI Pharmaceutical Roundtable Research Grant for partial support of this research. This research was funded in part by the Austrian Science Fund (FWF) [10.55776/P36538].

[1] M. S. Robescu et al., *ACS Catal*. **2022**, 12, 7396

[2] A. Tonoli et al., *ChemBioChem* **2023**, e202300146.

[3] A. J. Waldman, T. L. Ng, P. Wang, E. P. Balskus, *Chem. Rev*. **2017**, *117*, 5784

Fig. 1



IL4-2

Enzyme Cascades for Fuel Cell and Electrosynthesis Applications

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[2]Missouri University of Science nad Technology, Rolla, United States

Electrosynthesis of commodity and fine chemicals is an emerging area. Although bioelectrocatalysis has made great advances in the last 20 years for energy application, bioelectrocatalysis has only recently been considered for this application. This talk will discuss the advantages of bioelectrocatalysis for energy and electrosynthesis with examples ranging from fuel cells to ammonia production to pharmaceutical products and intermediates. In the last 5 years, there have been extensive studies and new materials designed for catalytic reduction of nitrogen to ammonia. This is a challenging reductive transformation for traditional electrocatalysts and photocatalysts, but nature can provide an inspiration. Nitrogenase is the only enzyme known to reduce nitrogen to ammonia. This talk will discuss electroanalytical techniques for studying nitrogenase electrochemistry, including both mediated bioelectrocatalysis and direct bioelectrocatalysis. Then, this talk will discuss electrode materials innovation for interfacing these complex proteins with electrode surfaces as well as using them for electrosynthesis of ammonia as well as other value-added products (i.e. chiral amines, chiral amino acids, etc.). Finally, this talk will discuss the use of enzyme cascades for enzymatic bioelectrosynthesis and synthetic biology for microbial bioelectrosynthesis of ammonia and other value-added products.

IL4-3

The Quest for Artificial Biocatalytic Reactivities: Metalloproteins as Mediators of Pericyclic Reactions

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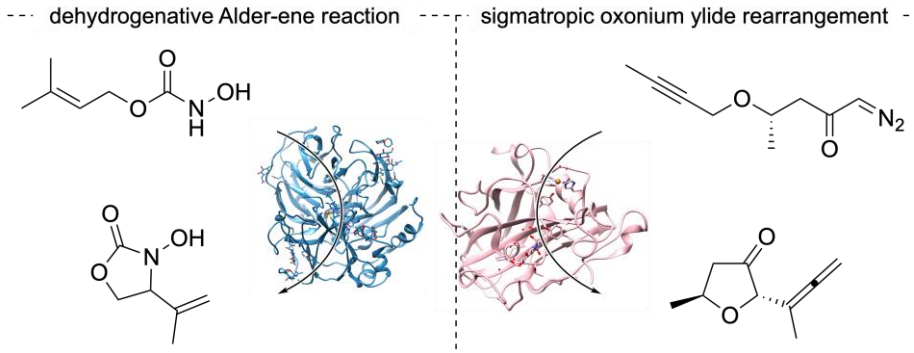
University of Helsinki, Helsinki, FI

Pericyclic reactions play a crucial role in traditional organic synthesis as they provide unique reactivity patterns and excellent selectivities over a broad range of carbon-carbon and carbon-heteroatom bond forming reactions. In contrast, pericyclic transformations take much more of a niche position in the world of biological chemistry, where the more prominent examples include the Claisen rearrangement catalyzed by chorismate mutase in the shikimate pathway or the non-enzymatic, light-driven electrocyclic ring opening en route to vitamin D. In fact, pericyclic reactions such as the Diels-Alder cycloaddition have been early on identified as attractive target for protein engineering campaigns aiming to use de novo protein designs to create new biocatalysts that can act in non-natural transformations.[1]

In search of biological mediators with abilities to address synthetically important reactions beyond the biosynthetic repertoire, our group has focussed on the exploitation of catalytic promiscuity of existing biocatalysts, and various wild-type metalloenzymes were identified as effective promoters in a range of unnatural transformations for the synthesis of O-heterocyclic compounds.[2-6] In this presentation, our most recent discoveries exploiting copper-dependent proteins as versatile biocatalysts in pericyclic reactions will be discussed. On one hand, blue multicopper enzymes (laccases) were identified to possess significant activity in the oxidative generation of reactive nitroso species, a novel enzymatic activation mode that enable the asymmetric C-N bond formation through ene-type reactions.[7] Furthermore, copper-dependent polysaccharide-degrading oxidoreductases are introduced as powerful mediators in sigmatropic rearrangements enabling the preparation of complex tetrahydrofurans in high stereoselectivities.



References

[1] Siegel, Zhanghellini, Lovick, Kiss, Lambert, St. Clair, Gallaher, Hilvert, Stoddard, Houk, Michael, Baker, *Science* 2010, *329*, 309.
[2] Naapuri, Rolfes, Keil, Sapu, Deska, *Green Chem.* 2017, *19*, 447.
[3] Thiel, Doknić, Deska, *Nature Commun.* 2014, *5*, 5278.
[4] Liu, Merten, Deska, *Angew. Chem. Int. Ed.* 2018, *57*, 12151.
[5] Liu, Rolfes, Björklund, Deska, *ACS Catal*, 2023, *13*, 7256.
[6] Kiefer, Liu, Gummerer, Jäger, Deska, *Angew. Chem. Int. Ed.* 2023, *62*, e202301178.
[7] Jäger, Haase, Koschorreck, Urlacher, Deska, *Angew. Chem. Int. Ed.* 2023, 62, e202213671

L4-1

Electrobiocatalytic Conversion of CO_2 with Formate Dehydrogenases

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Carbon dioxide (CO_2) is one of the key drivers and biggest contributor to global warming. The ability to catalyze the conversion of CO_2 and water into sustainable fuels and chemicals, utilizing renewable energy sources, is critical to mitigate the irreversible climate change and environmental degradation.

A direct pathway to convert thermodynamically stable CO_2 into high-in-demand commodity chemicals, such as formate, formaldehyde, and methanol, is *via* multi- enzyme cascade catalysis, utilizing the reverse catalytic abilities of three different types of enzymes- formate dehydrogenase (FDH), formaldehyde dehydrogenase (FalDH) and alcohol dehydrogenase (ADH). FDHs (EC 1.17.1.9) from *Rhodobacter capsulatus* (RcFDH) and *Cupriavidus necator* (CnFDH) are high molecular weight (heterotetrametric, MW ~360 kDa), metal-dependent and NAD^+- dependent multi-domain enzymes, coordinating molybdenum (Mo) in the active site with Flavin mononucleotide (FMN) as cofactor. Recent studies showed that the protein subunits connect a series of Fe-S clusters which are directly involved in the electron- transfer from FMN to Mo [1]. FDHs can be expressed in *Escherichia coli*, but their structural size, fragility, and the poor electron donation capacity of NADH hinder the efficient and robust CO_2 catalysis for sustainable large- scale implementation and industrial application.

The study aims to demonstrate that the enzymatic rates of FDHs can be improved *via* protein engineering and direct electron donation.

Hypothesizing that not all subunits of the enzyme are required for its catalytic activity, especially regarding the choice of the electron donor, different truncated variants were constructed. These constructs were produced in a high-throughput *E. coli* expression platform using different medias, strains and temperatures to identify their optimal expression conditions. Their electrocatalytic activity towards CO_2 was assessed in the presence of different electron donors. In addition, novel putative metal- dependent FDHs were identified using bioinformatic analyses.

This works provides novel insights into the molecular mechanism of FDHs towards CO_2 conversion and furthermore highlights the importance of the selection of the electron donor.

[1] C. Radon, et al., *Nat. Commun.*, **2022**, 11, 1912.

L4-2

One-step hydrogen-driven carboxylic acid reduction under non-explosive conditions

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The reduction of carboxylic acids to alcohols is a highly important transformation in industrial chemistry. For instance, the production of fatty alcohols in 2015 was 3 million tons worldwide. Reduction of carboxylic acid usually requires esterification of fatty acids, followed by metal-catalyzed hydrogenation at elevated temperatures and pressures. Direct reduction of carboxylic acids can be easily accomplished by carboxylic acid reductases (CARs) [1], which require the stoichiometric addition of electron donors such as glucose for recycling of the cofactors ATP and NADPH, respectively. In whole-cell biocatalysis, only a small part of the electrons of the molecule is directed to the biotransformation. This results in a poor atom economy, the accumulation of unwanted side-products and causes land-use conflicts in large scale.

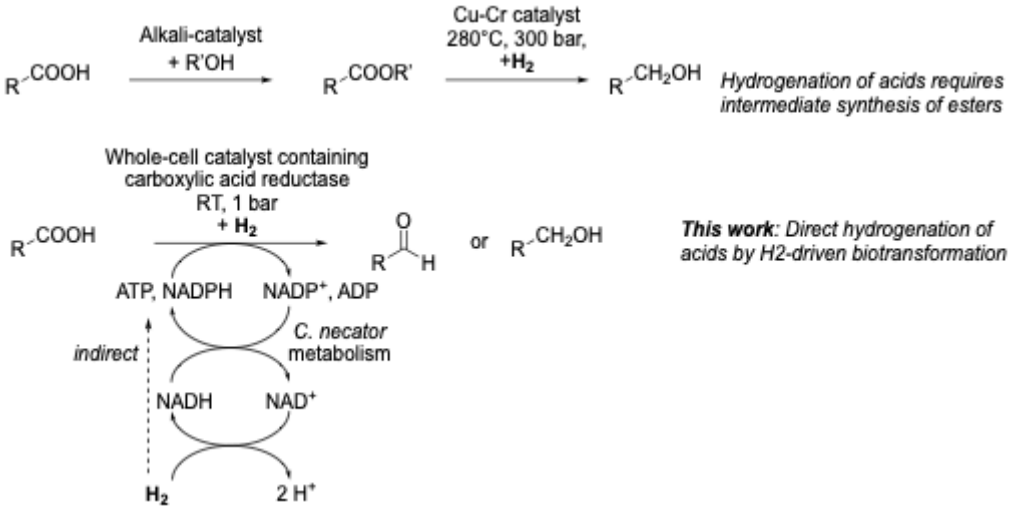
Figure 1: Hydrogen-driven whole-cell bioreduction allows the reduction of carboxylic acids in one step.

Herein we report the coupling of CARs to hydrogen-driven cofactor-recycling in recombinant cells of the hydrogen-oxidizing "Knallgas" bacterium *Cupriavidus necator* (Figure 1). The endogenous soluble [NiFe]-hydrogenase provides reduced NADH and subsequently NADPH [2]. Both the membrane-bound dehydrogenase and oxidation of NADH build up a proton gradient at the inner cell membrane, which is used for ATP synthesis. Yet, the oxygen that is required for respiration is a safety risk in presence of hydrogen. Our research therefore focused on the development of a system for the cultivation of the cells and the biotransformation under non-explosive conditions, thus combining efficient hydrogen-based biocatalysis with minimal safety concerns.

[1] B. Daniel et al., ACS Catalysis, 2022, 12, 15668–15674.

[2] L. Assil-Companioni et al., ChemSusChem, 12, 2361-2365.

Fig. 1



L4-3

Selective hydrogenation of nitro compounds to amines by coupled redox reactions over a heterogeneous biocatalyst

D. Sokolova[1], T. Lurshay[1], J. Rowbotham[1], G. Stonadge[1], H. Reeve[1], S. Cleary[1], T. Sudmeier[1], K. Vincent[1]
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Efficient and sustainable routes to amine synthesis remain in high demand for the production of pharmaceuticals (Fig. 1A) and agrochemicals as well as other areas of chemical manufacturing. This has led to a wide range of developments in selective methods for amine synthesis. Reduction of nitro-groups is a common synthetic route to amines, and is a key target for greener synthetic protocols as current methods often lack selectivity and rely on stoichiometric reductants or costly precious metals. (Fig. 1B). Although biocatalytic strategies for nitro reduction are often viewed as environmentally friendly and more selective alternative, they are still hindered by reliance on glucose to recycle cofactor and limited progression beyond intermediates.

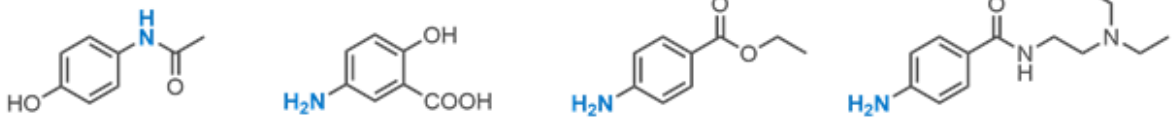
A new concept is emerging for electrochemical hydrogenation mechanism in heterogeneous catalysis, whereby H_2 oxidation at one metal site is coupled to a reduction process at a separate, but electronically linked, catalytic site. It has been noted recently that nitro group hydrogenations at Pd/C may proceed *via* an electrochemical mechanism whereby H_2 oxidation occurs at active sites on the Pd providing electrons for nitro reduction at the carbon support. However, the presence of Pd under H_2 can lead to a range of unwanted side reactions on a target substrate. In contrast, hydrogenase enzymes are able to split H_2 at a buried active site inaccessible to larger organic substrates releasing electrons to the protein surface *via* iron-sulfur clusters. This suggested to us that hydrogenase on carbon could be exploited as a *selective* catalyst for activation of H_2, to give Pd-free "electrochemical hydrogenation" of nitro compounds (Fig. 1C).

Inspired by the concept of electrochemical hydrogenation in heterogeneous catalysis, we have established a hybrid bio-chemo catalyst, which operates entirely *via* an electrochemical (coupled redox) mechanism. We show that this gives an easy-to-use, highly versatile catalyst for the synthesis of amines *via* hydrogenation of aromatic nitro compounds under mild conditions. The catalyst comprises a carbon black supported NiFe hydrogenase (Hyd-1) which enables use of H_2 at atmospheric pressure as an atom-efficient reductant, without need for a co-catalyst or cofactor. We confirm tolerance of the biocatalytic system to a wide range of functional groups by hydrogenating various derivatives of nitrobenzene to the corresponding aniline, with isolated yields from 78% to 96%. The catalyst is fully recyclable over 5 reaction cycles, and is applicable for a gram-scale hydrogenation, as demonstrated with procainamide synthesis. We further show that this approach can be extended to hydrogenation of aliphatic substrates by utilising another NiFe hydrogenase (Hyd-2), which we confirm by hydrogenation of 1-nitrohexane. Overall, this work represents a valuable addition to the suite of approaches available for amine synthesis.[1]

1. Sokolova D, et al. ChemRxiv. 2024, DOI: 10.26434/chemrxiv-2024-vqfc1

Fig. 1

A. *Examples of amine-containing pharmaceuticals*



Paracetamol
Pain reliever

Mesalazine
Inflammatory bowel disease treatment

Benzocaine
Local anaesthetic

Procainamide
Cardiac arrhythmia treatment

B. *Current state of the field for nitro hydrogenations and current challenges*

Pt-group-catalyzed hydrogenations
Pt, Pd, Rh, Ir

- *poor functional group tolerance*
- *high price and scarcity of the metal*
- *catalyst leaching*
- *often require modification for selectivity*

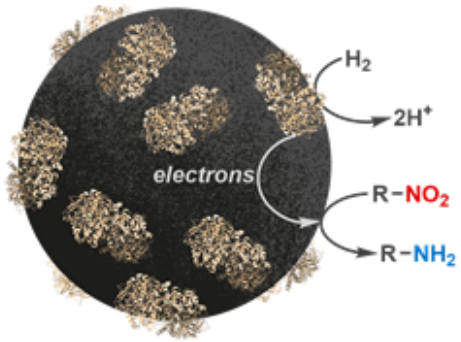
Non Pt-group-catalyzed reductions
Ni, Cu, Au, Ag, Co, Fe

- *harsh operating conditions*
- *lower activities than PGM catalysts*
- *possible toxicity*
- *often require modification for selectivity*

Biocatalytic reductions
nitroreductases

- *require expensive cofactors*
- *glucose-driven cofactor recycling*
- *co-catalyst needed*
- *limited demonstrated substrate scope*

C. *This work: new biocatalyst for hydrogenation of nitro compounds*



chemoselective

functional group tolerant

operates under mild conditions

does not require cofactor

L4-4

Electro-Driven Biocatalysis for Sustainable Chemical Synthesis in a Future Bioeconomy

P. Schoenmakers[1], G. Y. Lim[1], A. Al-Shameri[2], S. Guillouet[3], U. P. Apfel[4], L. Lauterbach[1]
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[2]Technical University Munich, Chemie Biogener Rohstoffe, Munich, Germany
[3]INSA Toulouse, Toulouse Biotechnology Institute, Toulouse, France
[4]Ruhr University Bochum, Inorganic Chemistry I, Bochum, Germany

Introduction:

A bioeconomy, essential for carbon neutrality, depends on rethinking industrial biotechnology processes. Traditional reliance on glucose from starch and sucrose is increasingly challenged by land use and food industry concerns. Bioelectrocatalysis offers an environmentally friendly alternative, enabling precise chemical transformations with minimal waste, crucial for both bulk and fine chemical production.

Objective:

Our research explores the integration of biocatalysis with electrolysis and covers enzymatic and whole-cell systems using electrical energy to create new possibilities for chemical synthesis.

Results:

We developed a system of immobilised biocatalysts including an O_2-tolerant hydrogenase [1,2] in a continuous flow setup using molecular hydrogen from water electrolysis for electro-synthesis of chemicals [3-5]. This approach allowed the reliable synthesis of chiral cyclohexanedione derivatives using flavin and enoate reductase, as well as N-heterocyclic derivatives utilizing NADH, putrescine oxidase and imine reductase [3,4]. We have integrated enzymatic systems into *Cupriavidus necator* for CO_2 sequestration and efficient H_2 oxidation, using a developed electro-fermentation reactor for safe chemical synthesis [5]. Our work gave valuable insights into interplay between biocatalysts and electrolysers, improving yield and efficiency and demonstrated scalable biotechnological advances.

Conclusion:

The integration of water electrolysers with H_2-driven enzymatic systems, together with advances in lithoautotrophic biorefineries represents a significant step forward in biocatalysis. This approach has potential for scale-up and is in line with the goals of a sustainable bioeconomy, paving the way for greener and more efficient chemical production.

References:

[1] Al-Shameri A, Siebert DL, Sutiono S, **Lauterbach L**, Sieber V. 2023. Nat Commun. 14(1):2693. doi:10.1038/s41467-023-38227-9

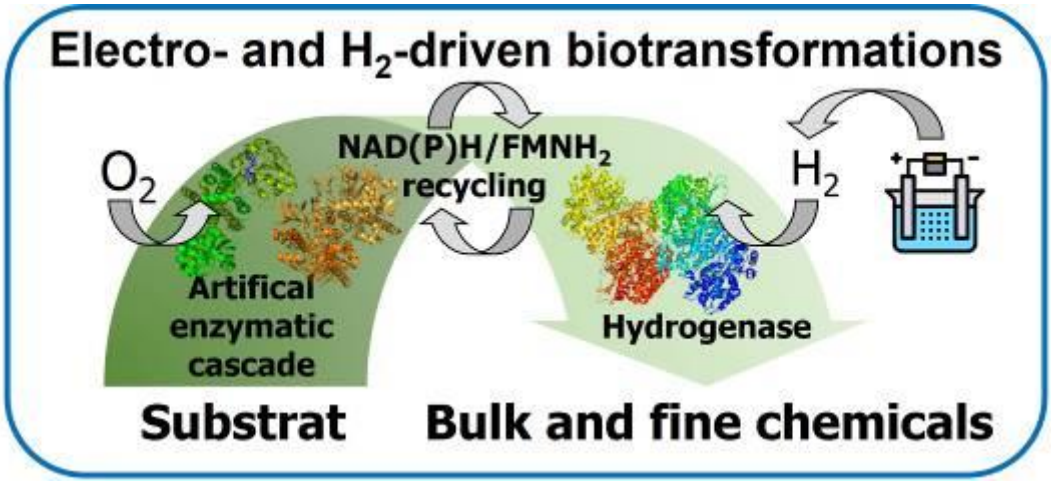
[2] Al-Shameri A, Willot SJ-P, Paul CE,§ Hollmann F, **Lauterbach L** 2020 Chem. Commun. 56, 9667–9670 doi.org/10.1039/D0CC03229H

[3] Al-Shameri A, M Petrich M-C, Puring Kj, Apfel U-P, Nestl BM, **Lauterbach L** 2020 Angew. Chem. Int. Ed. 59:10929–10933 doi.org/10.1002/anie.202001302

[4] Lim G., Cordero P, Calabrese D, Rother D., Paul C., **Lauterbach L** (manuscript in preparation)

[5] Schoenmakers P, Ihl A, Rad R., Weickardt I, Guillouet S, Apfe U.-P., **Lauterbach L** (manuscript in preparation)

Fig. 1


Electro- and H_2-driven biotransformations
O_2
NAD(P)H/$FMNH_2$ recycling
H_2
Artifical enzymatic cascade
Hydrogenase
Substrat
Bulk and fine chemicals

L4-5

Opportunities for Merging Chemical and Biological Synthesis

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Natural products and their derivatives have long been used as medicinal agents, and they still make up a significant fraction of clinically approved drugs. Natural product synthesis provides a rich and unparalleled opportunity to develop new synthetic transformations, conceive novel and general strategies to access complex structures and study the mechanism of action of bioactive targets. This lecture will illustrate the opportunities that lie at this interface between synthetic organic chemistry and chemical biology by describing a series of examples that we are actively working on in our laboratory at Peking University. We take inspiration from Mother Nature to study the biosynthesis of plant-derived natural products to elucidate new enzymatic mechanisms and apply the chemoenzymatic approach to prepare complex natural products and their derivatives. Moreover, we further use bioactive natural products to explore new biology and develop novel drug candidates for human diseases.

References:

1. Bin Jiang, Lei Gao, Haijun Wang, Yaping Sun, Xiaolin Zhang, Han Ke, Shengchao Liu, Pengchen Ma, Qinggang Liao, Yue Wang, Huan Wang, Yugeng Liu, Ran Du, Torben Rogge, Wei Li, Yi Shang, K. N. Houk, Xingyao Xiong, Daoxin Xie, Sanwen Huang, **Xiaoguang Lei***, Jianbin Yan* "Characterization and heterologous reconstitution of *Taxus* biosynthetic enzymes leading to baccatin III" ***Science*** **2024**, *383*, 622-629
2. Lei Gao, Yike Zou, Xiaojing Liu, Jun Yang, Xiaoxia Du, Jin Wang, Xinshui Yu, Junping Fan, Mingxuan Jiang, Yuli Li, K. N. Houk*, **Xiaoguang Lei*** "Enzymatic control of endo and exo stereoselective Diels-Alder reactions with broad substrate scope" ***Nature Catalysis*** **2021**, *4*, 1059-1069
3. Lei Gao, Cong Su, Xiaoxia Du, Ruishan Wang, Shuming Chen, Yu Zhou, Chengwei Liu, Xiaojing Liu, Runze Tian, Liyun Zhang, Kebo Xie, She Chen, Qianqian Guo, Lanping Guo, Yoshio Hano, Manabu Shimazaki, Atsushi Minami, Hideaki Oikawa, Niu Huang, K. N. Houk, Luqi Huang*, Jungui Dai*, **Xiaoguang Lei*** "FAD-dependent Enzyme-Catalysed Intermolecular [4+2] Cycloaddition in Natural Product Biosynthesis" ***Nature Chemistry*** **2020**, *12*, 620-628
4. Weilong Liu, Benke Hong, Jin Wang, **Xiaoguang Lei*** "New Strategies in the Efficient Total Syntheses of Polycyclic Natural Products" ***Acc. Chem. Res.*** **2020**, *53*, 2569–2586

L4-6

Batch and continuous operation of a fluidized bed electrode for in-situ co-factor generation in an electro-enzymatic process

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Introduction:
Conventional chemical synthesis often relies on fossil-derived feedstocks, posing substantial environmental and climatic challenges. Electro-biotechnology offers a promising alternative by synergistically combining electrochemistry with biotechnology to utilize renewable electrical energy and green biocatalysts (e.g. enzymes) for more sustainable chemical production.

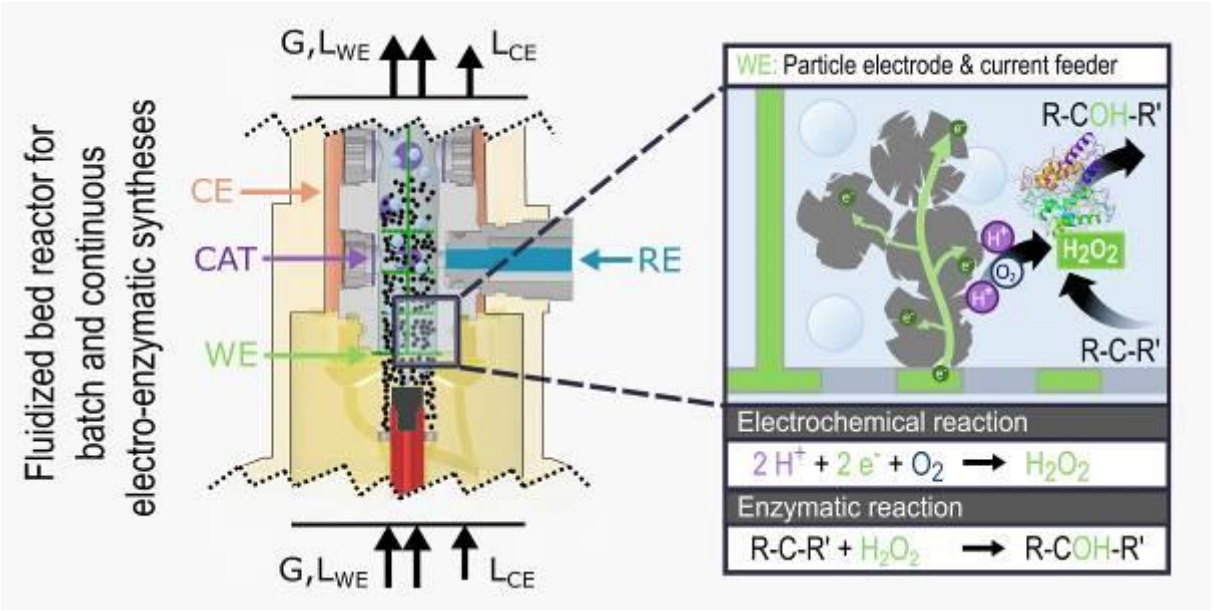
Objective:
This study aims to assess the operational efficiency of a novel fluidized bed electrode for electro-enzymatic processes in both batch and continuous operation. An electro-enzymatic process is demonstrated through the in-situ generation of hydrogen peroxide (H_2O_2), which serves as a co-factor in electro-enzymatic syntheses, specifically for the hydroxylation of non-activated C-H bonds by the peroxygenase *Aae*UPO.

Fig. 1: Illustration of a fluidized bed reactor for electro-enzymatic syntheses. In a model reaction, H_2O_2 is electrochemically generated through oxygen reduction and serves as co-factor for enzymatic hydroxylation reactions involving the peroxygenase *Aae*UPO. The reactor is divided by a cation-exchange membrane (CAT) into two compartments: the central compartment with a reference electrode (RE) and the fluidized particle electrode (WE) aerated by airflow (G_{WE}) for oxygen redelivery, and the outer compartment with the counter electrode (CE). Electrolyte flow in both compartments (L_{WE} and L_{CE}) supports both continuous and batch operation.

Results:
A fluidized bed reactor was developed, utilizing conductive graphite electrode particles that are fluidized by a gas and/or liquid flow. An innovatively designed current feeder allows for electrical contacting of these fluidized electrode particles to maximize the high electrode surface area and exploit the good mixing properties of the fluidized bed. This enabled the effective generation of H_2O_2 with current efficiencies of up to 60% at neutral pH and low salt concentrations, integral for the peroxygenase-catalyzed hydroxylation reaction. In batch operation, a space-time yield (STY) of 19.6 g/(L*d) was achieved for the hydroxylation of 4-ethylbenzoic acid to 4-(1-hydroxyethyl)benzoic acid. Transitioning to continuous operation resulted in a 40% increase in STY to 27.5 g/(L*d). The reactor system could maintain high enzyme stability through the on-demand co-factor production and demonstrated good total turnover numbers maximizing at 350.000 mol/mol.

Conclusion:
The fluidized bed electrode's capability to operate efficiently in both batch and continuous modes demonstrates the broad scope of application for electro-enzymatic syntheses. This innovative approach not only supports on-demand H_2O_2 generation but can also be adapted for other electro-enzymatic co-factor regeneration systems. The adaptability and efficiency of the fluidized bed reactor, as well as the inherent scalability of particle electrodes, make it a compelling candidate for sustainable and high-performance industrial applications.

Fig. 1


Fluidized bed reactor for batch and continuous electro-enzymatic syntheses
G,L_WE
L_CE
CE
CAT
RE
WE
G,L_WE
L_CE
WE: Particle electrode & current feeder
R-COH-R'
H2O2
R-C-R'
Electrochemical reaction
2 H+ + 2 e- + O2 → H2O2
Enzymatic reaction
R-C-R' + H2O2 → R-COH-R'

P4-1

Characterization of the enantioselective carbene free cyclopropanation reaction catalyzed by *E.coli* cyclopropane fatty acid synthase (ecCFAS)

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[2]King's College London, Chemistry, London, United Kingdom

The cyclopropane ring is an important structural element present in natural products and pharmaceuticals. Its rigid structure and its reactivity make it a desirable motif for medicinal chemistry and organic synthesis. Several different chemical methodologies for cyclopropanes racemic or enantioenriched synthesis have been developed but all of them require harsh reaction conditions, the use of stoichiometric metals, and hazardous diazo-compound reagents. Pioneering examples using P450- and myoglobin-based biocatalysts demonstrated great substrate versatility, often with high stereoselectivity and yields. Despite the importance of these achievements, the requirement of stoichiometric amounts of toxic and potentially explosive diazo-carbene substrates hampers their application on an industrial scale. Cyclopropane fatty acid synthases (CFAS) are a unique class of methyl transferases catalyzing the enantioselective methyl transfer and cyclopropane ring closure from S-adenosyl methionine, to the Δ9-*cis*-double bonds of unsaturated phospholipid fatty acyl chains. The proposed work describes the biocatalytic characterisation of E. coli CFAS (ecCFAS) enzymatic activity and its utilization for the stereoselective synthesis of cyclopropanated lipids. EcCFAS was exploited for the chemo-enzymatic transformation of phosphatidylglycerol (PG) to methyl dihydrosterculate with conversions up to 58% and 73% *ee* (9S,10R). Stereo- and regio-selectivity of the ecCFAS cyclopropanation activity on phospholipids was investigated. Moreover, substrate screening, site-directed mutagenesis and *in silico* studies uncovered intersting insights on the substrate properties/activity relationship (lipid head group and lipid macromolecular/supramolecular arrangement). Finally, an orthogonal multi-enzymatic cascade reaction combining pure ecCFAS with AtHMT enzyme was developed for the *in situ* regenaration of SAM and SAM-d3 and the biocatalytic synthesis of methyl dihydrosterculate and its deuterated analogue.

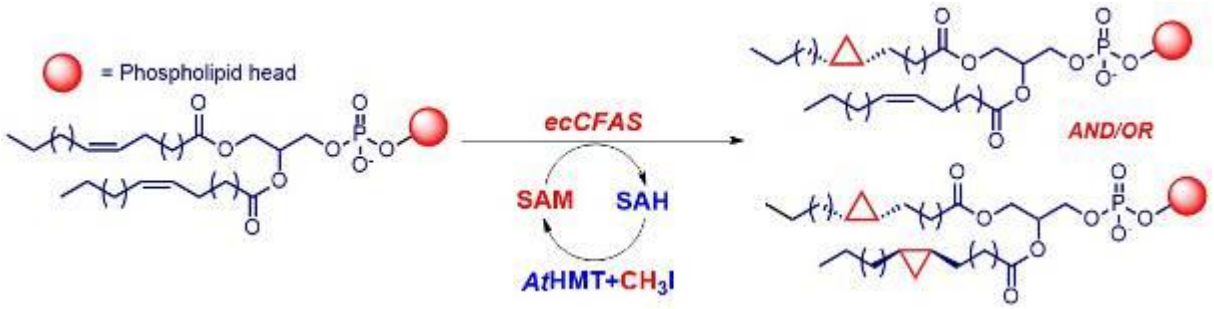
[1] M. Yasumoto et al., *J. Nat. Prod*. **2000**, 63, 1534-1536

[2] F. Micheli et al., *J. Med. Chem*. **2010**, 53, 4989-5001

[3] P. S. Coelho et al., *Science* **2013**, 339, 307-310

[4] P. Bajaj et al., *Angew. Chem., Int. Ed*. **2016**, 55, 16110-16114

Fig. 1



P4-2

Boron Catalysis in a Designer Enzyme

L. Longwitz[1], G. Roelfes[1]
[1]University of Groningen, Groningen, Netherlands

Introduction

Enzymes display outstanding reactivities and selectivities in many different biological processes and chemical reactions. Harvesting this immense potential of activity for abiological reactions and to apply biocatalysis in organic synthesis is a major challenge.[1] The introduction of non-canonical amino acids and unnatural functionalities as new active catalytic centers in enzymes allows for the design of hybrid catalysts by inheriting advantages of both biocatalysis and homogeneous catalysis.[2] Until recently, most non-canonical amino acids that have been used as catalysts have been focused on fine tuning the reactivity of natural amino acids with similar functional groups.[3]

Objectives

In this work, the high synthetic utility and catalytic activity of boronic acids is utilized in the creation and application of boronic acid containing designer enzymes for the first time.[4] Boron is absent in any form of natural biocatalysis, even though the element is relatively abundant in the earth curst and has found a lot of utility in organic synthesis. The unique chemistry of boronic acid derivatives, including their low valent character and reactivity towards diols is utilized in organocatalysis and would enhance the toolbox of activation modes in biocatalysis immensely.

Results

The boronic acid moiety was introduced into LmrR, a dimeric protein that has no native catalytic function, using stop codon suppression. The successful genetic incorporation of the low valent boron species was confirmed using several analytical techniques including high resolution mass spectrometry and 11B NMR spectroscopy. The overall structure of the protein scaffold changed significantly based on protein crystallography analysis compared to the wild type protein. Furthermore, certain amino acid side chains show cased strong hydrogen bond interactions with the boronic acid functionality. Based on the unique reactivity of the boronic acid residue, a kinetic resolution of hydroxyl ketones via condensation with hydroxyl amines was developed. The hydroxyl group acts as a directing group to favor the essential interaction with the boronic acid inside the biocatalyst. While initial selectivity with the parent protein were only moderate, a directed evolution campaign led to the development of highly improved mutants reaching E-values of over 100.

Conclusion

We developed a designer enzyme with a new-to-nature activation mode, made possible by the genetic incorporation of a boron functionalized amino acid.

References

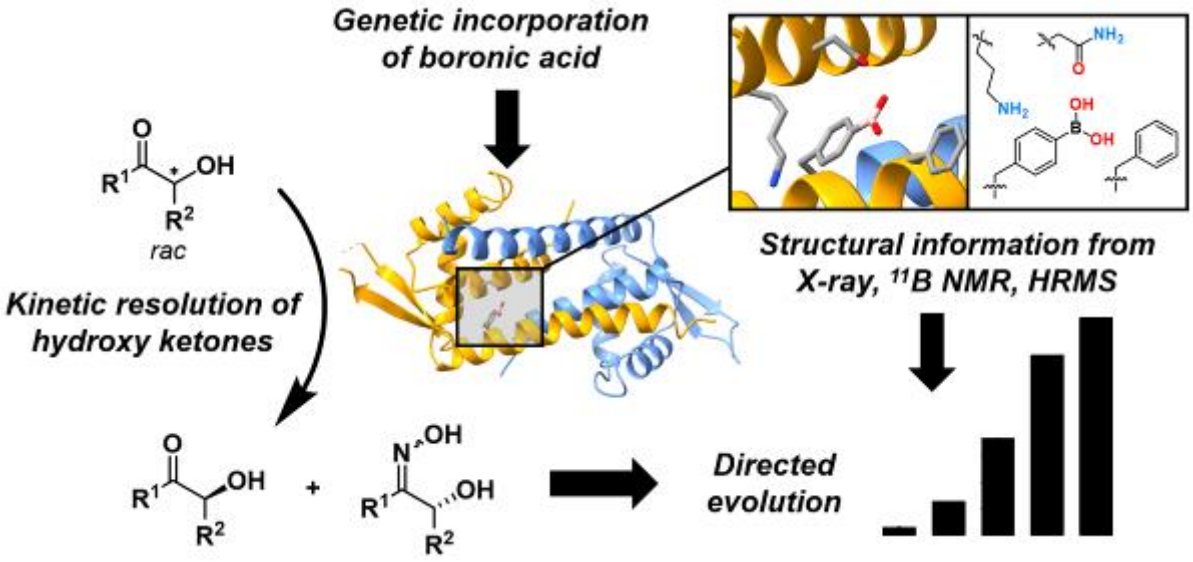
[1] K. Chen, F. H. Arnold, *Nat. Catal.* **2020**, *3*, 203-213.

[2] R. B. Leveson-Gower, C. Mayer, G. Roelfes, *Nat. Rev. Chem.* **2019**, *3*, 687-705.

[3] J. Zhao, A. J. Burke, A. P. Green, *Curr. Opin. Chem. Biol.* **2020**, *55*, 136-144.

[4] L. Longwitz, R. B. Leveson-Gower, H. J. Rozeboom, A.-M. W. H. Thunnissen, G. Roelfes, on ChemRxiv, 10.26434/chemrxiv-2023-10vcv: *Nature* **2024**, accepted.

Fig. 1


Genetic incorporation of boronic acid
Structural information from X-ray, 11B NMR, HRMS
rac
Kinetic resolution of hydroxy ketones
Directed evolution

P4-3

Co-substrate recycling for (*S*)-TAs in kinetic resolutions

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[1]Otto-von-Guericke University Magdeburg, Insitute of Chemistry, Magdeburg, Germany
[2]University of Bielefeld, Institute of Chemistry, Bielefeld, Germany
[3]University of Greifswald, Institute of Biochemistry, Greifswald, Germany

Introduction: Enantioselective production of chiral and enantiomerically pure amines is often realized in kinetic resolutions using transaminases (TAs). According to their catalytic cycle, stoichiometric concentrations of both substrates are required. Thus, high co-substrate amounts are used in large scale, possibly leading to enzyme inhibition, undesired reaction equilibria, high costs and low atomic efficiencies. While co-substrate recycling of (*R*)-selective TAs by d-amino acid oxidases (d-AAO) is well established, recycling by l-AAOs has not been common.

Objectives: To reduce co-substrate concentrations, a recycling system was targeted for (*S*)-selective TAs consisting of a highly active fungal l-AAO[1] and a catalase. Suitable co-substrates and enzyme ratios must be established. In addition, immobilization of all enzymes is targeted to improve the reaction, work-up and stability of the enzymes.

Results: An enzyme cascade was established for the kinetic resolution of racemic 1-phenylethylamine (*rac*-1-PEA) (Fig. 1).[2] All enzymes were immobilized on different supports either alone or together, with co-immobilization proving to work best.[3] In general, the recycling cascade could be used for several (*S*)-selective TAs, including an engineered TA for the production of a Sitagliptin intermediate. Preparative production (90 mg) of enantiomerically pure (*R*)-1-PEA proved the applicability of the cascade in both soluble and co-immobilized form.

[Figure 1]

Figure 1: Co-immobilized enzyme cascade. The co-immobilized enzyme cascade worked best in this setup, and the advantages of the enzyme cascade and the co-immobilization of the cascade are shown.

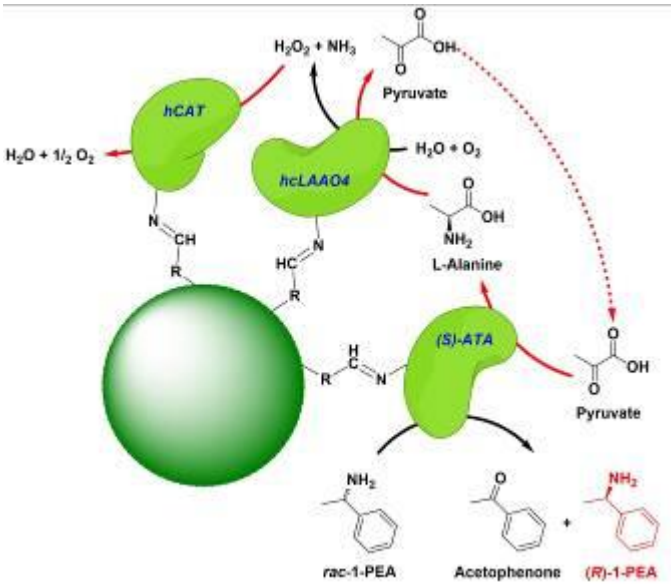
Conclusion: A recycling system was successfully established for the co-substrates of different (*S*)-selective TAs in kinetic resolutions, allowing catalytic rather than stoichiometric quantities. Co-Immobilization of the cascade significantly improved the reaction.

[1] M. C. Heß, S. Bloess, J. M. Risse, K. Friehs, G. Fischer von Mollard, MicrobiologyOpen, 2020, 9, e1112.

[2] T. Heinks, J. Paulus, S. Koopmeiners, T. Beuel, N. Sewald, M. Höhne, U. T. Bornscheuer, G. Fischer von Mollard, Chem-BioChem, 2022, 23, e202200329.

[3] T. Heinks, S. Koopmeiners, N. Montua, N. Sewald, M. Höhne, U. T. Bornscheuer, G. Fischer von Mollard, ChemBioChem, 2023, 24, e202300425.

Fig. 1



Optimization through enzyme cascading

- α-amino acids instead of α-keto acids
- 1.000-fold reduction of the co-substrate conc.
- No co-substrate inhibition
- Co-product removal
  - Impact on reaction equilibrium
  - Simplified product purification
- Improved atomic efficiency

Optimization through (Co-)Immobilization

- Simplified enzyme removal
- Re-use of the enzyme cascade
- Stabilization of enzymes
- Spatial proximity of the enzymes
  - Substrate channeling
  - Increased reaction rates

P4-4

Advancements in Cofactor Regeneration for Efficient Nucleotide Sugar Synthesis

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Nucleotide sugars are fundamental precursors in glycobiology that find extensive utility across various industrial sectors, particularly in pharmaceuticals, cosmetics, and food production. Despite their significance, their availability is often constrained by the limitations of conventional chemical synthesis methods, which tend to be both costly and inefficient. In this context, enzymatic synthesis pathways, particularly those harnessing salvage pathways, have emerged as a promising alternative meeting the growing demand for nucleotide sugars. Especially, UDP-GlcNAc and UDP-GalNAc, two prominent nucleotide sugars widely used in glycosylation processes and as precursor molecules for glycosaminoglycans are interesting synthesis targets. To achieve this, a salvage pathway cascade of enzymes was employed, consisting of NahK (*N*-acetylhexosamine 1-kinase), AGX1 (UDP-*N*-acetylglucosamine pyrophosphorylase), and PpA (Pyrophosphatase). A key challenge encountered in this enzymatic synthesis process was the inhibitory effect posed by high ATP and ADP concentrations, particularly with respect to NahK and AGX1 enzymes. The high costs for UTP are also an important factor for the economic efficiency of the process. To mitigate this inhibition and to make the synthesis more cost-effective, cofactor regeneration cycles for ATP and UTP facilitated by low-cost polyphosphate have been implemented adding three more enzymes; a PPK (Polyphosphatekinase), CMPK (Cytidine monophosphate kinase) and CDPK (Cytidine diphosphate kinase). By maintaining optimal enzyme and cofactor concentrations and employing kinetic modeling and parameter screening, inhibitory effects were overcome and high conversion rates within a relatively short timeframe were achieved. These regeneration systems can now be extended to other UDP-sugars, which also hold significant interest as substrates for glycosyltransferases involved in N- and O-glycosylation processes, as well as the synthesis of glycosphingolipids. Furthermore, it is intended to be expanded to GDP-sugars, which face similar limitations due to cofactor inhibition.

P4-5

Multifunctional artificial enzymes for tandem abiotic transformations

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[2]University of Basel, Department of Chemistry, Basel, Switzerland
[3]Protein Production and Structure Core Facility (PTPSP), School of Life Sciences, École polytechnique fédérale de Lausanne, Lausanne, Switzerland

Multi-enzyme concurrent cascades play a crucial role in the synthesis of biomacromolecules, pharmaceuticals, and natural products. However, they often require substantial protein engineering for each enzymatic component. This limitation has stimulated interest in multifunctional biocatalysts (MFBs)[1] as a potential solution to this challenge. While significant progress has been achieved in discovering multifunctional (engineered) natural enzymes (Fig. 1A), their chemistry remains constrained by natural activity. Artificial enzymes offer a promising alternative, as they can exhibit non-natural reactivity beyond the limitations of natural enzymatic activities. Despite advancements in artificial enzymes, realizing multifunctional versions remains challenging (Fig. 1B). In this study, we engineered multifunctional artificial enzymes relying on the biotin-streptavidin technology. By sequentially incorporating different catalytic cofactors, we demonstrate a programmable platform for constructing multifunctional artificial enzymes. This work introduces a versatile approach for the development of multifunctional artificial enzymes, which may find applications in synthetic biology and biocatalysis.

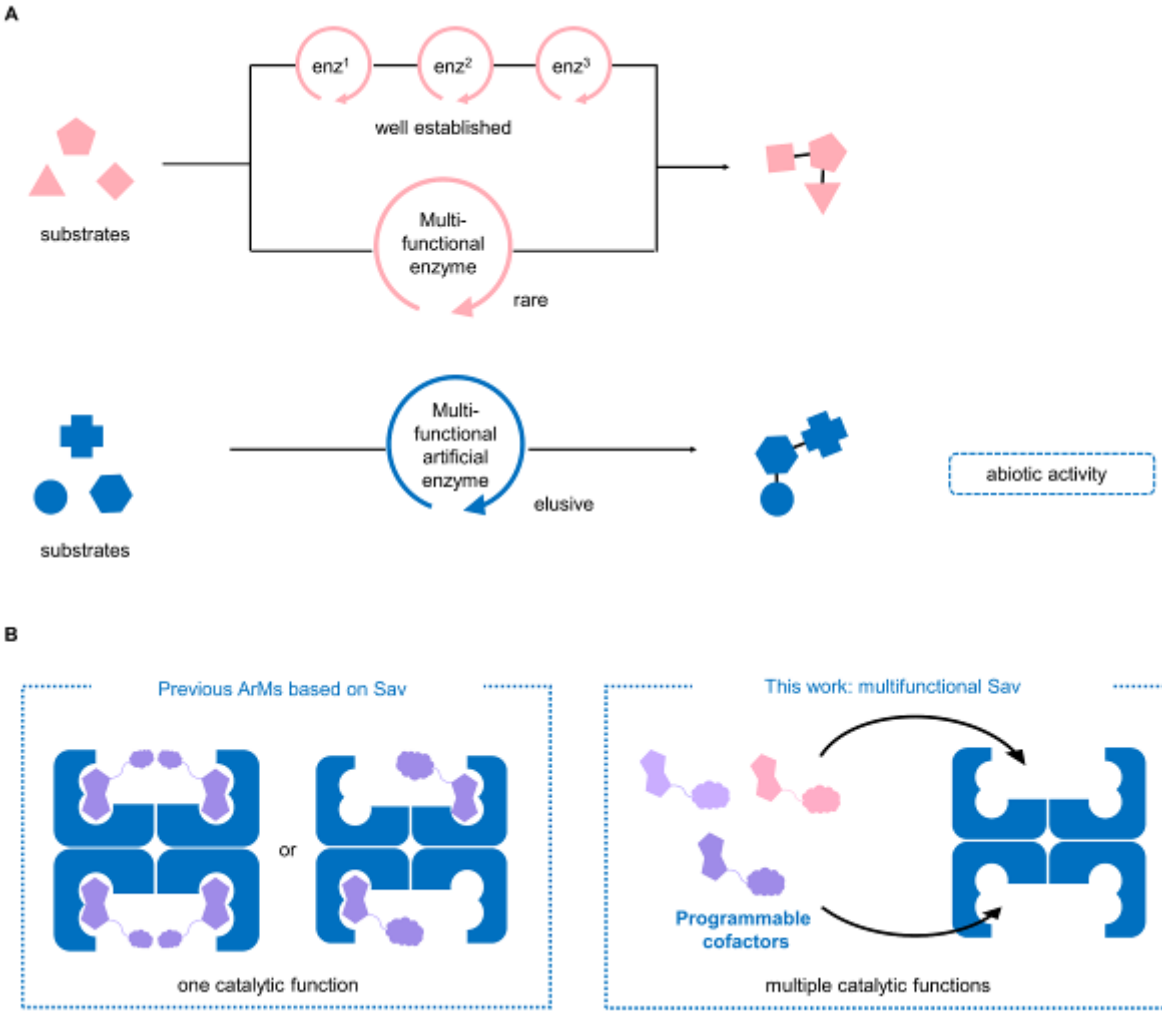
References:

1. Thorpe, T. W., Marshall, J. R. & Turner, N. J. Multifunctional Biocatalysts for Organic Synthesis. *J. Am. Chem. Soc.*, doi:10.1021/jacs.3c09542 (2024).

Fig. 1. Introduction of multifunctional biocatalysts and our design of multifunctional artificial enzymes.

A. Artificial multifunctional enzymes would provide attractive alternatives for multienzymes systems with non-natural activity. B. Our hypothesis for the design of multifunctional artificial enzymes.

Fig. 1


A
enz1
enz2
enz3
well established
substrates
Multi-
functional
enzyme
rare
Multi-
functional
artificial
enzyme
elusive
substrates
abiotic activity
B
Previous ArMs based on Sav
or
one catalytic function
This work: multifunctional Sav
Programmable
cofactors
multiple catalytic functions

P4-6

Biocatalytic cascade reactions featuring a designer enzyme for intramolecular Friedel-Crafts alkylations

C. Da Settimo Passetti[1], G. Roelfes[1]
[1]University of Groningen, Stratingh Institute, Groningen, Netherlands

Introduction

Enzymes are highly active and selective catalysts, work in mild reaction conditions and are evolvable, but their reaction and substrate scope are limited by the functional groups on the 20 natural amino acids.1 For the design of a sustainable catalyst with broader reaction scope, the Roelfes group employed the *Lactococcus lactis* multidrug resistance regulator LmrR as protein scaffold.2 Previously, we developed LmrR-based enzymes featuring the catalytic unnatural aniline residue para-aminophenylalanine (pAF) for iminium ion catalysis, such as the intermolecular Friedel-Crafts alkylation between α,ß-unsaturated aldehydes and indoles.3

Objectives

In this study, we now aimed to engineer highly efficient and selective artificial enzymes for the catalysis of intramolecular Friedel-Crafts alkylations to produce chiral tetrahydrocarbazole compounds, which are important pharmaceutical building blocks. This catalysis is possible thanks to a novel biocatalytic cascade that comprises the flavin-dependent oxidase HMFO and the engineered LmrR_based enzyme. Naturally HMFO catalyses the oxidation of 5-hydroxymethylfurfaral (**HMF**) into 2,5-furandicarboxylic acid (**FDCA**).4 Here we adopted it for the oxidation of a synthetically made allylic alcohol (**1**) to the corresponding α,β-unsaturated aldehyde (**2**), which is the substrate for the Friedel-Crafts reaction, but proved to be unstable upon storage. The engineered LmrR_pAF then converts it into the target chiral tetrahydrocarbazole product (**3**).

Results

The cascade reaction between HMFO and LmrR_V15pAF allows respectively the synthesis in situ of the substrate and the formation of the final tetrahydrocarbazole. Using an iterative site saturation mutagenesis approach in 96-well format , LmrR_V15pAF was evolved to improve both the activity and the enantioselectivity of the Friedel-Crafts alkylation. So far, LmrR _V15pAF_NLY is the best improved mutant with up to 77 % e.e. and more than three-fold increase in catalytic activity compared to LmrR_V15pAF. Currently, further rounds of directed evolution are underway.

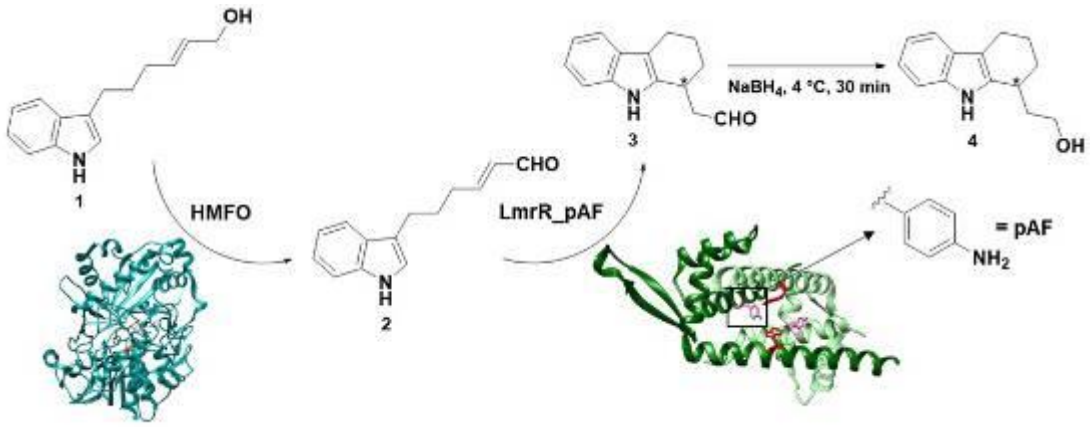
Conclusion

In conclusion, we have demonstrated that the efficient cascade reaction between a natural oxidase (HMFO) and a designer enzyme featuring a non-canonical pAF catalytic residue (LmrR_pAF) allows the development of a robust platform for the directed evolution of the latter. Moreover, this has given rise to the biocatalytic system for the enantioselective synthesis of tetrahydrocarbazole products. Currently, we are further extending this system towards further novel intramolecular Friedel-Crafts alkylation reactions for the synthesis of polycyclic indole derivatives.

References

1. I., Drienovská; G., Roelfes; *Nat. Catal.,* **2020**, 3, 193-202.
2. G., Roelfes; *Acc. Chem. Res*., **2019**, *52*, 545-556.
3. R. B., Leveson-Gower; I., Drienovská; Z., Zhou; G., Roelfes; *ACS Catal.,* **2021**, 11, 12, 6763-6770.
4. C., Martin; A., Ovalle Maqueo; H. J., Wijma; M. W. Fraaije; *Biotechnol. Biofuels Bioprod.,* **2018**, 11, 56.

Fig. 1


OH
NH
1
HMFO
CHO
NH
2
LmrR_pAF
NH
CHO
3
NaBH4, 4 °C, 30 min
NH
OH
4
= pAF
NH2

P4-7

Protein-polymer Conjugates as Artificial Enzymes for Catalysis

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Artificial enzymes, created by incorporating abiological catalytic species into protein hosts, are emerging as valuable biocatalysis tools. Despite their promise, they lag behind natural enzymes in activity and selectivity due to limited active sites.

Here, I introduce a new strategy for creating artificial enzymes by combining proteins with catalytically active polymers to create "artificial polyenzymes" (ArPoly). Unlike traditional artificial enzymes, ArPolys offer adjustable structure, composition, catalysis loading, and active sites, enabling high reactivity and unprecedented selectivity.

Our first study combines protein scaffolds with proline polymers via atom transfer radical polymerization (ATRP),[1] yielding highly water-soluble ArPolys capable of performing asymmetric aldol reactions in water — a first for water-soluble proline catalysts. This success stems from the synergistic interaction between proline catalysts and protein scaffolds.

Leveraging controllability, we fine-tune the polymer structure on ArPoly, including copolymerizing polystyrene and polyproline from protein surfaces.[2] This customization enhances catalytic efficiency and selectivity significantly, evidenced by a prototype ArPoly achieving 94% conversion and 98% enantiomeric excess (ee).

Expanding our approach, we introduce metal-containing artificial polyenzymes by polymerizing active ligands to proteins and coordinating them with metal ions. For example, ArPolys conjugated with proline polymers coordinated with Cu(II) act as efficient clickases for bioorthogonal chemistry without cytotoxicity.[3] Additionally, polymerizing chiral ligands and coordinating with Ru metal has yielded ArPolys capable of asymmetric hydrogenation with nearly 100% yield and 93% ee.[4] Remarkably, these metal-containing polymers enable new-to-nature reactivity, such as transforming amine to chiral alcohol.

In summary, my group's work focuses on developing ArPolys that integrate active polymers with proteins/enzymes, demonstrating enhanced activity, selectivity, and the ability to catalyze novel reactions. Our research has produced four distinct ArPolys, showcasing the potential of tailored polymer active units and structures for advanced catalysis.

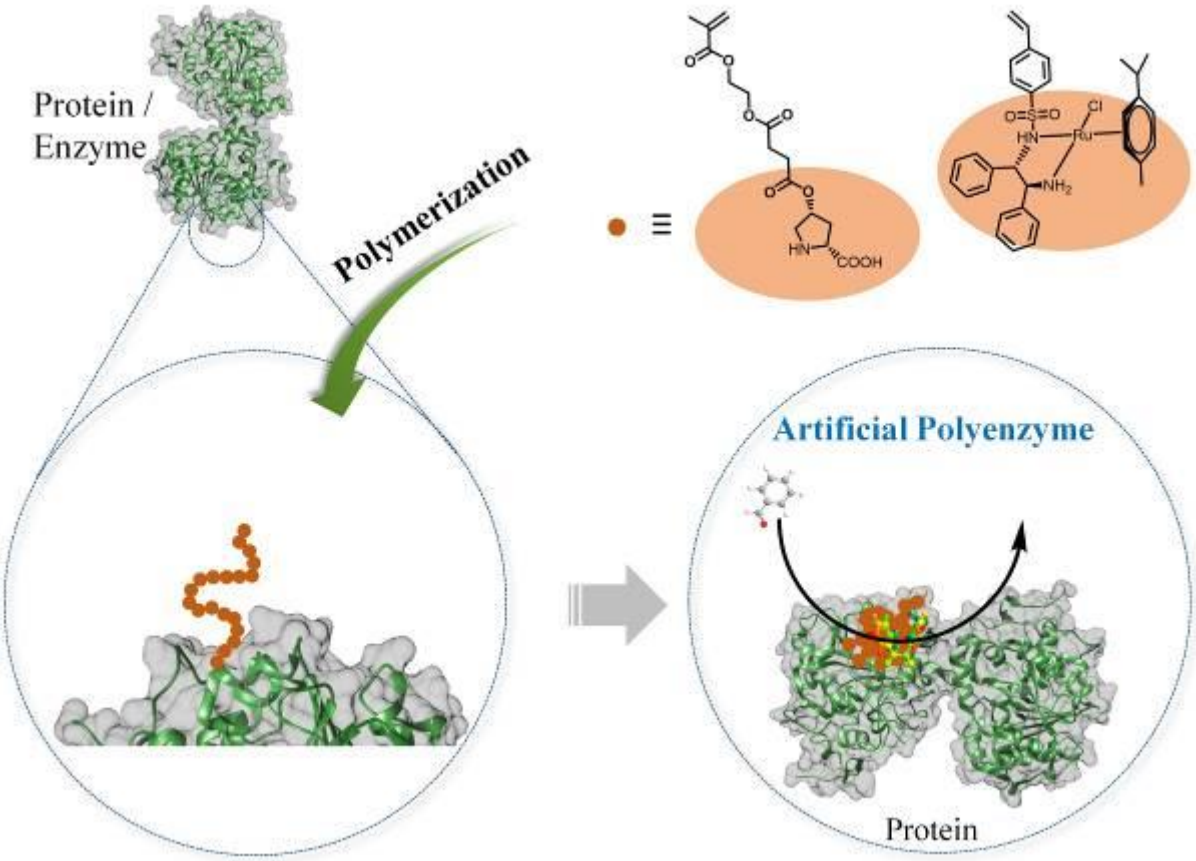
[1] N. Zhang, Z. Sun, C. Wu, *ACS Catal.* **2022**, *12*, 4777-4783.

[2] N. Zhang, C. Wu, *ACS Synth. Biol.* **2022**, *In press*.

[3] N. Zhang, P. Bessel, C. Wu, *Bioconjugate Chem.* **2022**, *33*, 1892-1899.

[4] S. Wang, C. Wu, *In preparation* **2023**.

Fig. 1


Protein /
Enzyme
Polymerization
Artificial Polyenzyme
Protein

P4-8

Biomimetic enzymatic cascade for fatty alkyl *p*-hydroxycinnamates synthesis

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Alkyl *p*-hydroxycinnamates (AHCs) are commonly found in plants as aliphatic esters of *p*-hydroxycinnamic acids (HCAs)[1], which are closely connected to suberin and cutin[2]. The presence of the aliphatic group on AHCs modifies their hydrophilic/lipophilic balance which enhances their incorporation in oil-based formulations as well as their biological activities, compared to their phenolic acid precursors[3]. Various methods for producing AHCs have been developed, including chemical, enzymatic, and chemoenzymatic approaches. However, these methods are either effective or environmentally friendly but not both simultaneously. In this study, we present a two-step biomimetic enzymatic cascade as a more environmentally friendly pathway to generate AHCs. This process involves activating HCA with a CoA: ligase (CL) enzyme and transferring the activated acyl group to an aliphatic moiety (acyl acceptor) using a BAHD acyl transferase (AT) enzyme. By optimizing the initial step and demonstrating the potential for CoASH recycling in the subsequent step, we demonstrated the potential of the proposed cascade for the *in vitro* synthesis of 16-feruloyloxy palmitate. To broaden the range of alkyl AHCs, we explored the promiscuity of acyl transferase and evaluated its permissiveness with various acyl acceptors through molecular docking. Our results indicate that the enzyme tested is a long-chain acyltransferase that favours hydroxy decanoic acid, but shows limited tolerance for alcohols bearing terminal alkynes. Overall, these findings demonstrate the feasibility of employing this cascade reaction as a sustainable alternative to traditional synthetic routes in the aim to access AHCs.

Key words: Alkyl hydroxycinnamates (AHCs), p-hydroxycinnamic acid (HCA), CoA: ligase (CL), Acyl transferase (AT), enzymatic cascade.

Figure 1. Reactions implicated in the enzymatic synthesis of AHCs in plants.

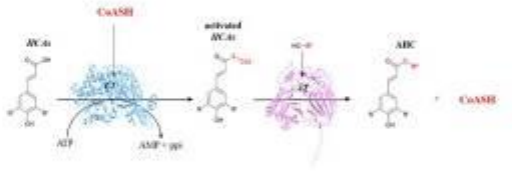
References

[1] D. A., Grajales-Hernández; M. A., Armendáriz-Ruiz; F. L., Gallego, and J. C., Mateos-Díaz, Applied Microbiology and Biotechnology, 2021, 105(10), 3901-3917.

[2] F., Domergue and D. K. Kosma, Plants, 6(3), 25-41.

[3] Y. G., Shi; Y.J., Zhu; S. Y., Shao; R. R., Zhang; Y., Wu; C. M., Zhu; X. R., Liang, and W. Q., Cai, Journal of Agricultural and Food Chemistry, 2018, 66 (45), 12088-12101.

Fig. 1


CoASH
HCAs
activated HCAs
HBC
CoASH
ATP
AMP + ppi

P4-9

Enzymatic cascade to natural ligustrazine

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Ligustrazine (2,3,5,6-tetramethylpyrazine; TMP), a Maillard reaction product that contributes to nutty, roasted, and toasted tonalities of heat-processed food, is widely used in the food industry as a flavor ingredient.[1,2] Additionally, TMP is a natural product found in *Ligusticum wallichii*, a herb used in traditional Chinese medicine. It contributes to cardio-and cerebrovascular health, has anti-cancer properties, and serves as a precursor for other pharmacologically active compounds.[3] The demand for natural TMP exceeds its supply from raw materials, favoring biotechnological methods.[2] Several chemoenzymatic methods rely on enhanced microbial production of acetoin followed by spontaneous condensation with ammonia, often at high temperatures.[1,2,4] We set out to develop an enzymatic cascade towards TMP where all the steps are performed under mild conditions (Fig. 1). A carboligase (CL) / transaminase (TA) sequence[5,6] was applied for the first time in synthesis of a short-chain vicinal amino alcohol. For the 3rd step, we tested the ability of a panel of alcohol dehydrogenases (ADHs) to catalyze the challenging oxidation of a vicinal amino alcohol to an aminoketone, which spontaneously forms TMP. 46% of TMP was produced from racemic amino alcohol standard, whereas a sequential one-pot cascade yielded up to 18% TMP. Further optimization will ensure enantiocompatibility of applied enzymes and complete consumption of acetaldehyde, which otherwise forms side-products.

Fig. 1 3-step 1-pot sequential enzymatic cascade for the synthesis of TMP.

Acknowledements: This work was funded by Axxence Slovakia s. r. o. We thank the COMET center acib: Next Generation Bioproduction is funded by BMK, BMDW, SFG, Standortagentur Tirol, Government of Lower Austria and Vienna Business Agency in the framework of COMET - Competence Centers for Excellent Technologies. The COMET-Funding Program is managed by the Austrian Research Promotion Agency FFG.

[1] K. Peng, D. Guo, Q. Lou, X. Lu, J. Cheng, J. Qiao, L. Lu, T. Cai, Y. Liu, H. Jiang, *ACS Synth. Biol.* **2020**, *9*, 2902–2908.

[2] Z. Xiao, X. Hou, X. Lyu, L. Xi, J. Zhao, *Biotechnology for Biofuels* **2014**, *7*, 106.

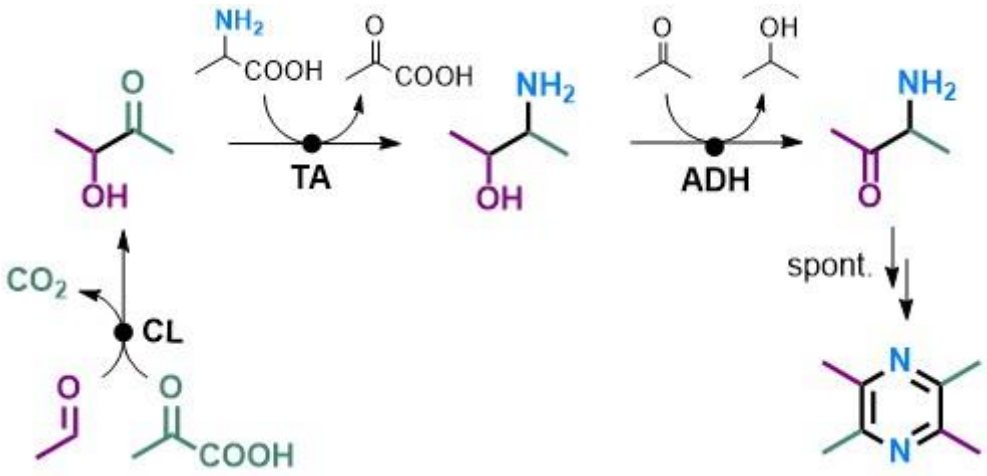
[3] J. Zou, P. Gao, X. Hao, H. Xu, P. Zhan, X. Liu, *European Journal of Medicinal Chemistry* **2018**, *147*, 150–162.

[4] G. Kłosowski, D. Mikulski, K. Pielech-Przybylska, *Biomolecules* **2021**, *11*, DOI 10.3390/biom11111736.

[5] V. Erdmann, T. Sehl, I. Frindi-Wosch, R. C. Simon, W. Kroutil, D. Rother, *ACS Catal.* **2019**, *9*, 7380–7388.

[6] N. Fracchiolla, S. Patti, F. Sangalli, D. Monti, F. Presini, P. P. Giovannini, F. Parmeggiani, E. Brenna, D. Tessaro, E. E. Ferrandi, *ChemCatChem* **2023**, e202301199.

Fig. 1


NH2
COOH
O
COOH
TA
NH2
OH
O
OH
O
ADH
NH2
O
spont.
N
N
CO2
CL
O
O
COOH

P4-10

A multi enzyme system for the generation of a new natural sugar syrup

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The dairy industry produces large quantities of sweet and sour whey every day, making the value-adding handling of these side streams important. However, only a small proportion of the total whey produced is currently converted into value-added products and a significant proportion of sour whey is sold as pig feed, for example, sometimes resulting in additional costs for dairy companies.

The aim of the project was to obtain a lactose concentrate from lactose-containing side streams such as sweet/sour whey or skimmed milk UF (SM-UF) permeate, which can then be converted into a syrup with comparable sweetness to sucrose by a multi enzymatic process.

Therefore, the lactose (RS = 16) contained in side streams from the dairy industry can first be hydrolyzed by means of a *ß*-galactosidase (EC 3.2.1.23) to the monosaccharides galactose (RS = 32) and glucose (RS = 74), both of which are sweeter than the starting sugar lactose. This sweetness can be further increased if glucose is isomerized to fructose (RS = 172) by means of a xylose isomerase (EC 5.3.1.5) and galactose is further isomerized to tagatose (RS = 92) by means of an L-arabinose isomerase (L-AI, EC 5.3.1.4) (see Figure 1).

While commercial preparations for *ß*-galactosidases and xylose isomerases were available, it was necessary to screen for L-arabinose isomerases that could be used for the corresponding process conditions, including application in sweet and sour whey. *In silico* screening was done to identify putative L-AIs. The most promising L-AI was recombinantly produced in *E. coli* BL21(DE3), purified by heat treatment and used to isomerize galactose in SM-UF permeate (pH 4.5 and 6.5). Starting from 95 g/L lactose in SM-UF permeate pH 6.5, a sugar solution containing glucose, fructose, galactose and tagatose (25 g/L each) were obtained by tri-enzymatic reaction. In addition, 95 g/L in SM-UF permeate pH 4.5 were converted to glucose (50 g/L), galactose and tagatose (25 g/L each) by means of bi-enzymatic reaction.

Figure legend

Figure 1: Enzymatic conversion of lactose (mod. after [1]). RS: relative sweetness; RS of sucrose = 100.

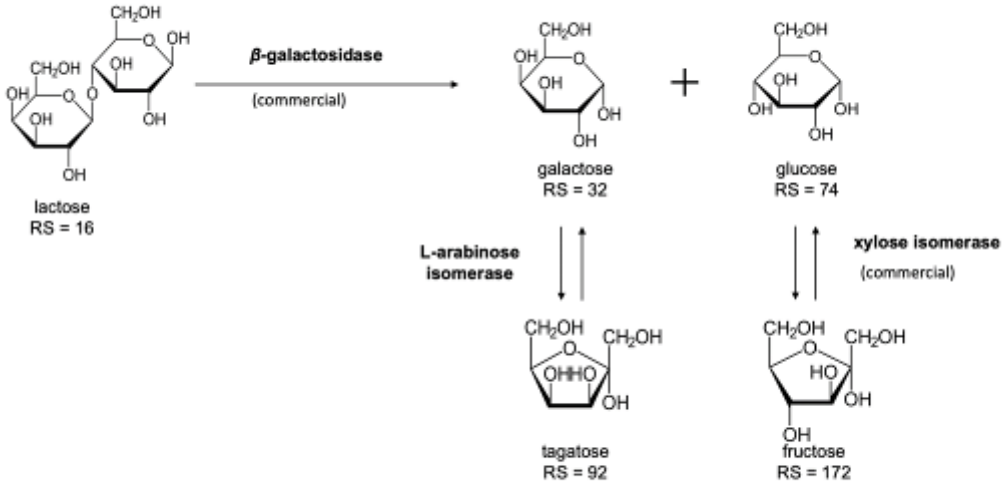
Reference

[1] Torres, P., & Batista-Viera, F. (2017). *Molecules*, *22*(2).

Acknowledgement

We would like to express our gratitude to the German Federal Ministry of Economics and Technology (AIF/FEI Project No. 21100 N) for the financial support of this research.

Fig. 1


β-galactosidase
(commercial)
lactose
RS = 16
galactose
RS = 32
glucose
RS = 74
L-arabinose
isomerase
xylose isomerase
(commercial)
tagatose
RS = 92
fructose
RS = 172

P4-11

Electrochemical characterisation of Formaldehyde dehydrogenase

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Human CO_2 emissions are mainly responsible for the climate changes and related problems that we see today. However, atmospheric CO_2 is also a potential valuable carbon resource. CO_2 can be converted into methanol using a three-step reaction catalysed by three enzymes. The first step catalyses the reduction of CO_2 into formate by a formate dehydrogenase (FDH, EC 1.2.1.2). The second step reduces formate into formaldehyde, this reaction is catalysed by a formaldehyde dehydrogenase (FaldDH, EC 1.2.1.46). Finally, formaldehyde is converted into methanol by an alcohol dehydrogenase (ADH, EC 1.1.1.1). Nature"s choice for electron donors is NAD(P)H however, these are ineffective and expensive in an industrial setting. As an alternative, electrons provided by electricity can be utilised and make the enzymes relevant for a Power-to-X plant. This work presents a comparison of the catalytic activity of FaldDH between electrochemical donated electrons and NADH.

The results will allow for determining the optimal method for further investigation of FaldDH and other redox-active enzymes. The kinetic parameters determined in this work will furthermore serve as a baseline for future optimization of the FaldDH.

P4-12

Enzymatic catalyzed esterification of small molecules in a biphasic process

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[2]RWTH Aachen University, ABBt, Aachen, Germany
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[5]RWTH Aachen University, ITMC, Aachen, Germany

Introduction:

Novel sustainable feedstocks for (industrial) production processes are required due to limited fossil resources, increasing greenhouse gas emissions, and global warming. One possible future feedstock is CO2, which can be integrated during a reaction or used directly as a feedstock.[1] In the latter, CO2 is often first converted into more reactive C1 molecules such as formic acid, formaldehyde, or methanol. These C1 molecules are building blocks for higher molecular products and different catalyst types, as enzymes, microbes, and chemical catalysts accept them as substrates. By combining chemical catalysts with biocatalysts and also microbes in one process, a more sustainable and efficient process can be developed than if only one type of catalyst is used. The advantages of all catalyst types are used and no downstream processing is required between the different process steps.[2]

Objective:

An already published process is a combined catalyst process between a *Saccharomyces cerevisiae* fermentation and a ruthenium catalyst, in which formic acid and ethanol are produced from renewable resources.[3] Now, we want to add an enzymatic step to valorize the previous products to ethyl formate.

Results:

It could be proven, that a carboxylic acid reductase A-domain catalyzes the esterification reaction between formic acid and ethanol in the aqueous phase. This is advantageous concerning the solubility of the substrates, however the product ethyl formate hydrolyses under these conditions. As a consequence, a synthesis under organic conditions is being further investigated concerning product stability. Lipase B from *Candida antarctica* was identified as a suitable enzyme for the synthesis of ethyl formate in various solvents such as 1,2-dichloroethane, dodecane, and CPME. CPME has appropriate cross-solubility of the substrates, is barely miscible with water, and is also declared as a green solvent. In CPME, ethyl formate is stably synthesized in a biphasic system with the applied lipase.

Conclusion:

We show that ethanol and formic acid can be enzymatically upgraded to ethyl formate in a biphasic system consisting of an equal aqueous phase and an organic phase of CPME. The reaction is synthesized by immobilized lipase B from *Candida antarctica*. When using CPME, the product stably formed.

References:

[1] Satanowski, *EMBO Reports*, **2020**, 21, 4, e50273.

[2] Mengers, H.G., Guntermann N., von Westarp W.G, Jupke, A., Klankermayer J., Blank L.M., Leitner W., Rother D., *Chemie Ingenieur Technik*, **2022**, 95, 485-490.

[3] Guntermann N, Mengers H.G., Franciò G., Blank L.M., Leitner W., *Green chemistry*, **2021**, 23, 9660–64.

Fig. 1

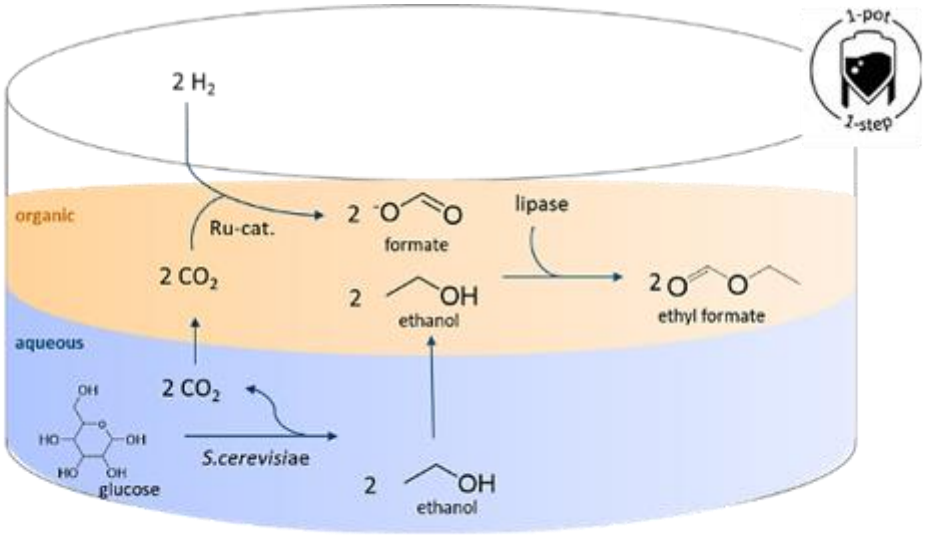

Figure 1: One-pot, one-step process with three catalyst types.

P4-13

Metal affinity immobilization of fusion enzymes for oxyfunctionalizations in micro-aqueous media

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The use of enzymes for lactonizations has been shown to be an environmentally friendly and resource-efficient alternative to conventional chemical methods.1 However, there are some technical drawbacks in enzymatic processes including cofactor dependency, enzyme instability, and oxygen limitation. In this work, to overcome these restrictions and to enable industrial applicability, a fusion enzyme of cyclohexanone monooxygenase (CHMO) and alcohol dehydrogenase (ADH) in an artificial redox-neutral linear cascade is applied in micro-aqueous media.2,3,4 The fusion enzyme was immobilized on a newly reported resin, Chromalite MIDA loaded with a variety of metal ions, via a one-pot metal affinity approach of simultaneous purification and immobilization (Fig. 1).5,6 The immobilization methodology was rationally optimized in terms of activity- and immobilization yields by Design of Experiments (DoE).

Figure 1: Linear cascade catalyzed by the immobilized fusion enzyme for a further scale-up and application in a rotating bed reactor (RBR). CHL: Cyclohexanol, CHO: Cyclohexanone, ECL: e-Caprolactone, TbADH: Thermoanaerobacter brockii alcohol dehydrogenase, TmCHMO: Termocrispum municipale cyclohexanone monooxygenase.

The immobilized fusion enzyme led to an improved productivity of ε-caprolactone (Fig. 1) in 99.5 vol.% cylopently methyl ether (CPME) than the buffer system. Meanwhile, higher productivity and operational stability in comparison with the free enzyme were achieved, allowing for a prospective enzyme reusability of seven times. The upscale of the cascade in a rotating bed reactor at 125 mL in micro-aqueous media was established, demonstrating the feasibility of further improvements on a technical scale.

[1] F. Hollmann *et al.*, *Chem. An Asian J.*, 13 (**2018**), 3601–3610.

[2] J. Engel *et al.*, *Org. Process Res. Dev.*, 25 (**2021**), 411–420.

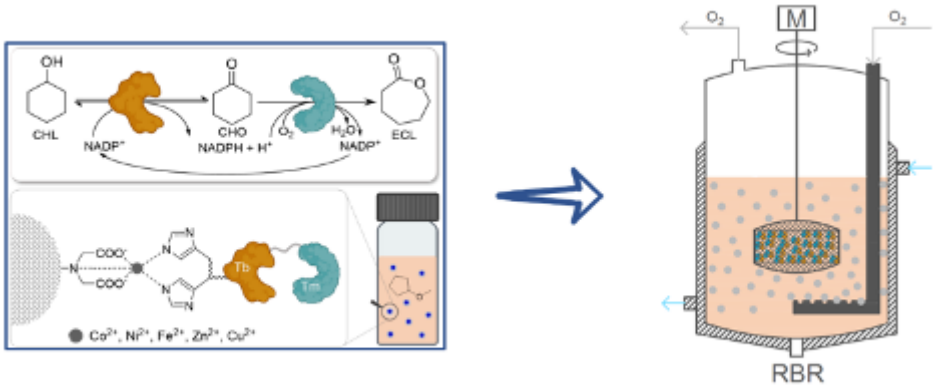
[3] Y. Ma *et al.*, *Front. Bioeng. Biotechnol.*, 10 (**2022**), 944226.

[4] G. Vernet *et al., ChemBioChem*, (**2023**), e202200794.

[5] G. Vernet *et al.*, *Curr. Opin. Green and Sustain. Chem.*, 38 (**2022**), 100692.

[6] A. Basso *et al.*, *Org. Process Res. Dev.*, 26 (**2022**), 2075–2084.

Fig. 1



P4-14

Analysis of protein changes induced by high taurine concentration with Aβ in NE-4C

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Introduction

Alzheimer's disease is a neurodegenerative disorder characterized by the deposition of amyloid-β protein (Aβ) in the brain. Accumulated Aβ induces reactive oxygen species causing cytotoxicity. Taurine has anti-oxidant properties and is considered to decrease cytotoxicity caused by Aβ. In contrast, high taurine concentrations reduce cell numbers and neurite outgrowth in primary cultured hippocampal cells as mature neurons with changes in protein expression. It means that the effect of cell death and protein fluctuations depends on the taurine concentration, however, the effect of high taurine concentration in the presence of Aβ remains unclear.

Objective

This study investigated the cytotoxicity and protein expression caused by Aβ and high concentrations of taurine in NE-4C as neural stem cells (NSCs) from mice.

Result

NE-4C cells were cultured in 3 mM taurine (LT), and 30 mM taurine (HT) in the presence or absence of 1 mM Aβ for 48 hours. Cell survival and protein expression levels under these conditions were evaluated using Cell Counting Kit-8 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining, respectively. The cell survival in the absence of Aβ showed no significant different by taurine concentration. Moreover, no differences in protein expression were observed in the HT conditions compared to the LT without Aβ according to SDS-PAGE. It was suggested that NSCs is more require taurine compared to mature neurons due to proliferation and differentiation, thereby, HT was not induced cytotoxic. However, HT with Aβ significantly reduced cell viability compared to cells with only added Aβ. Furthermore, the protein expression levels of a 16 kDa band decreased in the HT with Aβ condition compared to the other groups. It suggested that this 16 kDa protein may play a key protein in cell survival.

Conclusion

HT did not reduce cell numbers in NSCs. In contrast, HT with Aβ decreased cell survival and the level of 16 kDa protein. Identification of a 16 kDa protein may help to elucidate the mechanism of reduced cell survival under HT with Aβ conditions.

P4-15

Analysis of lipid-modification proteins in renal cells after treatment with Shakuyaku-Kanzo-to in response to changes in glucose concentration

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Introduction

Shakuyaku-Kanzo-to (SKT) is a major traditional medicine used in Japan to relieve cramps in patients with diabetic kidney disease undergoing hemodialysis. Glycyrrhizin acid (GA) is an active pharmaceutical ingredient in SKT that improves high blood glucose-induced sirtuin 1 downregulation (1). Sirtuin 1 is a cytoprotective enzyme that removes lipid modifications from proteins and regulates transcription. This suggests that SKT can modulate lipid modification of proteins via GA activity by improving sirtuin 1 expression. However, this SKT mechanism remains unproven.

Objectives

This study aimed to analyze the expression of lipid-modification proteins in renal cells treated with SKT components in the presence of high glucose concentrations.

Results

Synthesized 16-carbon fatty acid alkynes were applied to rat renal cells at a final concentration of 100 µM with GA or SKT in media containing different glucose concentrations. Cells were then cultured for 24 h. Cellular proteins were extracted and lipid-modified proteins were purified prior to electrophoresis. Electrophoresis results showed fluctuation in staining intensity of bands in the 35–210 kDa range with changes in glucose concentration. In particular, a decrease in staining intensity of a band at 210 kDa under GA treatment was significantly associated with increasing glucose concentration. In contrast, SKT treatment did not alter these bands. It suggested that GA induced upregulation of an enzyme for the removal of lipid modification, while SKT reduced the enzyme in cells by interaction of GA with paeoniflorin as another active pharmaceutical ingredient.

Conclusion

Our study provides insights into the removal of lipid modification on a 210 kDa GA-induced protein at high glucose concentrations. Whether these data depended on sirtuin 1 or not needs further investigation.

References

(1) S. Hou, et al., *Int. J. Mol. Sci.*, 15 (9), 15026-15043, 2014

P4-16

Investigation of the combinability of Unspecific Peroxygenase from *Myceliophthora thermophila* with L-amino acid oxidase from *Hebeloma cylindrosporum*

S. Last[1], T. Heinks[1], N. Dietz[2], S. Koopmeiners[3], G. Fischer von Mollard[3], M. Weissenborn[2], J. von Langermann[1]
[1]Otto-von-Guericke-Universität Magdeburg, Magdeburg, Germany
[2]Martin-Luther University Halle-Wittenberg, Institute of Chemistry, Halle, Germany
[3]University of Bielefeld, Faculty of Chemistry, Bielefeld, Germany

Introduction. Unspecific peroxygenases (UPOs) act as catalysts in a wide variety of chemical reactions, such as hydroxylations, epoxidations or one-electron oxidations, with high regioslectivity.[1] This type of enzyme is capable of using H_2O_2 as co-substrate for oxyfunctionalisations, in which water is the only by-product.[2,3]

Objectives. An important factor for the usa of these enzymes on an industrial scale is their stability. Unfortunately, too much hydrogen peroxide will quickly deactivate any unspecific peroxygenase through a Haber-Weiss reaction.[4] To avoid unwanted side reaction, it is necessary to provide very low concentrations of H_2O_2. In this study, this was achieved by in situ generation of H_2O_2 using L-amino acid oxidase from *Hebeloma cylindrosporum* (LAAO).[5] H_2O_2 concentrations were additionally quantified in parallel by an electrochemical sensor.

Results. We were ably to achieve a significant increase of UPO-stability due to the constant addition of H_2O_2 through LAAO.

Conclusion. We expect that this cascade will be useful for other forms of cascades as well.

[1] K. Scheibner, R. Ullrich, J. Kiebist, H. Kellner, M. Hofrichter, *BioSpektrum* **2020**, *26*, 103–106.

[2] J. Münch, P. Püllmann, W. Zhang, M. J. Weissenborn, *ACS Catal* **2021**, *11*, 9168–9203.

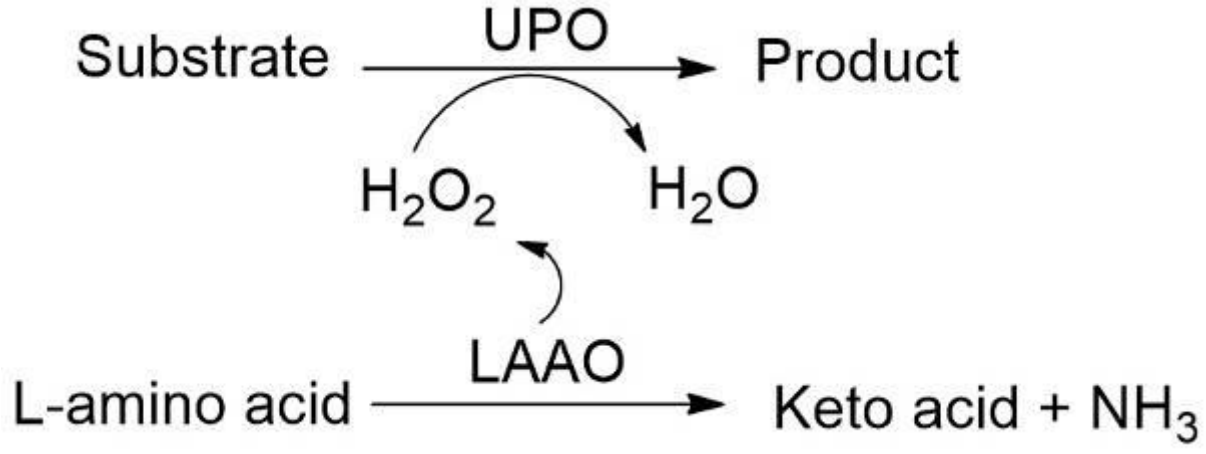
[3] J. Münch, N. Dietz, S. Barber-Zucker, F. Seifert, S. Matschi, P. Püllmann, S. J. Fleishman, M. J. Weissenborn, *ACS Catal* **2024**, 4738–4748.

[4] J. P. Kehrer, *The Haber-Weiss Reaction and Mechanisms of Toxicity*, **2000**.

[5] S. Bloess, T. Beuel, T. Krüger, N. Sewald, T. Dierks, G. Fischer von Mollard, *Appl Microbiol Biotechnol* **2019**, *103*, 2229–2241.

Fig. 1: Reaction scheme for a enzyme cascade using L-amino acid oxidase for the generation of of hydrogen peroxide to feed an Unspecific peroxygenase oxyfunctionalizing a substrate.

Fig. 1



P4-17

Optimizing the coenzyme regeneration in alcohol dehydrogenase catalyzed rhododendrol oxidation

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Introduction

Raspberry ketone is a natural phenolic aroma compound and is widely used in the food and cosmetic industries. The amount of raspberry ketone in the fruit is low, which makes direct isolation of the compound economically unviable. A promising production method is a biocatalytic process that uses as substrate rhododendrol glycosides, precursors found in the inner birch bark. Hydrolysis of rhododendrol glycosides results in the (R)- and (S)- rhododendrol, which can be enzymatically converted to raspberry ketone.

Fig. 1: The reaction scheme – coenzyme regeneration by NOX

Fig. 2: The reaction scheme – coenzyme regeneration by acetone reduction

Objectives

In this study, optimization of coenzyme regeneration in the reaction of (R)–rhododendrol oxidation catalyzed by alcohol dehydrogenase (ADH) in a batch reactor was studied. Two enzymatic methods of coenzyme regeneration were tested: NADPH oxidation by NAD(P)H oxidase (NOX) (Figure 1), acetone reduction catalyzed by ADH already used for the main reaction (Figure 2). The kinetic parameters were estimated under the optimal conditions T = 25 °C, KPi buffer pH = 8. Based on the kinetic studies, a mathematical model was developed and validated in the batch reactor. Reactions were carried out with commercial R-rhododendrol present in racemic mixture and the with rhododendrol obtained in the reaction of rhododendrol glycosides hydrolysis. Rhododendrol yielded from the hydrolysis reaction was not isolated but the process was carried out as sequential two-step, one pot reaction.

Results

The kinetic parameters for the ADH enzyme revealed that the reaction equilibrium is shifted to the reverse reaction. This means that the coenzyme regeneration is essential not only to reduce the cost of NADP+ but also to move the process in the desired direction. Coenzyme regeneration by NOX proved to be better in the reaction in which commercial substrate was used. Main drawback of coenzyme regeneration by NOX is significant inhibition by rhododendrol glycosides and glucose which makes it unsustainable for one pot reactions. Good alternative for crude substrate is shown to be coenzyme regeneration by acetone reduction catalyzed by ADH where the maximum yield was 85 %.

Conclusion

Statistical analysis provided by Scientist software proved that the proposed model describes the experimental results well, and thus can be a useful basis for future predictions of this system.

Acknowledgement: This project has received funding from the European Union"s Horizon 2020 research and innovation programme under grant agreement No 101000560 (RADICALZ).

Fig. 1

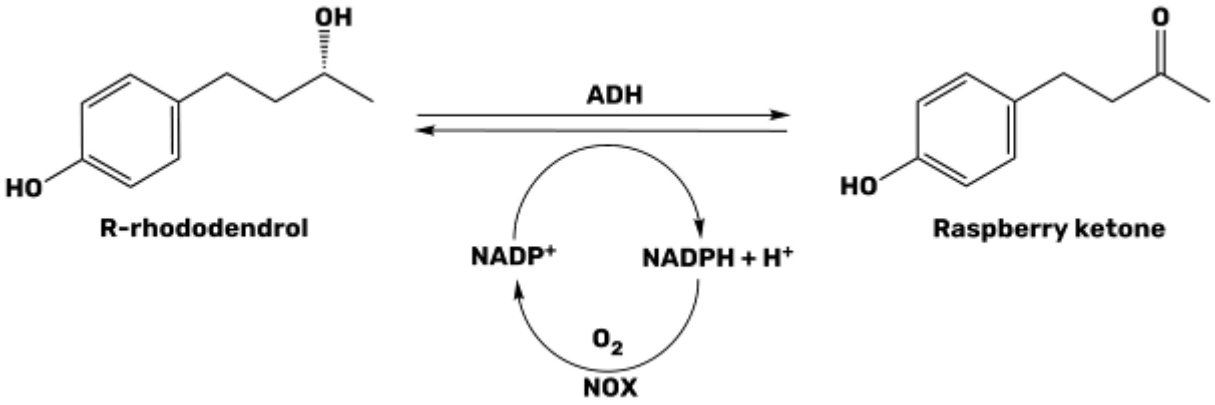
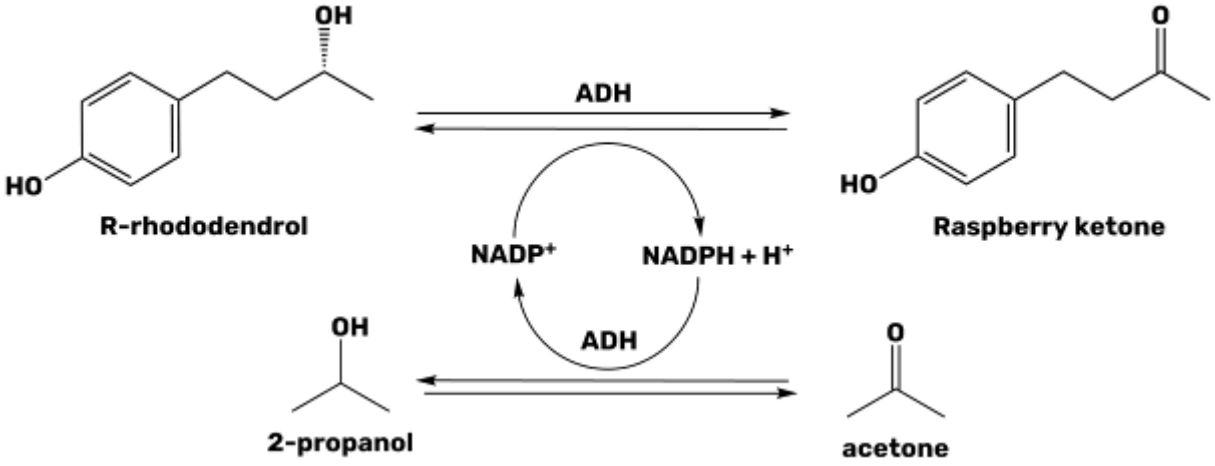

OH
HO
R-rhododendrol
ADH
NADP+
NADPH + H+
O2
NOX
O
HO
Raspberry ketone

Fig. 2


OH
HO
R-rhododendrol
ADH
NADP+
NADPH + H+
O
HO
Raspberry ketone
OH
2-propanol
ADH
O
acetone

P4-18

Electrochemical H_2O_2-Stat Mode as a Reaction Concept to Improve the Process Performance of an Unspecific Peroxygenase

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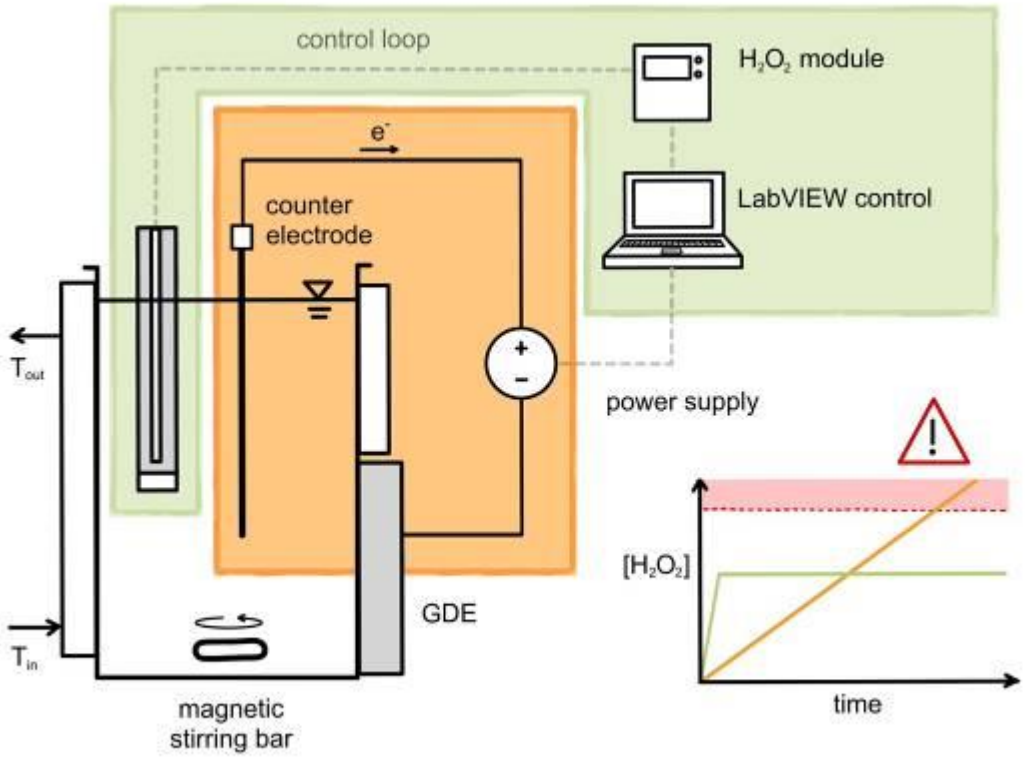
Hydrogen peroxide (H_2O_2) is a valuable co-substrate in biocatalytic processes due to its strong oxidation power. However, the toxicity of H_2O_2 towards H_2O_2-dependent enzymes limits its widespread use. Precise dosing of H_2O_2 is crucial yet challenging. Recently, the application of gas diffusion electrode (GDE) has been expanded, particularly for the electrochemical generation of H_2O_2. Unlike other methods, the electrochemical *in situ* H_2O_2 generation does not increase the reaction volume and avoids the formation of complex by-products. However, constant *in situ* H_2O_2 generation (galvanostatic) leads to an accumulation of H_2O_2 in the medium as the enzyme activity constantly decreases due to its catalase malfunction reaction. This study proposes the implementation of H_2O_2-stat mode to keep the H_2O_2 concentration constant (**Fig. 1**), independent from the enzyme activity. The effect of this approach on the enzyme operational stability was investigated. The goal is to find the optimal H_2O_2 concentration limit under the H_2O_2-stat mode, which would enable a high turnover frequency (TOF) while maintaining a high total turnover number (TTN).

The hydroxylation of 4-ethylbenzoic acid (EBA) catalyzed by the unspecific peroxygenase from *Agrocybe aegerita* (*Aae*UPO) was performed in a GDE system. Two electrogeneration modes were employed to supply the H_2O_2 *in situ*: 1) a H_2O_2-stat mode at a concentration limit between 0.06 mM and 0.28 mM, 2) a galvanostatic mode at a current density between 0.8 mA cm^{-2} and 6.4 mA cm^{-2}, which served as an internal benchmark. For H_2O_2 production and EBA conversion, a maximum of 5.5 µM min^{-1} cm^{-2} and 10.5 g L^{-1} d^{-1} were achieved under the galvanostatic condition at 6.4 mA cm^{-2}. Meanwhile, the highest TTN of 710,000 mol mol^{-1} and TOF of 87.5 s^{-1} were obtained under the H_2O_2-stat mode at concentration limits of 0.15 mM and 0.28 mM, respectively. The most favorable outcome was found under the H_2O_2-stat mode at concentration limit of 0.2 mM. Here, a TTN of 655,000 mol mol^{-1}, a TOF of 80.3 s^{-1} and a productivity of 6.1 g L^{-1} d^{-1} were achieved.

The results demonstrate that each mode has its own advantages and disadvantages. On the one hand, galvanostatic mode offers a higher productivity at a higher current density but suffers from a faster enzyme deactivation due to a constant increase of H_2O_2 concentration. On the other hand, operation under H_2O_2-stat condition enables achievement of high TOF and TTN, albeit at a lower productivity. The advantage of the H_2O_2-stat mode lies in its ability to adapt to the changes of the H_2O_2 consumption rate over time, in accordance to the progress of the reaction.

Fig 1. Schematic representation of the electroenzymatic reaction system. The control unit (automation) is used to regulate the current output sent to the electrodes to adjust the H_2O_2 productivity and to maintain a constant H_2O_2. Dashed line: working cycle of the automation system. Solid line: power supply – electrode"s electric circuit.

Fig. 1



GDE = gas diffusion electrode

P4-19

Multi-Enzyme Cascade in Packed bed Reactors for the Production of 3'-Sialyllactose

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3'-Sialyllactose (3'-SL) is one of the most abundant sialylated human milk oligosaccharides (HMOs) and plays an important role in the growth and development of infants, especially in the first months of their life [1]. Unfortunately, 3'-SL is rarely added to infant formula due to the high cost, whereas other HMOs such as 2'-fucosyllactose or lacto-*N*-tetraose are more present [2]. A cost-effective biocatalytic process could help to facilitate the use of 3'-SL in the infant formula market. An optimised, continuous process using stable enzymes could be promising.

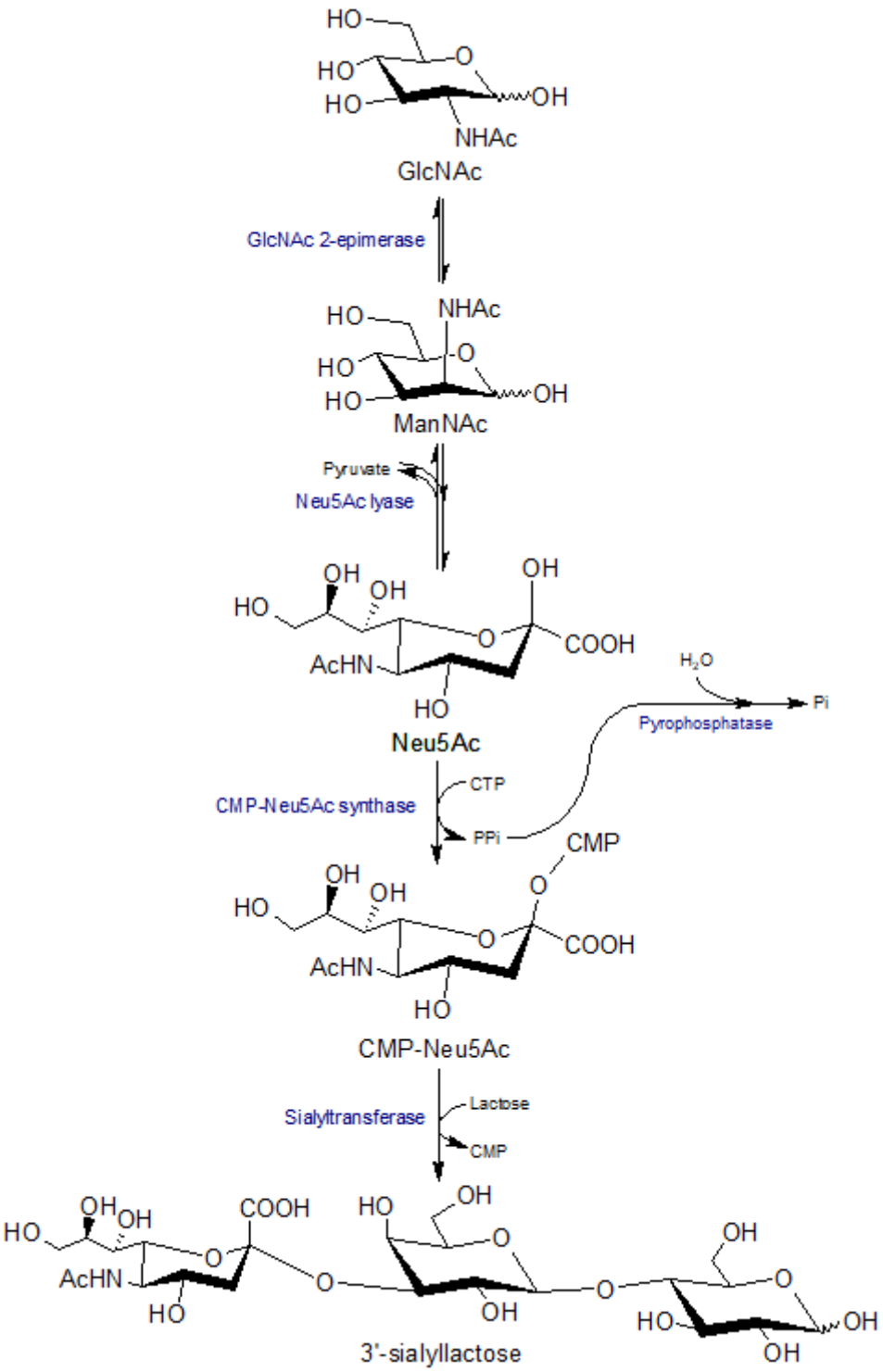
Multi-enzyme cascades in industrial processes are often challenging due to low enzyme stability and the difficulties in recovering and reusing the enzymes. Immobilisation of enzymes can increase enzyme stability, affect specificity and selectivity, and potentially reduce inhibition [3]. A variety of reactors, such as packed bed reactors (PBR), can be used for immobilised enzymes [4]. Covalent enzyme immobilisation offers the advantage of stable bonds. This prevents leaching of the enzyme [5].

A biocatalytic process for the production of 3'-SL starting from GlcNAc was developed using five covalently immobilised enzymes in a PBR (Fig. 1). All five immobilised enzymes showed high stabilities, making the biocatalysts suitable for use in a continuous PBR [6][7]. A Michaelis-Menten kinetic process simulation model was developed to optimise the enzyme cascade. A customised reactor configuration makes it possible to use all five enzymes in a single process. Financial funding was provided by the Federal Ministry of Education and Research (founding number: 031B1080C and 031B1370) and is greatly appreciated.

References:

1. Bruggencate, S.J. Ten et al.; Functional Role and Mechanisms of Sialyllactose and Other Sialylated Milk Oligosaccharides. *Nutrition Reviews* **2014**, *72*, 377–389, doi:10.1111/nure.12106.
2. Walsh, C.. et al.; From Lab Bench to Formulated Ingredient: Characterization, Production, and Commercialization of Human Milk Oligosaccharides. *Journal of Functional Foods* **2020**, *72*, 104052, doi:10.1016/j.jff.2020.104052.
3. Bolivar, J.M., et al.; Is Enzyme Immobilization a Mature Discipline? Some Critical Considerations to Capitalize on the Benefits of Immobilization. *Chemical Society Reviews* **2022**, *51*, 6251–6290, doi:10.1039/d2cs00083k.
4. Federsel, H.J., et al.; Recent Trends in Enzyme Immobilization—Concepts for Expanding the Biocatalysis Toolbox. *Molecules* **2021**, *26*, 2822, doi:10.3390/molecules26092822.
5. De Santis, P., et al.; The Rise of Continuous Flow Biocatalysis – Fundamentals, Very Recent Developments and Future Perspectives. *Reaction Chemistry & Engineering* **2020**, *5*, 2155–2184, doi:10.1039/d0re00335b.
6. Reich, J.A., et al.; Shift of Reaction Equilibrium at High Pressure in the Continuous Synthesis of Neuraminic Acid. *Beilstein* **2022**, *18*, 567–579, doi:10.3762/bxiv.2022.11.v1.
7. Hölting, K., et al.; Resilient Enzymes through Immobilisation: Stable NDP Polyphosphate Phosphotransferase from *Ruegeria Pomeroyi* for Nucleotide Regeneration. *Catalysts* **2024**.

Fig. 1


GlcNAc
GlcNAc 2-epimerase
ManNAc
Pyruvate
Neu5Ac lyase
Neu5Ac
H_2O
Pi
Pyrophosphatase
CTP
CMP-Neu5Ac synthase
PPi
CMP
CMP-Neu5Ac
Sialyltransferase
Lactose
CMP
3'-sialyllactose

P4-20

The biocatalytic production of pseudouridine, a key mRNA vaccine ingredient

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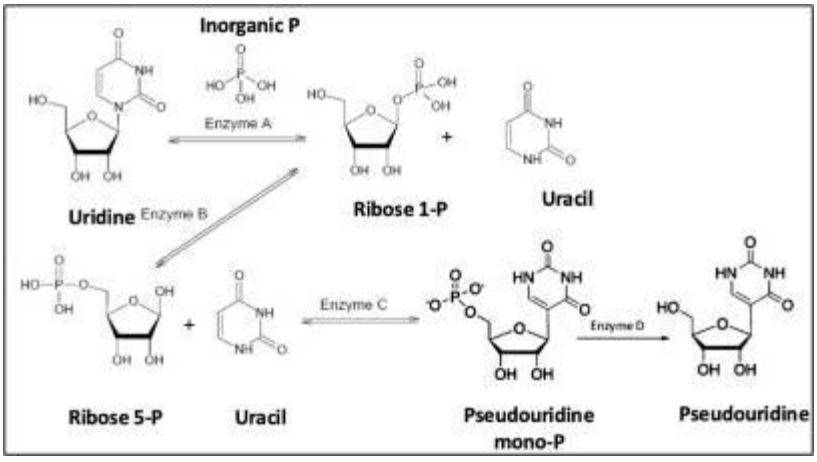
A key aspect of mRNA vaccines is the use of the modified nucleobases to increase their effectiveness, by reducing the immunogenicity of the mRNA and increasing antigen stability and expression. Quick adoption of mRNA-based vaccines and expansion of their application into different clinical trials areas have generated high commercial demands for pseudouridine and its derivatives.

Pseudouridine (5-ribosyluracil) is the most abundant modified nucleoside in cellular RNA. Although pseudouridine was discovered more than half a century ago, its biosynthesis and biological roles are still not fully elucidated. Organic synthesis of pseudouridine has been challenging because of the stereochemistry requirement and the sensitivity of reaction steps to moisture. Herein, we present multi-step enzymatic pathway to form pseudouridine from easily available substrates, such a uridine and inorganic phosphorous.

The reaction consists of 4 enzymatic conversions in a one-pot system. During the first part of the cascade, 3 enzymes are simultaneously added to generate pseudouridine monophosphate via a glycosyltransfer (uridine phosphorylase) followed by phosphotransferase (phosphopentomutase) and finally a C-glycosylation (pseudouridylate synthase). Once sufficient formation of pseudouridine monophosphate is achieved, the fourth and final enzyme (hydrolase) is added in order to form pseudouridine.

Here we present the proof of concept, reaction conditions optimization and scaling of the one-pot multi-step enzymatic pathway to 1 L. The overall process had a conversion of 98.7% of pseudouridine monophosphate and after an efficient dephosphorylation step a pseudouridine yield of 96.2%. Thereby, an effective scalable enzyme cascade reaction with potential industrial applications is here demonstrated.

Fig. 1



P4-21

Multi-enzyme cascade reaction under high hydrostatic pressure: a novel approach for the continuous production of human milk oligosaccharides

F. Lopez Haro[1], J. Reich[1], M. Schmalle[1], M. Aßmann[2], K. Hölting[2], P. Bubenheim[1], A. Liese[1]
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Enzymatic transformations often require multiple steps to go from cost-effective raw materials to target products. While enzymes themselves are typically active under mild conditions of temperature and pH, as well as moderate substrate and buffer concentrations, these various conditions seldom align between different enzymes. Thus, the maximum activity of an individual enzyme is rarely achieved. Instead a compromise of operation conditions must be implemented to ensure all enzymes remain active and stable. This is still one of the obstacles in the widespread implementation of multi-enzyme cascade reactions [1].

This loss of activity in the reaction system, could be mitigated by hydrostatic pressure. Elevated pressures lead to changes in the perfomance of a system. As such the activity of the enzymes in a cascade can be regulated to maximize productivity, even when other parameters (temperature, pH, etc.) are constrained. Furthermore, it is a non-thermal treatment that preserves bioactive compounds, while inactivating microorganisms [2].

One application for cascade reactions is the production of human milk oligosaccharides (HMOs). These complex sugars are a family of compounds predominantly found in human breastmilk [3]. They act as prebiotics, helping establish a healthy microbiome in babies" digestive tract. Currently some HMOs are supplemented into infant formula to obtain their prebiotic effects, however, critical oligosaccharides such as α2,3- and α2,6-sialyllactose (3"-SL and 6"-SL, respectively), are still largely absent from commercial formulations [4].

The current work presents a cascade reaction [5] for the production of 3"-SL, and assesses the use of immobilized enzymes within a series of packed bed reactors for its continuous synthesis [6]. Additionally, it evaluates the potential of using high hydrostatic pressure as a paramenter to optimize the overall productivity of the system in the range between 0.1 and 100 MPa.

Experimental analysis of the individual reactions suggest that operation under high hydrostatic pressure results in a measurable improvement in the yield of the reactions of between 14% to 25% when comparing low pressure and high pressure operation. Thus far, the semi-continuous production of 3"-SL has been consistently achieved at a concentration of 10 mM (6.3 g/L) with 24% analytical yield. This value is a first proof of principle result and is expected to increase as the reaction system is further optimized.

The results suggest that it is possible to repeatedly and reliably produce 3"-SL using a reaction cascade in pressurized packed bed reactors with immobilized enzymes. The implementation of high hydrostatic pressure increases the yield of the system, without the need to alter any of the other operating conditions of the system. The use of high hydrostatic pressure as a parameter may therefore represent a strategy to enhance the productivity of this and potentially other multi-enzyme cascade reactions.

Fig. 1

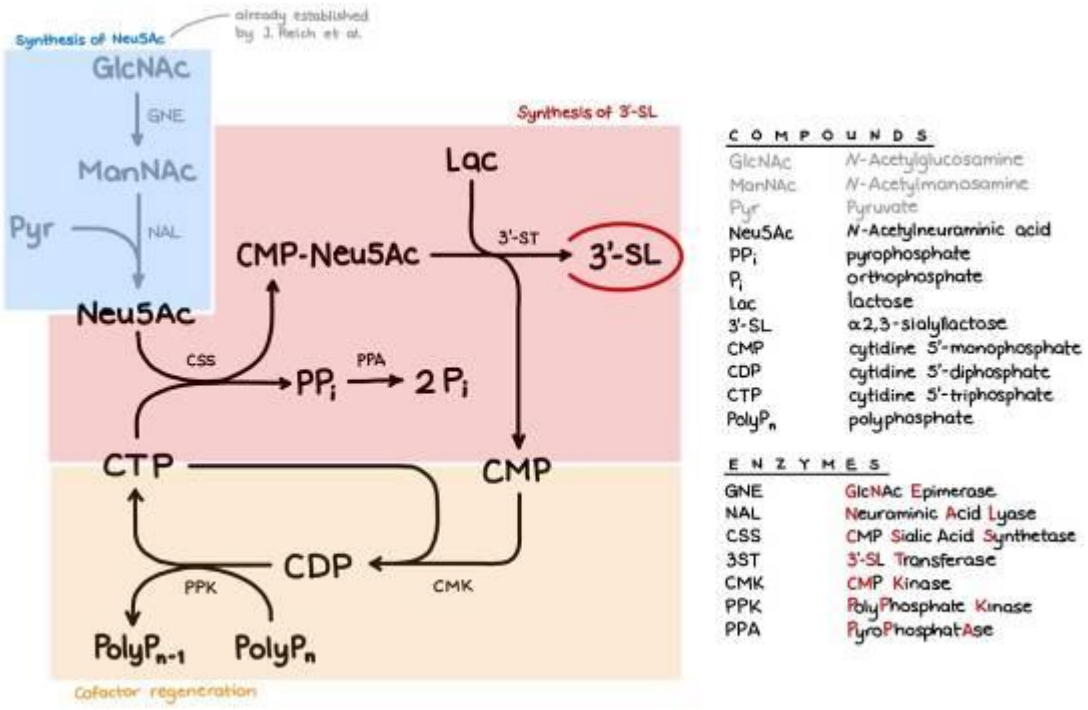
Synthesis of Neu5Ac
already established by J. Reich et al.
GlcNAc
GNE
ManNAc
Pyr
NAL
Neu5Ac
Synthesis of 3'-SL
Lac
CMP-Neu5Ac
3'-ST
3'-SL
CSS
PPA
PPi
2 Pi
CTP
CMP
CDP
CMK
PPK
PolyPn-1
PolyPn
Cofactor regeneration
COMPOUNDS
GlcNAc N-Acetylglucosamine
ManNAc N-Acetylmanosamine
Pyr Pyruvate
Neu5Ac N-Acetylneuraminic acid
PPi pyrophosphate
Pi orthophosphate
Lac lactose
3'-SL α2,3-sialyllactose
CMP cytidine 5'-monophosphate
CDP cytidine 5'-diphosphate
CTP cytidine 5'-triphosphate
PolyPn polyphosphate
ENZYMES
GNE GlcNAc Epimerase
NAL Neuraminic Acid Lyase
CSS CMP Sialic Acid Synthetase
3ST 3'-SL Transferase
CMK CMP Kinase
PPK PolyPhosphate Kinase
PPA PyroPhosphatAse

Fig. 2

[1] Q. Ji, B. Wang, J. Tan, L. Zhu, and L. Li, “Immobilized multienzymatic systems for catalysis of cascade reactions,” *Process Biochem.*, vol. 51, no. 9, pp. 1193–1203, Sep. 2016, doi: 10.1016/J.PROCBIO.2016.06.004.

[2] H. Scepankova, D. Galante, E. Espinoza-Suaréz, C. A. Pinto, L. M. Estevinho, and J. Saraiva, “High Hydrostatic Pressure in the Modulation of Enzymatic and Organocatalysis and Life under Pressure: A Review,” *Molecules*, vol. 28, no. 10, p. 4172, May 2023, doi: 10.3390/MOLECULES28104172/S1.

[3] L. Bode, “Human milk oligosaccharides: Every baby needs a sugar mama,” *Glycobiology*, vol. 22, no. 9, pp. 1147–1162, Sep. 2012, doi: 10.1093/glycob/cws074.

[4] Z. Li, Z. Ni, X. Chen, G. Wang, J. Wu, and J. Yao, “Multi-Enzymatic Cascade One-Pot Biosynthesis of 3’-Sialyllactose Using Engineered Escherichia coli,” *Molecules*, vol. 25, no. 16, p. 3567, Aug. 2020, doi: 10.3390/molecules25163567.

[5] K. Hölting, S. Götz, M. Aßmann, P. Bubenheim, A. Liese, and J. Kuballa, “Resilient Enzymes through Immobilisation: Stable NDP Polyphosphate Phosphotransferase from Ruegeria pomeroyi for Nucleotide Regeneration,” *Catalysts*, vol. 14, no. 3, p. 165, Feb. 2024, doi: 10.3390/CATAL14030165/S1.

[6] J. A. Reich, M. Aßmann, K. Hölting, P. Bubenheim, J. Kuballa, and A. Liese, “Shift of the reaction equilibrium at high pressure in the continuous synthesis of neuraminic acid,” *Beilstein J. Org. Chem. 1859*, vol. 18, no. 1, pp. 567–579, May 2022, doi: 10.3762/BJOC.18.59.

P4-22

Enzyme Immobilization on Carbon Cloth for an Electroenzymatic System

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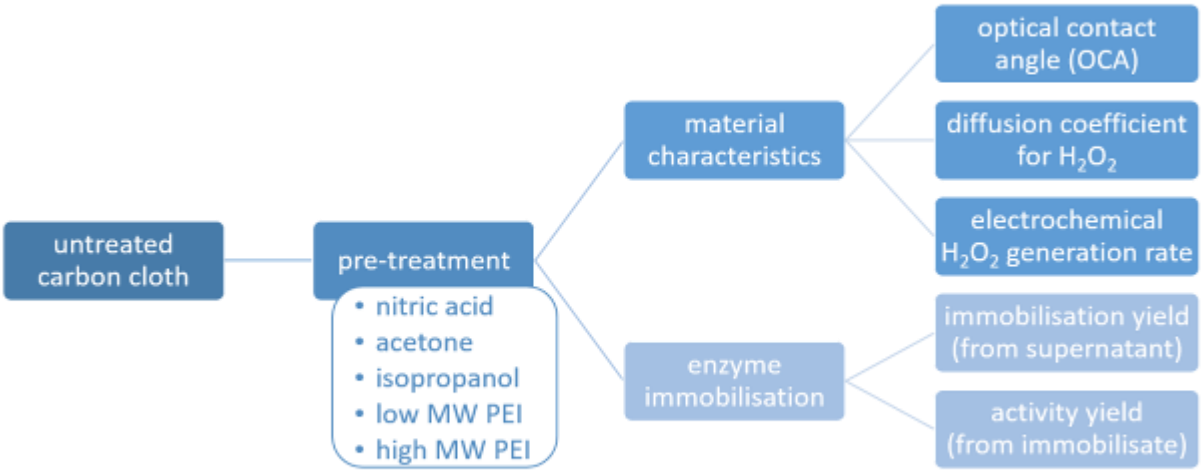
[2]Hamburg University of Technology, Institute of Polymers and Composites, Hamburg, Germany

Carbon cloth is a widely used material for microbial and enzymatic bioelectrochemical systems (BES), acting as an electrochemical anode or cathode. It is also often used as a support material for immobilisation of cells or enzymes in BES. Carbon cloth is popular not only because of its electrochemical properties, but also because of its low cost and commercial availability. It exhibits highly hydrophobic properties, which is not ideal for immobilisation of many enzymes, e.g. unspecific peroxygenase (UPO) from *Agrocybe aegerita* and lipase from *Candida antarctica* (CalB). Immobilisation by adsorption is one of the simplest forms of immobilisation, relying only on physical forces to maintain a bond between the enzyme and the support surface. To increase the affinity of a potential support material for adsorption of an enzyme, physical or chemical pre-treatment methods can be applied.

In this study, the adsorptive immobilisation of two different enzymes (UPO and CalB) on carbon cloth was investigated. Different pre-treatments were applied to carbon cloth, the enzymes were adsorptively immobilised and the immobilisation was analysed by immobilisation and activity yields. In addition, the effect of the pre-treatment was quantified using optical contact angle (OCA) measurements with water. While all five different pre-treatments (nitric acid, acetone, isopropanol, low and high molecular weight polyethyleneimine) increased the immobilisation yield, the low molecular weight polyethyleneimine (low MW PEI) pre-treatment showed by far the highest activity yield. This is in good agreement with the results of the OCA measurements. Here, the contact angle was reduced from an initial 129° for the untreated carbon cloth, indicating strong hydrophobicity, to 0° for the sample treated with low MW PEI, indicating strong hydrophilicity. The switch from hydrophobic to hydrophilic properties was not only found to be beneficial for immobilisation, but also improved the electrochemical performance of the carbon cloth for hydrogen peroxide production by a factor of three due to better contact with the aqueous electrolyte solution (buffer). This is supported by the determined diffusion coefficients of hydrogen peroxide through the carbon cloth. Again, the low MW PEI treated carbon cloth showed a three times higher diffusion coefficient than the untreated carbon cloth.

This study demonstrates how adsorptive enzyme immobilisation on carbon-based materials can be improved by a simple pre-treatment and how this interacts with and influences other performance indicators in an electroenzymatic system.

Fig. 1


untreated carbon cloth
pre-treatment
• nitric acid
• acetone
• isopropanol
• low MW PEI
• high MW PEI
material characteristics
optical contact angle (OCA)
diffusion coefficient for H_2O_2
electrochemical H_2O_2 generation rate
enzyme immobilisation
immobilisation yield (from supernatant)
activity yield (from immobilisate)

P4-23

Valorization of Glycerol to 1,3-propanediol via a Bioelectrochemical Enzyme Cascade Reaction

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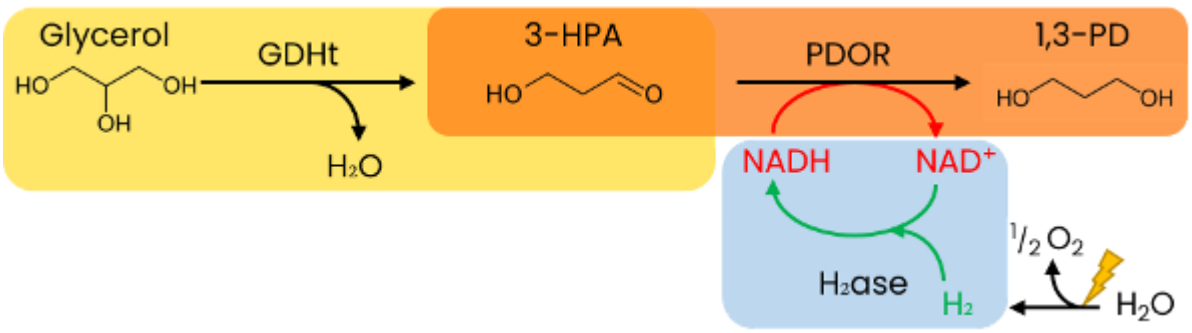
One of the major advantages of petrochemical based raw materials is their consistent composition and quality. In contrast, renewable and sustainable raw materials obtained from biomass, for use in biotechnological processes, vary in their composition and quality. This is due to differences in growth conditions such as soil quality, temperature and sunlight, which vary according to location, season or year, affecting biomass composition and quality. These variations in the substrate feed inevitably lead to variations of the process conditions, and therefore greatly impact the efficiency and feasibility of biotechnological processes. To solve this challenge, we are developing a "SMART" reactor and reaction system, as the purification of the feed substances may not be economically viable. This reactor has the ability to adapt its properties to the changing feed composition, ensuring the reaction is always conducted under optimal process conditions. To establish this reactor, we are using a model reaction of glycerol to 1,3-propanediol (1,3-PD) via a bioelectrochemically driven enzymatic cascade reaction, as shown in figure 1.

Figure 1: Reaction cascade for the valorization of glycerol by a glycerol dehydratase (GDHt), propanediol oxidoreductase (PDOR) and hydrogenase (H2ase) for integrated cofactor regeneration.

This cascade reaction involves three enzymes and an electrolysis cell. Glycerol is converted by a glycerol dehydratase (GDHt) to 3-hydroxypropionaldehyde (3-HPA) under separation of water. Then, 3-HPA is reduced to 1,3-PD by a propanediol oxidoreductase (PDOR) using NADH as a hydrogen donor. As NADH is an expensive cofactor, its regeneration *in situ* is necessary for economic viability of the cascade. Therefore, a hydrogenase is utilized, which regenerates the NADH using molecular hydrogen produced via *in situ* electrolysis of water. Three different kinds of GDHt and PDOR were taken from *Clostridium pasteurianum, Citrobacter freundii* and *Klebsiella pneumoniae*. They were labelled with a His6-tag and transformed into *E. coli* for protein expression. The enzymes are produced via fermentation and purified using immobilized metal ion chromatography (IMAC). To determine the kinetic parameters of the involved enzymes, enzyme activity tests were established. For the PDOR and H2ase, the enzymatic activity can be determined in a spectrophotometric assay by measuring the change in NADH concentration, which correlates to a change in absorption at 340 nm. The GDHt activity is determined in a coupled assay with alcohol dehydrogenase (ADH). ADH is supplied in excess and fills the role of the PDOR, reducing the 3-HPA using NADH, which can be measured as described above.

Using these assays, the enzyme kinetics will be determined and subsequently used to model and optimize the reaction cascade. This will serve as a starting point to investigate the integration of smart materials, supplied by the cooperation partners of the CRC 1615, into the enzyme cascade.

Fig. 1



P4-24

Continuous operation of enzyme membrane reactor with free enzyme reaction cascade for production of nucleotide sugars

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Introduction

Nucleotide sugars (NS) are biological intermediates, acting as donor substrates for the production of human milk oligosaccharides, glycosylation of therapeutics, biopolymers etc. [1]. However, the scarcity and high cost of NS limit their widespread usage. The development of enzymatic cascades, mimicking salvage pathways, provides a promising opportunity for synthesizing NS from monosaccharides [2].

Figure 1: Reaction cascade for the production of two nucleotide sugars (NS). First, NahK (*N*-acetylhexosamine 1-kinase from *B. longum*) performs the phosphorylation of GlcNAc (*N*-acetylglucosamine) and *N*-acetylgalactosamine (GalNAc) using the cofactor ATP (adenosine triphosphate). In the second step, AGX1 (UDP-*N*-acetylglucosamine diphosphorylase from *H. sapiens*) performs the transformation into the respective NS using UTP (uridine triphosphate). PmPpA (pyrophosphatase from *P. multocida*) hydrolyses the byproduct PPi (pyrophosphate), which acts as an inhibitor of the cascade.

Objective

We aim to develop a robust process for the continuous production of NS with high space-time yield (STY) and cofactor regeneration at kg scale. Currently, our focus is on the production of UDP-GlcNAc using a three-enzyme cascade (Figure 1) in a continuously operated enzyme membrane reactor (EMR).

Results

The continuous synthesis of UDP-GlcNAc was carried out in a 10 ml EMR equipped with a 3 kDa ultrafiltration (UF) membrane, at 37 °C (Figure 2). Purified enzymes obtained from fermentation, were mixed in different ratios based on specific activities and injected into the EMR. The flow rate of the substrate (GlcNAc, UTP, ATP and MgCl2 dissolved in aq. sodium carbonate buffer), was adjusted for different residence times (τ). Samples were analyzed by HPLC.

Figure 2: Flow scheme of the EMR setup for continuous synthesis of UDP-GlcNAc.

Continuous operation with NahK: 1 U/ml, AGX1: 2 U/ml and PmPPa: 20 U/ml, showed increasing conversion and decreasing STY with τ as expected, achieving UDP-GlcNAc yield as 35% at τ = 100 min with STY of 39 g/l/day for a 50 h run. On reducing AGX1: NahK ratio from 2 to 1 the yield decreased to 25%, inferring a bottleneck in the reaction cascade. A sudden decline in performance was seen after 33 h of stable operation, implying differing deactivation rates of enzymes.

Conclusion and outlook

The results from the continuous synthesis of UDP-GlcNAc in the EMR give a benchmark for process optimization. Detailed stability and kinetics study will be done individually for the cascade enzymes. Cofactor regeneration will also be introduced to make the process industrially attractive.

References

[1] Frohnmeyer H & Elling L,.2023. Carbohydr Res. 523:108727. doi:10.1016/j.carres.2022.108727

[2] Fischöder, T., Wahl, C., Zerhusen, C., Elling, L., 2019.. Biotechnol J 14, doi:10.1002/biot.201800386

Acknowledgement

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Fig. 1

Fig. 2

P4-25

PRODUCTION OF BIOBASED ETHYLBENZENE VIA CASCADE BIOCATALYSIS WITH AN ENGINEERED PHOTODECARBOXYLASE

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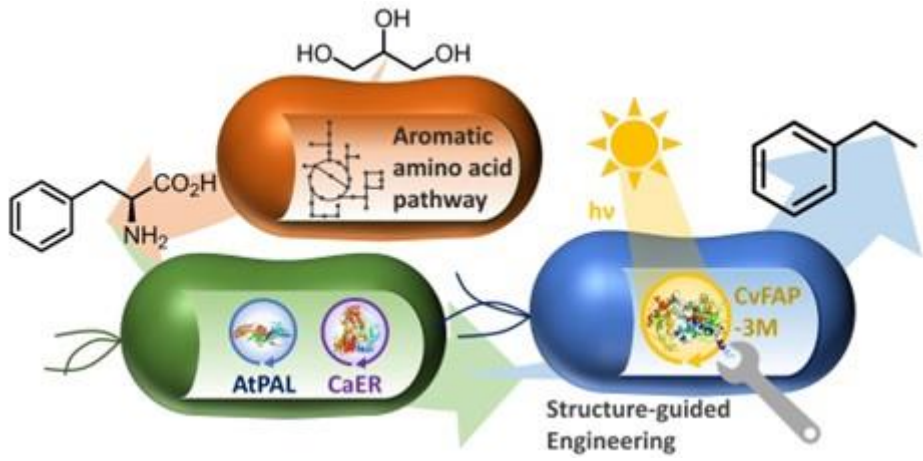
Key Words: Cascade Biocatalysis, Enzyme Cascades, Biobased Chemicals, Photodecarboxylase

Green manufacturing of commodity chemicals from renewable resources is vital for dealing with the challenges of global climate change and fossil fuel depletion. Ethylbenzene, as one of BTEX, is currently manufactured from fossil fuel-based materials in a very large capacity. However, there is no biocatalytic approach for production of biobased ethylbenzene. To change this situation, we designed a non-natural three-enzyme cascade for one-pot conversion of biobased L-phenylalanine into ethylbenzene. The key enzyme, a fatty acid photodecarboxylase (CvFAP) was subjected to three rounds of directed evolution and a triple mutant P460A/G462I/Y466T was obtained with significantly improved activity towards 3-phenylpropionic acid. With this CvFAP mutant, combined with a phenylalanine ammonia lyase (AtPAL) and an enoate reductase (CaER), a-three enzyme cascade was constructed for conversion of L-phenylalanine (10 mM) into ethylbenzene in 80% yield. Further integration of an L-phenylalanine-producing strain, enabled a coupled fermentation-biotransformation process for one-pot production of ethylbenzene in 850 mg/L from biobased glycerol. The study demonstrated the power of combining enzyme engineering and cascade biocatalysis to address problems in sustainable development.

Figure 1 –Production of biobased ethylbenzene via cascade biocatalysis with an engineered photodecarboxylase

Z. Qin, Y. Zhou, Z. Li, M. Höhne, U. T. Bornscheuer, S. Wu. *Angew. Chem. Int. Ed.* **2024**, *63*, e202314566.

Fig. 1



P4-26

Biocatalytic Synthesis of Macrocyclic Lactones

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The formation of medium-sized, *i.e.* 8- to 11-membered, as well as large-sized, *i.e.* >12 membered cyclic molecules presents significant challenges in chemical synthesis.[1] Both entropic and enthalpic hindrance impede efficient cyclization using conventional synthetic methods.[2] Despite their biological relevance, exploration and assessment of such molecules is thus drastically limited in medicinal chemistry.[3][4] In Nature, thioesterases are known to facilitate the efficient intramolecular lactonization of thioesters.[5]

In the presented project, we investigate the potential of thioesterases in synthesizing macrocyclic lactones. A library of SNAc or SCoA thioesters are investigated as substrates for thioesterases from different families. In the envisioned final process, the thioester substrates will initially be synthesized by ligases in a cascade reaction (**Figure 1**).

Figure 1. Cascade reaction to transform hydroxy acids into lactones. In module A, carboxylic acids are converted to thioesters by cofactor dependent ligases, that are subsequently cyclized by thioesterases in module B.

[[1]] Sartori, S. K., Diaz, M. A. N., Diaz-Munoz, G., *Tetrahedron*, **2021**, 84, 132001, DOI: 10.1016/j.tet.2021.132001 0040-4020

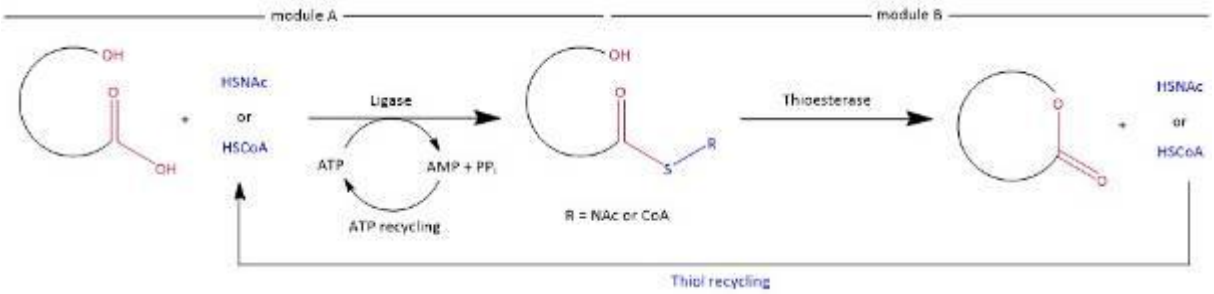
[[2]] Gao, D.-W., Jamieson, C. S., Wang, G., Yan, Y., Zhou, J., Houk, K. N., Tang, Y., *J. Am. Chem. Soc.*, **2021**, 143, 80-84, DOI: 10.1021/jacs.0c11226

[[3]] Vitaku, E., Smith, D. T., Njardarson, J. T., *J. Med. Chem.*, **2014**, 57, 10257-10274, DOI: 10.1021/jm501100b

[[4]] Mordhorst, S., Maurer, A., Popadic, D., Brech, J., Andexer, J. N., *ChemCatChem*, **2017**, 9, 1-6, DOI: 10.1002/cctc.201700848

[[5]] Adrover-Castellano, M. L., Schmidt, J. J., Sherman, D. H., *ChemCatChem*, **2021**, 13, 2095-2116, DOI: 10.1002/cctc.202001886

Fig. 1



IL5-1

Scale-down reactors for design of biocatalysts and processes

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All reactors, no matter their scale, can yield valuable information for subsequent optimization. The more direct measurements are made of concentration changes for example over time, the more can be inferred about (bio)catalyst kinetics and stability, to build suitable models. These are so-called smart reactors. In bioprocesses in particular the need to operate within a narrowly defined window of operating space makes control of utmost importance. Hence, smart (bio)reactors are of great importance for optimization, and consequently alongside such activities modelling is also needed to help understand and predict the effect of any changes.

In recent years" it has become clear that with enzyme-based biocatalysts understanding the effects of the industrially relevant conditions is not only important but also difficult. Indeed, industrial reaction conditions are rarely found in nature, because they involve the use of very high substrate and product concentrations, which can also lead to multiphasic mixtures. As reactors are scaled-up, the concentrations may exhibit gradients in tanks, meaning that even measuring biocatalyst performance under a single set of conditions is not adequate.

In an attempt to study some of these problems we have begun research using scale-down reactors where enzymes are tested under industrial conditions, but at a lab scale in order that experiments can be run cheaper and faster. In this lecture, I will outline some of the approaches we are taking and give a perspective for the future.

L5-1

Enhanced One-Pot Reaction for the (Bio)Synthesis of Sucrose Monopalmitate: Leveraging Imidazolium-Based Ionic Liquid for Efficient Transesterification

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The long-chain fatty acid esters of sucrose represent a class of non-ionic surfactants with a wide range of applications. Especially in the field of food, cosmetic and pharmaceutical additives, the potential superiority of sucrose over alternative acyl acceptors such as glucose and xylose has been emphasized.

The synthesis of these compounds is complex and requires a delicate balance between efficiency and selectivity, whether by chemical or biocatalytic means. Side reactions during saponification, which lead to undesirable darkening and caramelization of sucrose substrates, pose a purification challenge during chemical synthesis. While industrial advances have improved purification techniques, they have also introduced complexities such as hydroxyl group protection and deprotection, which often prolong production. Enzymatic approaches using lipases in organic solvents are promising as they have milder conditions and minimal ester cleavage. However, they are associated with challenges such as enzyme stability and water content management. Lab-scale synthesis has evolved and offers potential for process intensification in sucrose ester synthesis using methods such as ultrasonic intensification, microwave heating and the use of water-immiscible ionic liquids.

Here we investigate novel approaches that could be used for an intensified one-pot process to synthesize long-chain fatty acid esters. Starting from common strategies to intensify the reaction in carbohydrate (trans)esterification, such as microdispersion of the substrate in an organic solvent 2-methyl-2-butanol (here: 2M2B with 20 vol% DMSO) or the use of low solvent content conditions, were found to be far less efficient with sucrose in terms of conversion and monoester selectivity in intensified processes. We demonstrate the transesterification of sucrose (200 mM; ~70 g/L) from vinyl palmitate (>1 molar equivalence) in dry 2M2B containing variable amounts of the ionic liquid 1-butyl-3-methylimidazolium acetate ([Bmim][OAc]) (5-60% v/v) in the absence or presence of immobilized lipase (*Candida antarctica*; *Thermomyces lanuginosus*). The [Bmim][OAc] (≥ 20% v/v) was sufficient to efficiently promote transesterification without any additional benefit of the enzyme, including for product selectivity and initial rate, due to its combined effect on sucrose solubility and acceleration of the catalytic rate. Using ≥ 2 molar equivalents of vinyl palmitate, sucrose was almost completely converted (93%) with little hydrolysis of the palmitoyl donor (~7%) in 72 h at 60°C. A sucrose ester product was obtained consisting of ~75% monoester.

These results illustrate that the [Bmim][OAc]-driven synthesis of palmitoyl sucrose (mono)esters can be intensified with the desired hydrophilic-lipophilic balance (product ≥ 110 g/L; productivity ≥ 1.5 g/L h).

Ref.: Choi J.& Nidetzky B. (2022), 10.1016/j.mcat.2022.112371; Teng Y. et al.(2021), 10.1080/10408398.2020.1798346; Itol T.(2017),10.1021/acs.chemrev.7b00158.

L5-2

Maximizing Efficiency of Multi-Enzyme Cascades: A Data-Driven Optimization Strategy

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[3]Constructor University of Bremen, School of Science, Bremen, Germany

The combination of several enzymes in multi-step reaction cascades enables the synthesis of complex molecules with often shorter reaction pathways compared to chemical routes, thus supporting the development of ecologically sustainable processes [1]. Recent advancements have led to the development of efficient synthetic pathways with potential significance in producing anti-viral and anti-cancer compounds [2].

In the development of enzyme cascades, the cooperative effects between several design parameters must be taken into account, which involves high experimental effort. One way to overcome the challenge of optimizing such multi-parameter systems is to combine *in silico* models with experimental design, model calibration and validation. However, the more complex the reaction cascade becomes, the more interactions take place between enzymes, (co)substrates, and intermediate compounds, which are difficult to describe by mechanistic models. We therefore applied machine learning methods to identify optimal reaction conditions for enzyme cascades by using empirical surrogate models for Bayesian optimization [3]. As proof-of-concept systems, we have used enzyme cascades consisting of the phosphorylation of mevalonate to mevalonate phosphate complemented with synthesis and regeneration systems for the supply of ATP. Bayesian optimization was used to adapt the enzyme and co-substrate concentrations in order to improve the synthesis rate of mevalonate phosphate. With this approach, we were able to iteratively optimize the initial starting composition of the enzyme cascade components to increase the product synthesis rate.

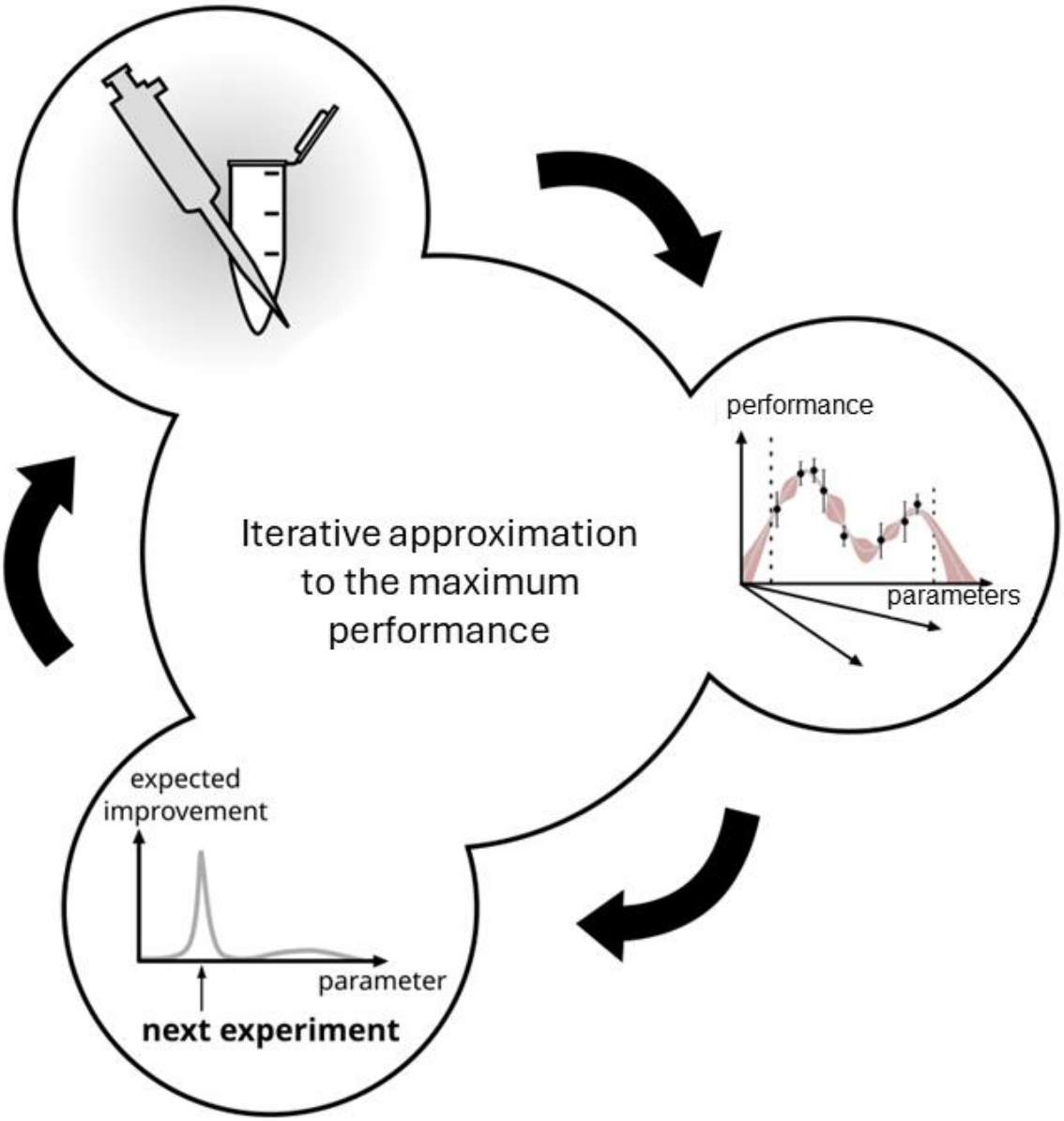
In conclusion, our data-driven approach offers a promising strategy for optimizing enzyme cascades. Our study demonstrates the potential of Bayesian optimization to streamline cascade optimization processes. Moving forward, this approach can contribute to the development of sustainable synthesis pathways for various applications, including pharmaceuticals and fine chemicals.

[1] Siedentop R., Rosenthal K., *Int. J. Mol. Sci.* **2022**, 23(7), 3605.

[2] Rosenthal K., Bornscheuer U., Lütz S., *Angew. Chem.Int. Ed.* **2022**, 61, e2022083.

[3] Siedentop R., Siska M., Möller N., Lanzrath H., von Lieres E., Lütz S., Rosenthal K., *Catalysts* **2023**, 13(3), 468.

Fig. 1


Iterative approximation
to the maximum
performance
performance
parameters
expected
improvement
parameter
next experiment

L5-3

Enzymatic Synthesis of Human Milk Fat Substitute by Immobilized Lipases: Role of Enzyme Carriers and Regulatory Implications

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[1]Sunresin, Xi'an, China

The industrial relevance of immobilised enzymes is primarily application driven, in the sense that there must be a differentiating advantage offered by such biocatalyst over soluble enzymes, whole cells or chemical catalysts. Therefore, the use of immobilised enzyme biocatalysts requires a good understanding of both technical and economic factors.

The manufacture of lipids used in baby formula is an application where immobilized enzymes are widely used. Human milk fat substitute (HMFS) is a structured lipid designed to simulate human milk fat. It contains 60-70% palmitic acid at the *sn*-2 position and unsaturated fatty acids at the *sn*-1,3 positions in triacylglycerol structure. HMFS is synthesized by the enzymatic interesterification of vegetable oils, animal fats or a blend of oils. The efficiency of HMFS synthesis can be enhanced through the selection of appropriate substrates, immobilized enzymes and reaction conditions.

Sunresin has developed specific enzyme carriers suitable for lipase immobilization used in the manufacture of HMFS. In addition to technical suitability such as high enzyme activity and extensive recycling, the enzyme carrier has to comply with stringent worldwide regulations. HMFS is the only source of food for human babies and infants in their first months of life therefore rigorous regulatory requirements are in place for its manufacture. Here, we review the global regulatory requirements for enzyme carriers and immobilized enzymes, by considering the chemical structure of the carrier and its combined effect with the lipase for an efficient and safe biocatalyst to use for HMFS manufacture.

L5-4

One-Pot Crystallization-Assisted Dynamic Kinetic Resolutions of a Beta-Chiral Amine and a Chiral Hydroxycarboxylic Acid

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[2]University of Rostock, Institute of Chemistry, Rostock, Germany
[3]Bielefeld University, Faculty of Chemistry, Bielefeld, Germany

Introduction:

Dynamic kinetic resolution (DKR) is a crucial technique, frequently used for the synthesis of optically pure products from racemic substrates, as it circumvents the limitation of 50% maximum yield of standard kinetic resolutions by racemizing the less-reactive substrate enantiomer.[1] Many possible approaches to DKR involving different techniques, such as e.g. organo- and metallocatalysis, have been investigated over the last decade, however, enzymatic approaches present several benefits in comparison to those (mild conditions, environmental tolerability etc.).[2]

Objectives:

Within two case studies, we aim to highlight the efficiency of DKR in a combination of biocatalysis with selective crystallization to obtain enantiopure compounds (Figure 1).[3] Case study A focuses on a transaminase-catalyzed conversion of a self-racemizing alpha-branched aldehyde and the subsequent crystallization of the corresponding enantiopure amine, which prevents product inhibition.[4] Case study B features racemization of mandelic acid by a mandelate racemase and the diastereoselective crystallization of the product salt.

Results:

Both studies facilitate product loadings of >100 mM and enantiomeric excesses of >90% with easy downstream-processing via a solid/liquid separation step (see Fig. 1 A/B).

Conclusion:

We are able to show, that selective reactive crystallization is a powerful tool for overcoming different reaction limitations when paired with biocatalysis in DKR-based reaction settings.

[1] Turner N. J., *Curr. Op. Chem. Biol*. **2004**, 8 (2), 114-119

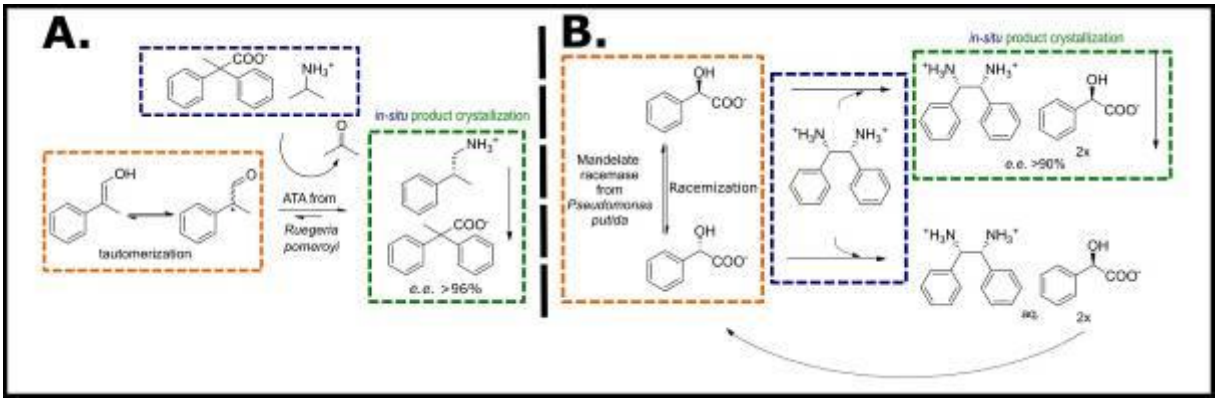
[2] Thejashree G. L., Doris E., Gravel E., Namboothiri I. N. N., *Eur. J. Org. Chem.* **2022**, e202201035; Harwood L. A., Wong L. L., Robertson J., *Angew. Chem. Int. Ed.* **2021**, *60*, 4434; M.Hall, *RSC Chem. Biol.* **2021**, **2**, 958-989; Musa M.M., *Chirality* **2020**, 32(2), 147–157

[3] Hülsewede, D.; Meyer, L.-E.; von Langermann, J., *Chem. Eur. J.* **2019**, 25, 4871-4884

[4] Fuchs, C.S., Hollauf, M., Meissner, M., Simon, R.C., Besset, T., Reek, J.N.H., Riethorst, W., Zepeck, F. and Kroutil, W., *Adv. Synth. Catal*. **2014**, 356, 2257-2265; Belov F., Gazizova A., Bork H., Gröger H., von Langermann J., *ChemBioChem* **2024**, e202400203.

Fig.1: Case studies of crystallization-assisted enzymatic DKR approaches in this work. Reached enantiomeric excesses of the product salts are shown. **A.** Transaminase-catalyzed conversion of 2-phenylpropanal and simultaneous reactive crystallization of (R)-2-phenylpropane-1-amine. **B.** Dynamic kinetic resolution of mandelic acid and selective crystallization of one enantiomer.

Fig. 1


A.
COO⁻
NH3+
in-situ product crystallization
OH
O
tautomerization
ATA from
Ruegeria
pomeroyl
NH3+
COO⁻
e.e. >96%
B.
OH
COO⁻
Mandelate
racemase
from
Pseudomonas
putida
Racemization
OH
COO⁻
+H3N
NH3+
in-situ product crystallization
+H3N
NH3+
OH
COO⁻
2x
e.e. >96%
+H3N
NH3+
OH
COO⁻
2x

P5-1

Optimization of Enzymatic Membrane Reactors for Effective Continuous Flow Biocatalysis

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1. Introduction

Enzymatic membrane reactor (EMR) is an emerging type of continuous flow bioreactors – combining enzyme catalysis and membrane separation in a single unit. EMRs offer easy reuse of the enzyme while giving opportunities to improve its activity and stability via immobilization. Yet, designing an EMR with simultaneously high biocatalytic activity and good mass transfer properties poses a challenge which limits their large-scale applications to this day. In this work, we propose a new method of design and optimization of EMRs – by selective modification of the membrane support with polyelectrolytes prior to enzyme immobilization to control enzyme-support interactions and thus regulate the overall EMR performance.

2. Objectives

- Understand the interconnection between biocatalytic activity, enzyme loading, enzyme stability, and membrane permeability in EMR with alcohol dehydrogenase (ADH) immobilized by physical adsorption.
- Design modification protocol with polyelectrolytes which would enable the optimal balance between desired EMR properties.
- Assess versatility of the method with the other enzymes.

3. Results

Applying higher enzyme loading on the same immobilization support (via longer immobilization time) did not result in higher biocatalytic activity of the reactor, but only decreased the flux (Fig. 1) – proving that further attempts to increase productivity should be done via support modification rather than increasing the enzyme load. Indeed, the threshold of biocatalytic activity of EMR was overcome (increased 8 times) by optimizing concentrations, time, and ionic strength upon membrane modification with polyallylamine (by polydopamine-assisted co-deposition method).

The final reactor showed biocatalytic activity 254±40 U/m^2 (at the flux 40.9±7.5 LMH) which also corresponded to immobilization efficiency 140±22% implying that EMR can also enhance activity of free enzyme due to opportunity of residence time control (by regulation of the operating pressure upon filtration) (Fig. 2). Immobilization of glycerol dehydrogenase showed similar performance - proving good versatility of the method for other systems.

4. Conclusion

EMRs hold a great potential for facilitating industrial biocatalytic processes once their overall performance is optimized. This work demonstrates that it can be efficiently done by thorough investigation of trade-offs in EMR performance and selection of the right method of support modification.

Fig. 1

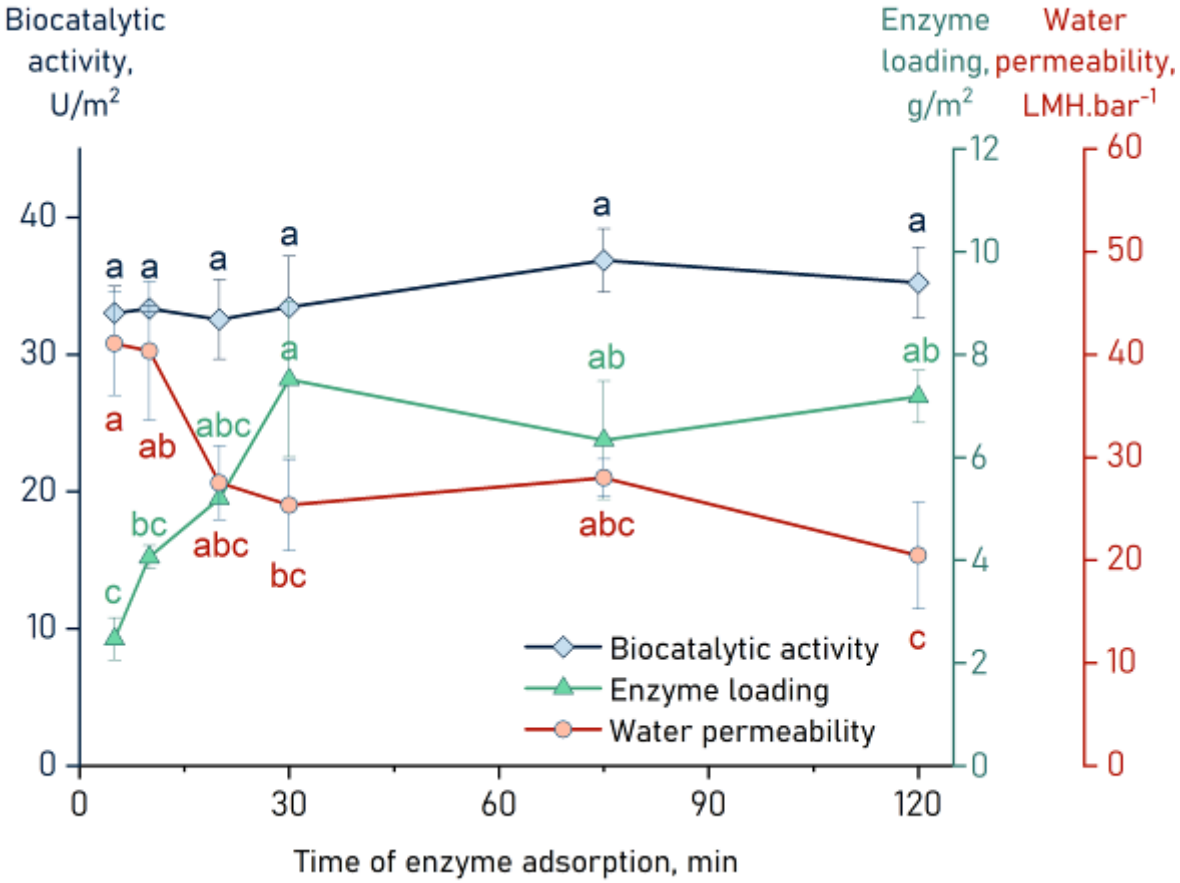
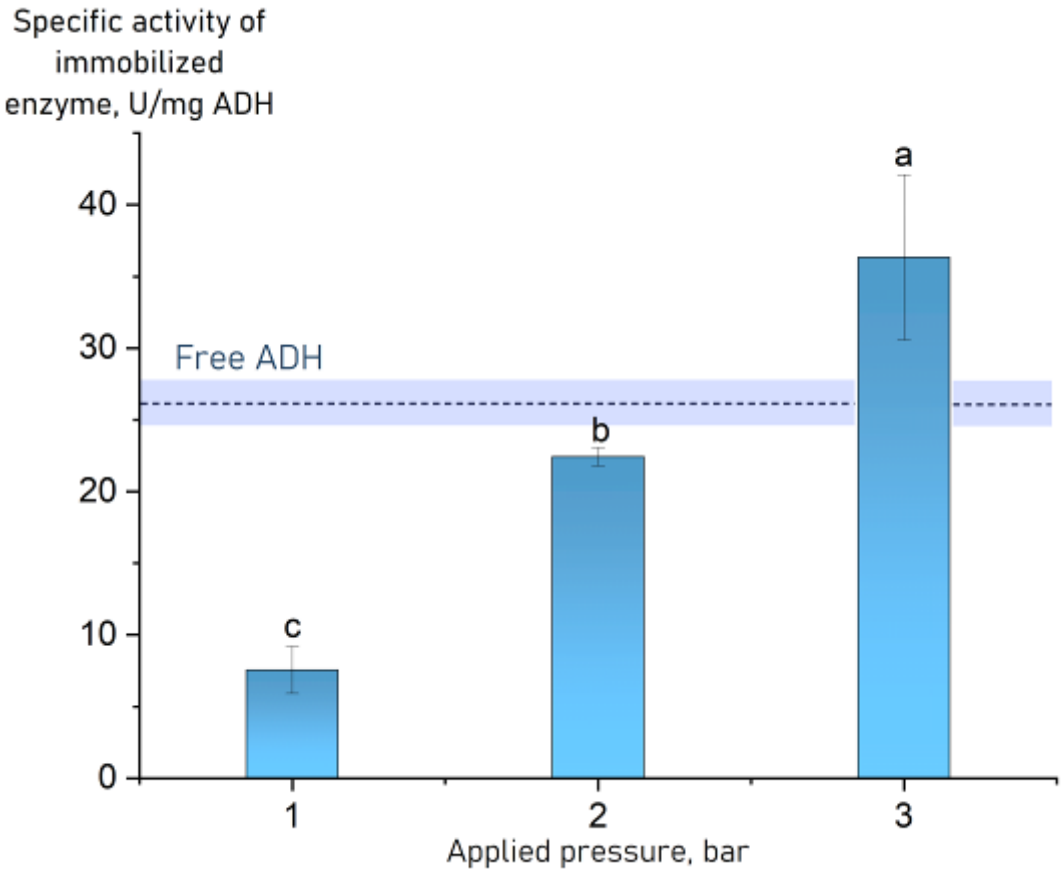

Biocatalytic activity, U/m²
Enzyme loading, g/m²
Water permeability, LMH.bar⁻¹
Time of enzyme adsorption, min
Biocatalytic activity
Enzyme loading
Water permeability

Fig. 2


Specific activity of immobilized enzyme, U/mg ADH
40
30
20
10
0
Free ADH
a
b
c
1
2
3
Applied pressure, bar

P5-2

Reaction Dynamic of Enzymatic Butane Hydroxylation in a Bubble Column Reactor

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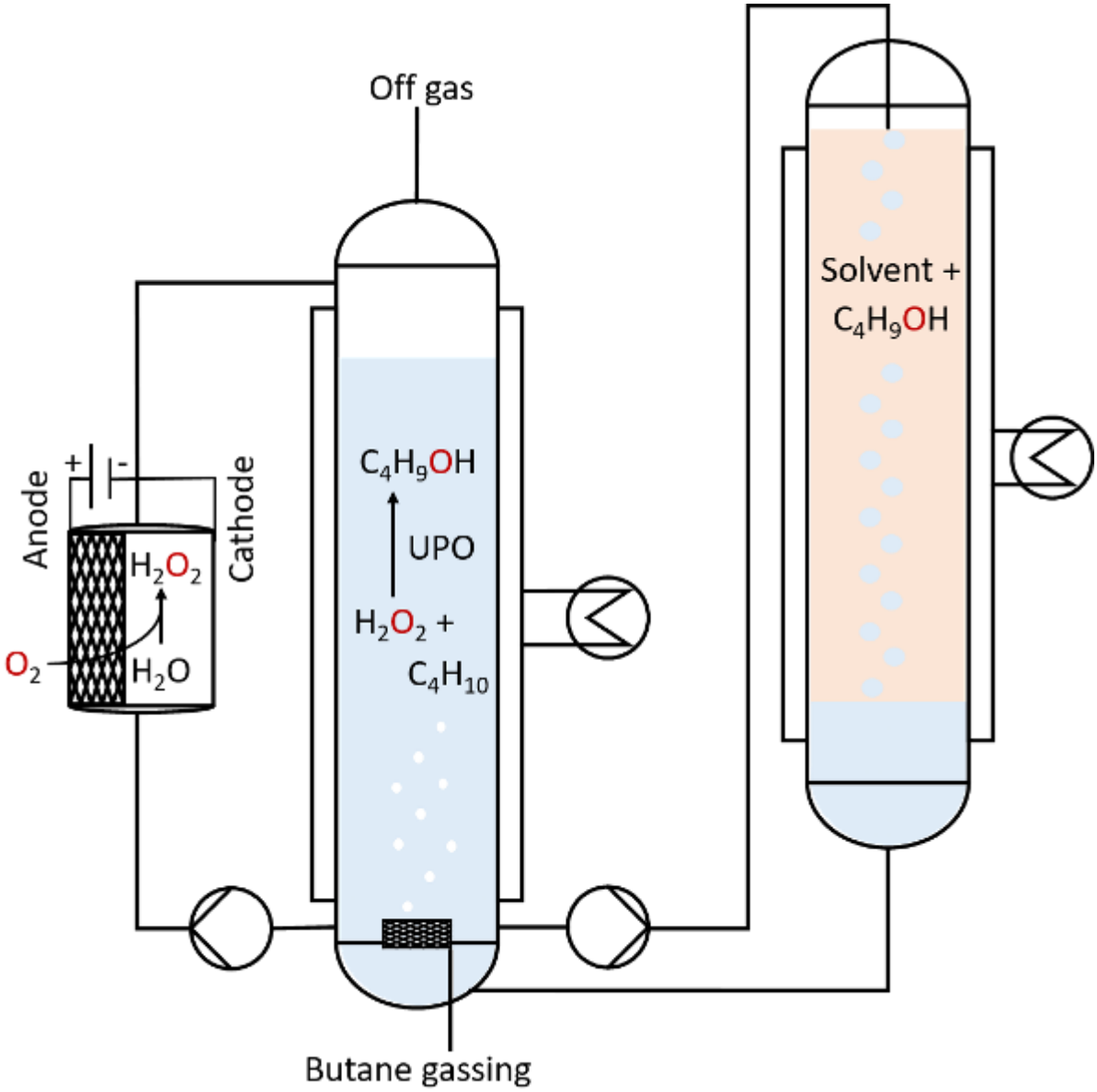
In accordance with the United Nations sustainable development goals, global energy politics leads away from naphtha-based fuels towards sustainable and holistic resource utilization. One recent approach is the enzymatic activation and valorization of alkanes. Those relatively inert hydrocarbons and their existing infrastructure open up a huge potential for novel raw materials.
This study investigates the reaction engineering of an enzymatic n-butane hydroxylation reaction to harness the chemical potential of these abundant bulk chemicals.
A recently discovered group of enzymes, the unspecific peroxygenases (UPO), provides this potential. Known to oxidize a broad variety of different organic substrates, they are capable to selectively convert aliphatic C-H bonds to alcohols, utilizing hydrogen peroxide (H_2O_2).[1]
Applied in a two-liter bubble column reactor (Fig), the gaseous substrate butane is converted into butan-2-ol and butan-2-one, as previously initiated by Perz et al.[2]
The integration of in-line H_2O_2 measurement, controlled electrochemical in-situ generation of H_2O_2 as well as in-situ product removal provides a comprehensive overview of the reactor setup.
Operational parameters such as temperature, pressure, gas flow rate, and catalyst concentration are being optimized to achieve efficient process windows.
Notably, micro-scale screenings showed the scalability of this process and are employed to optimize operational conditions in a safe and resource-efficient way.
However, this research extends beyond the characterization of a two-liter bubble column reactor and explores a practical implementation approach of peripheral components.
This understanding of butane oxidation highlights its potential for scalable and sustainable biochemical processes of novel raw materials.

[1] Aranda C, et al. 2021; doi.org/10.1016/j.biotechadv.2021.107703
[2] Per z, F. et al. 2020; doi.org/10.1002/cctc.202000431

Fig. 1


Off gas
Solvent +
C_4H_9OH
+
-
Anode
Cathode
H_2O_2
O_2
H_2O
C_4H_9OH
UPO
H_2O_2 +
C_4H_{10}
Butane gassing

P5-3

Expanding the Applicability of Pickering Emulsions for Biocatalysis

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- Pickering emulsions (PE) are colloidal systems consisting of two immiscible liquids stabilised by solid particles1 and have been extensively studied for industrial applications2. More recently, they have been shown to be an efficient medium for catalysis3,4 due to their exceptional stability and the constant and extensive contact between both phases, facilitating rapid exchange of reactants5.

  To use PE as a reaction medium, a water-in-oil (w/o) PE is typically prepared5, where the catalyst is dispersed in the aqueous phase (w), i.e. dispersed phase, to protect it from inactivation. However, this does not ensure that all catalysts are at the interface and participating in the reaction. Moreover, hydrophobic particles are required to form a w/o PE. This limits the range of particles that can be used and requires specific modifications before they can be used as emulsifiers.

  Therefore, this study aimed to develop silicone coatings of different hydrophobicities and apply them on particles with immobilised enzymes in order to; (i) evaluate the effect of coatings on the emulsifying ability of different particles within w/o PE; and (ii) analyse the reaction efficiency of particles with immobilised enzymes.

  The coated particles were characterised in terms of wettability, size and surface modification. The particles were used in w/o PEs with potassium phosphate buffer and methoxycyclopentane (CPME) as dispersed (w) and continuous (o) phases, respectively. Lipase B from *Candida antarctica* was then immobilised by absorption on the particles, followed by coating and formation of the PE. The efficiency of the catalysed transesterification reaction in this system was determined by gas chromatography. The stability of the PEs was evaluated from the variation of the droplet size distribution over time by microscopic inspection.

  The results suggest that amphiphilic coatings impart greater stability to PEs than the other coated particles. Furthermore, we could demonstrate the feasibility of using coated particles with immobilised enzymes for catalytic reactions within PE systems. Overall, our investigation highlights the role of silicone coatings in PE stability and in enhancing catalytic activity by preventing enzyme inactivation, thus contributing to the advancement of colloidal systems as catalytic media.

Keywords: Pickering emulsion, Surface modification, Stability, Lipase B, Catalysis

References

1. Pickering, S. U. (1907). CXCVI.—Emulsions. Journal of the Chemical Society, 91(0), 2001–2021.
2. Yang, Y., Fang, Z., Chen, X., Zhang, W., Xie, Y., Chen, Y., Liu, Z., & Yuan, W. (2017). *Frontiers in pharmacology*, *8*, 287.
3. Wei, L., Zhang, M., Zhang, X., Xin, H., & Yang, H. (2016). ACS Sustainable Chemistry & Engineering, 4(12), 6838–6843.
4. Heyse, A., Plikat, C., Ansorge-Schumacher, M. B., & Drews, A. (2019). Catalysis Today, 331, 60–67.
5. Ansorge-Schumacher, M. B., & Plikat, C. (2022). Frontiers in Catalysis, 2.

P5-4

Functionalized poly(aspartic acid) hydrogel particles as a carrier for covalent enzyme immobilization

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Enzymes have played a significant role in organic synthesis, evolving from early development to proof-of-concept at small scales to industrial applications.[1] This success story can also be attributed to different enzyme immobilization strategies.[2] The use in non-conventional media has become an important addition to organic synthesis due to potential substrate and product solubility issues and in this field enzyme immobilization strategies become more visible owing to stability advantages.[3] Enzymes require a certain amount of water activity in organic solvents to function. In this context, tailor-made, synthetic hydrogel materials have recently gained interest in immobilizing enzymes due to their ability to ensure the desired aqueous microenvironment.[4, 5] Poly(aspartic acid) hydrogels (PASP, Figure 1) provide not only modifiable surface areas but also the required enzyme-friendly conditions for biocatalytic reactions in non-aqueous media, due to the hydrophilic nature of these hydrogels.

In this work, we synthesized activated PASP hydrogel particles with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride / *N*-hydroxysuccinimide (Fig. 1, Carrier A), functionalized the activated particles with ethylenediamine (EDA) and glutardialdehyde (Fig. 1, Carrier B), or activated the PASP hydrogels with glycidol (GLY), followed by a functionalization with EDA and glutardialdehyde (Fig. 1, Carrier C). For comparison of these novel hydrogel-based enzyme carriers for targeted covalent attachments, three different model enzymes were applied: *Candida antarctica* lipase B (*Ca*lB), horse liver alcohol dehydrogenase (HLADH), and unspecific peroxygenase (UPO).[5]

Figure 1. Concept of enzyme immobilization onto poly(aspartic acid) hydrogels.

[1] Wandrey, A. Liese, D. Khimbu. *Org. Proc. Res. Dev.* **2000**, *4*, 4, 286–290.

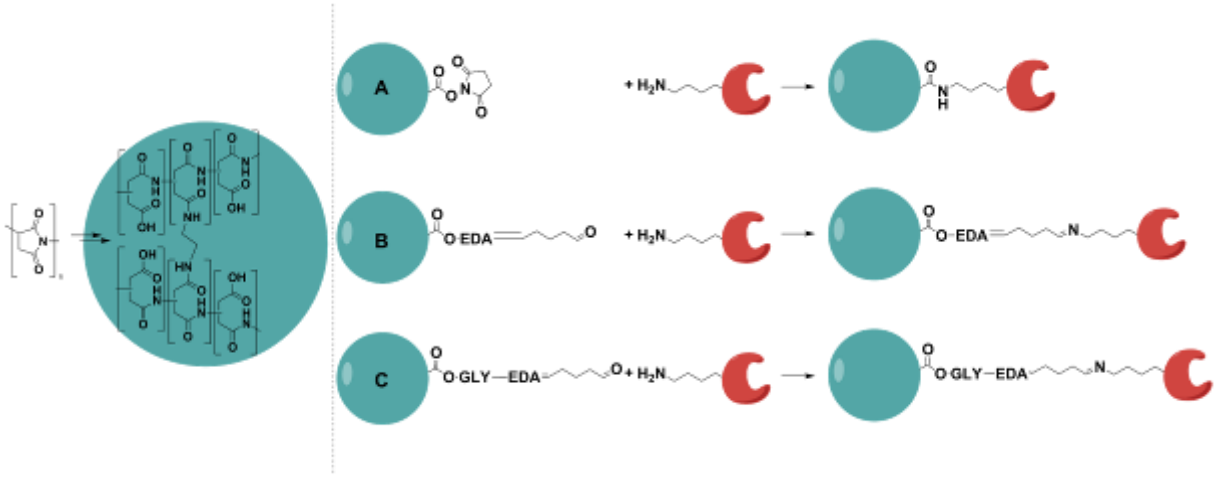
[2] Sheldon, S. van Pelt. *Chem. Soc. Rev.* ***2013***, ***42***, 6223-6235.

[3] Kourist, J. González-Sabín. *Chem.Cat.Chem.* **2020**, *12*, 7, 1903-1912.

[4] Meyer, L.-E. Meyer, S. Kara. *Eng. Life Sci.* **2022**, *22*, 165–177.

[5] -E. Meyer, D. Horváth, S. Vaupel, J. Meyer, M. Alcalde, S. Kara. *React. Chem. Eng.* **2023**, *8*, 984–988.

Fig. 1



P5-5

Characterization of linen fabric Immobilized thermostable β-glucosidase

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Thermostable β-glucosidase derived from a hydrothermal spring metagenome has a potential for applications in the lactose-containing food and side-stream processing industry [1,2]. This enzyme was successfully covalently immobilized onto linen cloth. Functional groups on support material were activated by using potassium periodate or further subsequently modified with ethylenediamine. Two types of immobilized enzymes have been created: enzyme molecules bound to oxidized potassium periodate linen cloth (Lf-ß-glucosidase) and enzyme molecules bound to modified linen cloth with an ethylendiamine - glutaraldehyde spacer (LfEG-ß-glucosidase). Immobilized β-glucosidase retained around 60% of its original activity for both types of immobilized enzymes. The activity of Lf-ß-glucosidase during storage was superior to that of free enzyme and was stable during the storage at 4 oC for 1 year whereas the free enzyme loses 88 % of its initial activity after 2 months of storage under the same condition. More than 80 % of lactose hydrolysis was achieved after 3 h of operation in the reactor over 3 runs in a row. The Michaelis constant (*K*m = 92 mM), a maximum velocity (*V*max = 106 µmol/min) and D-glucose inhibition constant (*K*i = 35 mM) of the system were determined.

LfEG-ß-glucosidase showed greater relative activity and operational stability compared to Lf-ß-glucosidase. A modified biocatalyst with repeated use over 15 runs in a batch reactor for the hydrolysis of lactose retained its initial activity at the level of 30 %. Therefore, ethylendiamine-glutaraldehyde immobilized recombinant thermostable β-glucosidase has the potential for application in the production of lactose-hydrolyzed milk and cheese whey.

References

1 Poladyan A, Trchounian K, Paloyan A, Minasyan E, Aghekyan H, Iskandaryan M, Khoyetsyan L, Aghayan S, Tsaturyan A & Antranikian G (2023) Valorization of whey-based side streams for microbial biomass, molecular hydrogen, and hydrogenase production. *Appl Microbiol Biotechnol* **107**, 4683–4696.

2 Schröder C, Elleuche S, Blank S & Antranikian G (2014) Characterization of a heat-active archaeal β-glucosidase from a hydrothermal spring metagenome. *Enzyme and Microbial Technology* **57**, 48–54.

P5-6

Crystallization-assisted enantiopure amine synthesis using transaminase-membrane reactor

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Introduction

Chiral amines are key building blocks for the pharmaceutical industry. Their synthesis often involves multi-step processes requiring costly organometallic homogeneous catalysts. Transaminases (TAs) may provide greener routes to produce chiral amines with excellent enantioselectivity. Yet, industrial applications of TAs remain restricted to batch processes in which TAs are employed as soluble enzymes (non-reusable). One can design more versatile heterogeneous biocatalysts amenable to flow processes, by immobilizing TAs on solid supports [1]. Importantly, most of the targeted transaminations are limited by unfavourable thermodynamics. One strategy to shift the equilibrium towards high amines yields, is to set-up combined reaction-purification processes, e.g. by selective product removal. Hence, coupling TAs with membrane technology is of particular interest.

Objectives

We present the immobilization of a R-selective TA onto polypropylene membrane (PP) and its application to catalyze an industrially relevant transamination, the asymmetric synthesis of R-2-fluoromethylbenzylamine (R-FMBA), coupled with a product separation (**Fig. 1**). Here, crystallization of R-FMBA was attempted (using 3,3-diphenylpropionic acid (DPPA) as crystallizing agent [2]) aiming to push the transamination towards completion and allowing to recover the pure amine by simple filtration. For TA immobilization, the PP membrane was coated with polydopamine to provide amine functions at its surface, which were further functionalized with epoxy groups to covalently graft the TA.

Results

The obtained immobilized TA showed enhanced activity and stability towards the studied transamination compared to the free enzyme. It also exhibited superior specific activity than widely employed heterogeneous TAs (i.e. Relizyme™-immobilized TAs). Importantly, the presence of DPPA in the reaction medium enabled to displace the thermodynamic limit **(Fig. 2a)** and to produce pure FMBA:DPPA crystals with excellent yields (98 %). The PP-immobilized TA does not leach and was able to perform high-yield successive asymmetric syntheses with only minor deactivation **(Fig. 2b)**.

Fig. 1. Crystallization-assisted heterogeneous transamination

Fig. 2. a) Asymmetric syntheses without (full curves) or in presence of 50 mM DPPA (dotted curves), employing soluble or PP-immobilized TAs. b) Recyclability test of PP-immobilized TA based on initial specific activity (also expressed in relative activity (%))

Conclusion

We demonstrate the use of a novel, robust and highly stable immobilized TA, well-suited for upcoming flow operations and paving the way to intensified processes for chiral amines production. The transfer of this technology from batch to flow is primed to solve many challenges associated with transamination reactions.

References

1. H. Meersseman Arango *et al*., *React. Chem. Eng.* ***2023****, 8*, 1505
2. J. von Langermann *et al., Eur. J. Org. Chem.* ***2018****, 18*, 2130–2133

Fig. 1

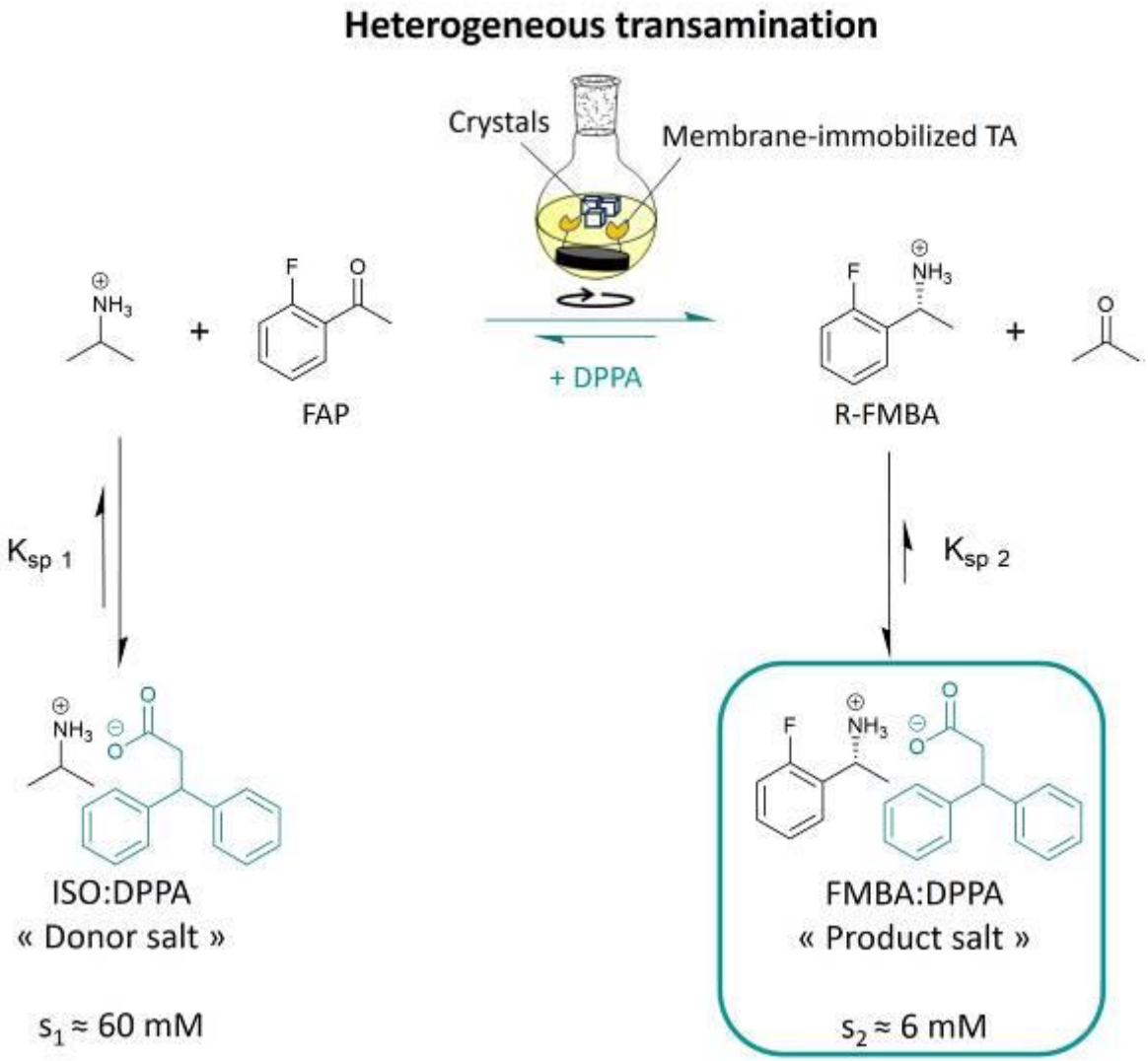
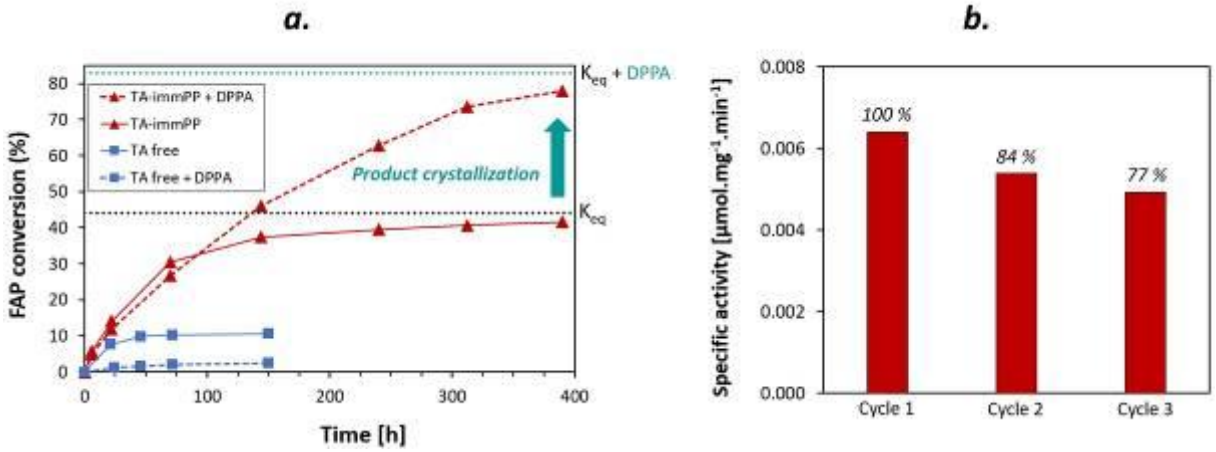

Heterogeneous transamination
Crystals
Membrane-immobilized TA
NH3
F
O
FAP
+ DPPA
R-FMBA
Ksp 1
Ksp 2
ISO:DPPA
« Donor salt »
s1 ≈ 60 mM
FMBA:DPPA
« Product salt »
s2 ≈ 6 mM

Fig. 2


a.
b.
FAP conversion (%)
Time [h]
TA-immPP + DPPA
TA-immPP
TA free
TA free + DPPA
Product crystallization
Keq + DPPA
Keq
Specific activity [µmol.mg-1.min-1]
100 %
84 %
77 %
Cycle 1
Cycle 2
Cycle 3

P5-7

Immobilization of enological pectinase by absorption on polyamide 6 microparticles and its application in the clarification of wine must

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Introduction: Enzymes are widely used as efficient biocatalysts in the food industry for various purposes. In winemaking, a specialized sector where enzymes play a crucial role, pectinases occupy a central position. These enzymes significantly influence the sensory properties of wines by catalyzing the hydrolysis of pectin, responsible for wine cloudiness. This contributes to enhancing clarification and filtration processes without compromising valuable wine components. However, native enzymes are very sensitive to extreme conditions, e.g., pH and temperature, and cannot be re-used. To overcome these limitations, enzymes can be immobilized on polymer supports. Polyamides could be good supports because they are biocompatible and possess appropriate physical and chemical properties that allow covalent and not-covalent enzyme immobilization.

Objectives: Immobilization of a commercial pectinolytic preparation on highly porous polyamide 6 microparticles (MPA6), with and without magnetic properties; Application of the new complexes for the clarification process of industrial rosé must.

Results: MPA6 with and without magnetic properties, were synthesized with controlled shape, size, and porosity by activated anionic ring-opening polymerization of caprolactam in solution. The pectinase immobilization was performed at room temperature, 24 hours, at pH 4.5. The activity of the new complexes against pectin was determined, as well as their kinetic parameters. In comparison with the free enzyme, the PA6-immobilized pectinase displayed a slightly higher affinity to the pectin substrate while acting as faster catalysts, being more resistant to inhibition. Furthermore, the immobilized complexes were applied in the clarification process of industrial rosé must samples and demonstrated enhanced performance, as compared to the free enzyme. Additionally, the immobilized pectinase biocatalysts offered the potential for 3 consecutive cycles of reuse.

Conclusion: The newly developed PA6-pectinase complexes exhibited enhanced activity compared to the free enzyme. Kinetic investigations revealed a slight increase in their affinity towards the substrate resulting in higher maximum velocities of pectin hydrolysis. In terms of clarification, these complexes demonstrated a slight acceleration in the process compared to the free enzyme, and they also proved capable of enduring multiple consecutive cycles of use.

P5-8

Formulation of Enzyme-Based Biomaterials for Flow Biocatalysis

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Introduction
In flow biocatalysis, the concept of interconnected, continuously perfused reaction chambers with integrated biocatalysts is used to improve reaction control and the efficiency of chemical syntheses. To align such concepts for industrial use, an efficient and stable immobilization of enzymes and enzyme cascades into biocatalytically active materials is essential. For this purpose, we have developed enzyme-based biomaterials that can self-assemble into all-enzyme hydrogels (AEHs).[1] Such AEH-materials are created by fusing genetically encoding coupling systems (SpyCatcher or SpyTag) to the enzyme of interest.

Objectives
To show the broad applicability of the AEH-technology a variety of enzymes from different classes, different multimerization states as well as cascades should be formulated into highly stable materials.

Results
By fusing enzymes, such as a phenolic acid decarboxylase (PAD),[2] or an alcohol dehydrogenase (ADH) and a glucose 1-dehydrogenase (GDH),[1] or a xylose reductase (XR) and a galactitol dehydrogenase (GalDH),[3] with the SpyCatcher (SC) or the SpyTag (ST), the site-specific mediated enzyme conjugation allowed the formulation of very stable and highly active biomaterials which consist almost exclusively of enzymes in contrast to other carrier-based immobilization techniques. AEH based on the bienzymatic ADH and GDH cascade can also be formulated directly inside cells[4] and recently we further enhanced the storage and process stability by formulating the material as a foam.[3] The novel AEH-materials were extensively characterized by a number of methods such as dynamic light scattering (DLS), scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray tomography, fluorescence microscopy, laser scanning microscopy (LSM), IR spectroscopy and thermogravimetry. Moreover, the gels showed excellent stereoselectivity, stable conversion rates, high space-time yields (STY) and cofactor retention for more than four days in continuous flow experiments.[5-6]

Conclusion
AEHs, especially in their novel formulation as foam, are a promising material with high application potential for sustainable and environmentally friendly industrial biocatalysis, compatible with a wide range of enzyme classes. Continuous improvement through innovative formulations will further increase the stability and expand the application possibilities.

References
[1] T. Peschke et al., C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2018**, *57*, 17028- 17032.
[2] E. Mittmann et al., K. S. Rabe, *Micromachines* **2019**, *10*, 795.
[3] J. S. Hertel et al., C. M. Niemeyer, *Adv. Mater.* **2023**, *35*, e2303952.
[4] P. Bitterwolf et al., C. M. Niemeyer, *Chem. Eur. J.* **2022**, *28*, e202202157.
[5] P. Bitterwolf et al., C. M. Niemeyer, *Micromachines* **2019**, *10*, 783.
[6] T. Peschke et al., C. M. Niemeyer, *Catalysts* **2019**, *9*, 164.

P5-9

Design of laccase coated membranes as promising reusable filtration materials for enzymatic bioremediation

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Micro-pollutants entering natural and human ecosystems have become a worldwide issue of increasing environmental concern. Most of these pollutants cannot easily be removed from wastewater by conventional water purification methods. Enzymatic catalysis offers a more environment-friendly option, on account of its lower energy requirements, moderate operation conditions and non-toxic products. More precisely, laccase is a class of oxidoreductase enzymes with broad substrate range that meet all the conditions to become a promising option for future water purification. However, design of sustainable laccase-based wastewater treatment processes requires their immobilization onto solid material. Porous membranes are seducing candidates because of their high specific surface, and as they can be used as filtration material.

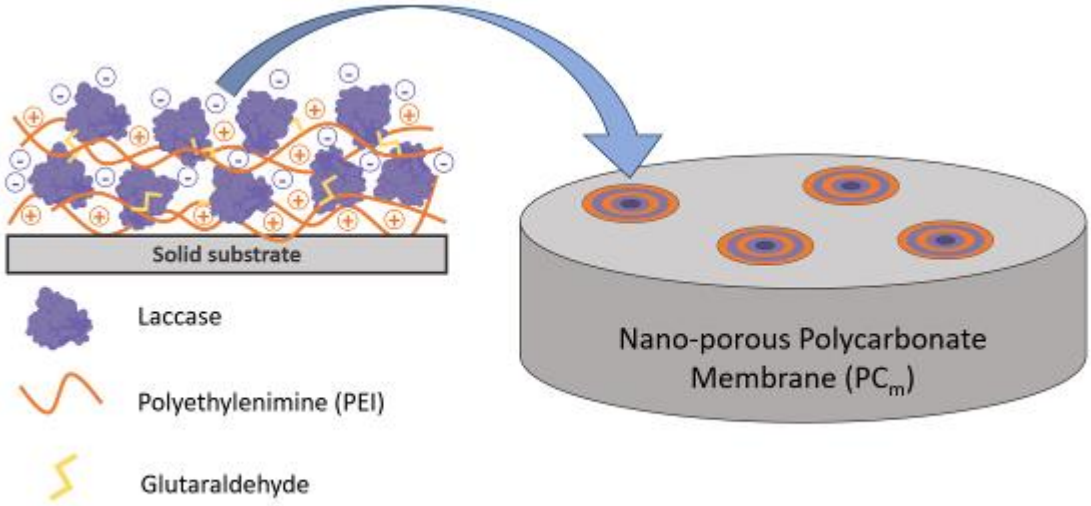
First, this work aims at evaluating and optimizing the well-known layer-by-layer (LbL) assembly technique for the immobilization of laccase from *Trametes versicolor* into nano-porous polycarbonate membranes (PCm) *(figure 1).* Secondly, flow-kinetic parameters and stability of these membranes were evaluated.

We demonstrated that using polyethyleneimine (PEI) as oppositely charged entity for LbL, and MES buffer as assembly media, resulted in more efficient laccase immobilization. We also highlighted that crosslinking each PEI-laccase bilayer with glutaraldehyde not only increased the multilayers stability, but also served as driving force for more efficient immobilization. When this method was used onto PCm, more than 50% of initially introduced laccase was successfully immobilized. We showed that adapting flow rate through the membrane allows to reach the desired degree of conversion, regarding the amount of substrate to be treated. PCm featured remarkable stability, as 85% of their initial activity remained after 40 days, while between 70 to 95% remained when operated continuously for 3h, depending on the applied flow-rate.

To conclude, we designed laccase-coated filtration membranes by introducing an easy and efficient immobilization strategy consisting of crosslinked PEI-laccase bilayers. These membranes featured excellent catalytic properties as well as remarkable stability when used in flow reactors. The evaluation of their ability to degrade pollutants is currently under progress and should allow to pave the way towards practical application.

Figure 1. Crosslinked PEI-laccase ([[PEI-lac]-glu]n) bilayers assembled on flat surface (left). Internal surface of pores from polycarbonate membranes coated with [[PEI-lac]-glu]n (right).

Fig. 1



P5-10

Effect of oxygen mass transfer on the kinetics of Baeyer-Villiger oxidation using a recombinant whole-cell biocatalyst

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This study investigates the performance of a whole-cell biocatalyst, employing genetically modified *Escherichia coli* BL21(DE3) resting cells expressing cyclohexanone monooxygenase, in the Baeyer-Villiger oxidation of a bicyclic ketone to a racemic mixture of bicyclic lactones. The primary objective was to examine the impact of oxygen mass transfer on bicyclic lactone production and oxygen metabolic consumption at varying biocatalyst and substrate concentrations. Over 120 initial rate measurements were conducted in a stirred aerated batch bioreactor with volumetric mass transfer coefficient of oxygen (k_La) ranging from 19 h^{-1} to 83 h^{-1}. Results varied notably depending on the initial bicyclic ketone concentration. Below 4 g/L, BV oxidation followed zero-order kinetics for both substrates, ketone and oxygen. In the absence of mass transfer resistance, specific rates for bicyclic lactone production and metabolic oxygen consumption remained constant at 1.4 mmol/g/h and 1.7 mmol/g/h, respectively. Mass transfer limitations intensified with higher biocatalyst concentrations and lower k_La-values, although BV oxidation faced more severe mass transfer limitations than oxygen metabolic consumption. From these findings, we proposed a refined conceptual model of oxygen demand for metabolism and BV oxidation. Substrate inhibition of BV oxidation was evident above the threshold of 4 g/L, while metabolic oxygen consumption rate was primarily affected at low biocatalyst concentrations. Similar trends were observed for the selectivity of bicyclic lactone production. Additionally, bicyclic ketone consumption rates indicated intracellular ketone accumulation.

Acknowledgements

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P5-11

In-line NMR Monitoring: A Window into Biocatalytic Transformations

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[3]University of Bielefeld, Industrial Organic Chemistry and Biotechnology, Bielefeld, Germany

***Introduction**:* Chiral purity is vital in pharmaceuticals, with biocatalytic processes emerging as sustainable alternatives to traditional methods. Threonine aldolases can be utilized to synthese pharmaceutical components like L-erythro-β-phenylserine (Baer et al., 2010). High diastereoselectivity is crucial for safe production. Understanding reaction kinetics is essential for efficiency. Although L-threonine aldolases can synthesize L-erythro-β-phenylserine, controlling diastereomers at the β-carbon is challenging (Baer et al., 2010; Fesko et al., 2016). In-line analytical methods offer efficient reactand control, promising swift and thorough analysis, especially in pharmaceutical components. Achieving high diastereoselectivity is imperative. Studies emphasize the need for efficient biocatalytic processes for pharmaceutical synthesis, given their potential to ensure chiral purity and enhance overall production efficiency.

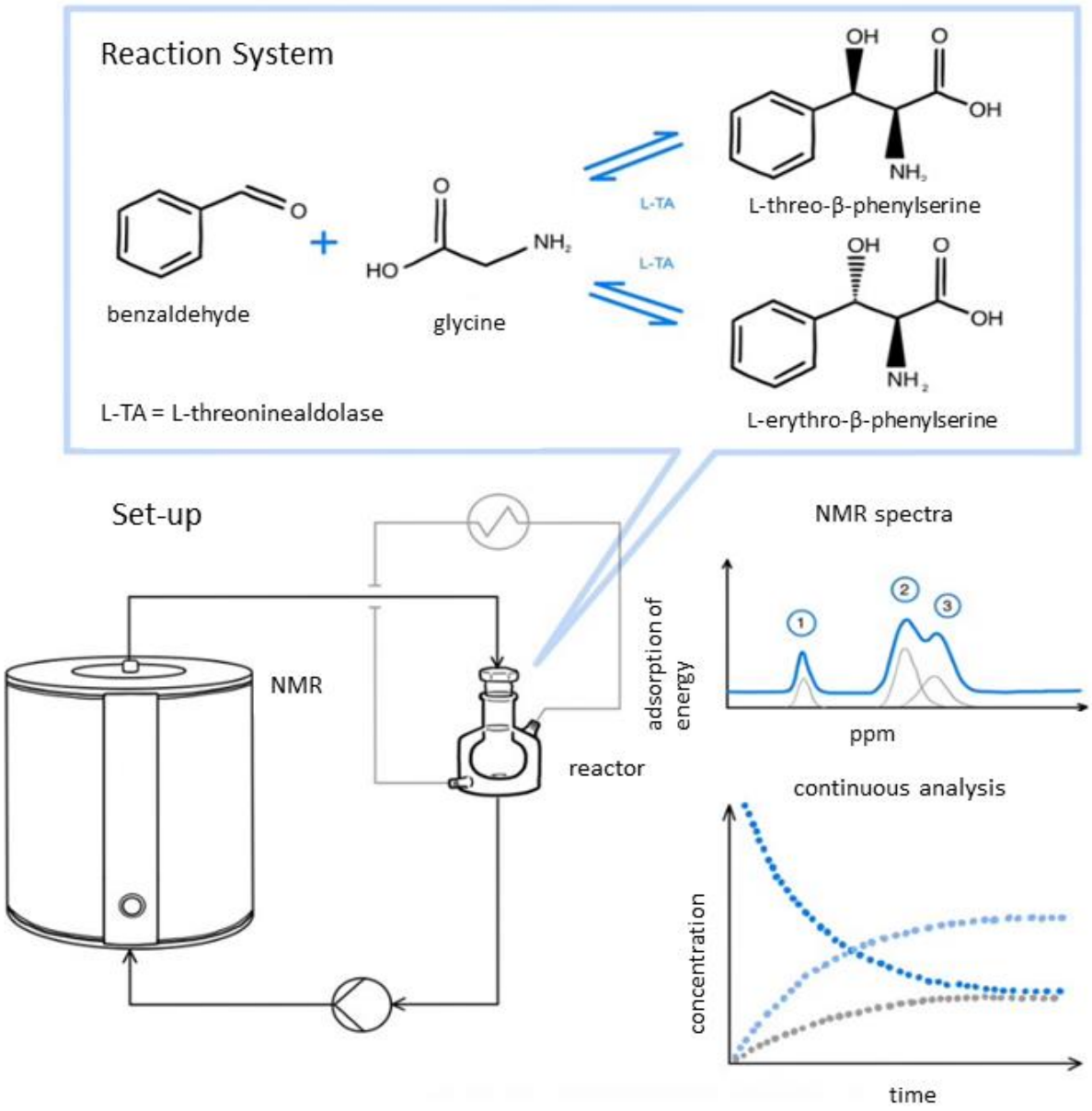
***Results - Analytical Validation**:* During the course of the aldol reaction, in-line measurements were conducted to assess diastereoselectivity, employing the Spinsolve 80 Carbon Ultra NMR system. Encountered challenges related to signal overlap, were overcome by the application of complementary hard modeling (CHM) approach facilitating the successful differentiation between L-threo- and L-erythro-β-phenylserine. This in-line methodology enables the determination of thermodynamic equilibrium data concurrently with rate data obtained from the enzyme-catalyzed aldol reaction. The thermodynamic equilibrium was reached at a benzaldehyde conversion rate of 30 % after 2 hours of reaction time. In-line measurements yielded a diastereomeric excess of 37:63 (erythro/threo), a result consistent with the findings from offline validation procedures.

***Enzyme Immobilization and Reactor Concepts**:* Apart from traditional batch reactors, this research investigates alternative reactor configurations such as fed-batch and continuous stirred-tank reactors (CSTR), employing immobilized enzymes to enhance the efficiency of pharmaceutical ingredient synthesis. A novel method of NMR relaxometry is employed to assess the efficiency of enzyme immobilization.

***Conclusion**:* This study thoroughly investigates in-line NMR's application in biocatalysis, showcasing a benchtop NMR's real-time assessment of diastereoselectivities and thermodynamic equilibrium. Exploring immobilization strategies and reactor configurations contributes significantly to efficient pharmaceutical synthesis. These findings highlight widespread adoption potential in biocatalytic process development, paving the way for more sustainable pharmaceutical production.

Figure 1: Schematic structure of the experimental concept

Fig. 1


Reaction System
benzaldehyde
glycine
L-TA
L-TA
L-threo-β-phenylserine
L-erythro-β-phenylserine
L-TA = L-threoninealdolase
Set-up
NMR
reactor
NMR spectra
adsorption of energy
ppm
continuous analysis
concentration
time

P5-12

Enzymatic sorbityl laurate production in dissolved and neat systems under conventional and microwave heating

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[3]Karlsruhe Institute of Technology, Technical Biology, Karlsruhe, Germany

Glycolipids such as sugar alcohol esters have been demonstrated relevant for numerous applications across various domains of specialty. However, many challenges and limitations remain such as extensive time of production and relatively low productivities amongst others which have to be solved in order to strengthen their biocatalytical production for industrial applications. Therefore, combinations of two heating methods (conventional and microwave) and three solvent systems (organic solvents, DES and neat) were evaluated for the intensification of sorbityl laurate production, as a model biocatalyzed reaction using Novozym 435®. By increasing the reaction temperature from 50°C to 90°C, space time yield and product yield were considerably enhanced for reactions in DES and the organic solvent 2M2B, irrespective of the heating method (conventional or microwave heating). However, positive effects in 2M2B were more pronounced with conventional heating, as 98% of conversion yield was reached within 90 minutes at 90°C equating thus to a nearly four-fold increase in performance yielding 117.96 ± 3.64 g/(L.h) productivity.[1]

With DES, overall yield and space-time yield were lower with both heating methods. However, the effects of microwave heating where more pronounced than in 2M2B. A 7-fold increase in space time yield at 50°C was observed and a 16-fold increase at 90°C when microwave was used instead of conventional heating. Furthermore, microwave irradiation enabled the usage of a neat, solvent free system, representing an initial proof of concept with productivities of up to 13.34 ± 2.34 g/(L.h). Thus, a space-time yield greater than 10 g/(L.h) could be achieved and thus the process can be considered as economically interesting for production of bulk chemicals. However, yields of the solvent-free reaction, as well as the reaction in DES need to be improved even further.

The space-time yield is a widely used metric determining the capital costs and energy requirements to achieve a given productivity. For the production of bulk chemicals the threshold for the space-time yield to achieve a feasible process is 10 g/(L.h). Regarding the results of this study, 10times higher space-time yields than the threshold were achieved for the synthesis of sorbitly laurate in 2M2B. This is complemented by a yield of 98%. This metric yield indicative for the impact of raw material costs has a threshold for bulk chemicals of 95%. The purification costs are normally estimated using the product concentrations. Under the optimized conditions of this study, product precipitation occurs which is of great advantage. In summary, these three process metrics indicate therefore a promising intensification of SL production in 2M2B with respect to feasibility of an industrial process.

[1] Delavault A., et. al., Intensification of enzymatic sorbityl laurate production in dissolved and neat systems under conventional and microwave heating. *ACS Omega* **2024,** accepted manuscript.

P5-13

Systematic methods for improved modelling of enzyme kinetics

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Introduction

Biotechnology is undoubtedly one of the most promising branches of science of our time and is regarded as one of the key technologies of the 21st century [1]. However, like any breakthrough technology, it is not without its limits. One of the central challenges is to make processes more efficient and thus economically competitive with existing chemical processes [2]. Mathematical models are key to evaluate the efficiency and compare different process alternatives [3] as well as optimizing and scaling up enzymatic reactions [4].

Objectives

Nonlinear regression (NLR) is a central element to estimate kinetic model parameters from experimental observations. However, NLR faces several challenges in the context of enzymatic reactions [5]. Especially poor initial guesses can lead to suboptimal solutions and parameter values that do not reflect the underlying biology. Finding appropriate initial values is particularly challenging when dealing with complex biological systems [6]. Systematic methods, such as proper regularization via spline interpolation or the deconvolution of the experimental data in the context of the adsorption energy distribution (AED) can help overcoming some of these hurdles for modelling enzyme kinetics.

Results

First three case studies of uni-uni reactions are evaluated, to show that the estimated parameters by spline interpolation are less dependent of (poor) initial value guesses than common numerical integration methods or the analytical expression of the progress curves.

Second the use of an AED-based estimation for initial slope experiments is illustrated, showcasing that the AED-base approach is able to estimate kinetic parameters with limited data and without the dependence on initial values of common NLR methods.

Conclusion

Both methods provide improved tools for modelling enzymatic reactions that reduce the dependence of the results on the initial values in a significant way.

References

[1] Presse- und Informationsamt der Bundesregierung. *Pressemitteilung 146;* Berlin, 2023.

[2] Gavrilescu, M. and Chisti, Y. Biotechnol. Adv. 2005, 23, 471–499.

[3] Roush, D. *et al*. *Biotechnol. Bioeng.* 2020, *117*, 3986–4000.

[4] Woodley, J.M. *Appl. Microbiol. Biotechnol.* 2019, *103*, 4733–4739.

[5] Sin, G. *et al*. *Biotechnol. Prog.* **2009**, *25*, 1529–1538.

[6] Duggleby, R.G. *Methods Enzymol.* **1995**, *249*, 61–90.

P5-14

Reductase-driven cofactor regeneration cascade in deep eutectic solvents

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After the pioneering work from Gorke et al. (2008)[1], deep eutectic solvents (DESs) have been gaining wide interest as promising non-conventional media for biocatalysis. Due to the tunability and the straightforward preparation, DESs could be potentially applied in all the steps of a biocatalytic process, ranging from upstream to downstream[2]. Furthermore, DESs could be rationally designed as 2-in-1 systems, acting as (co)solvents and substrates at the same time, due to the high variety of compounds that could be used for the generation of the hydrogen bond network[2]. This approach can be used to increase the efficiency and the atom economy of oxidoreductase (EC1) catalysis. The use of oxidoreductases in DESs, such as alcohol dehydrogenases (ADHs) or Baeyer-Villiger monooxygenases (BVMOs), shows promising results for the synthesis of value-added products[3]. To overcome the limitation given by the high cost of the nicotinamide cofactors (NAD(P)H), 2-in-1 DESs could be used for cofactor regeneration cascades[4]. Following this route, a process involving reducing agents as hydrogen bond donors, and Thioredoxin-1/Thioredoxin reductase (Trx-1/TR) from *Thermus thermophilus*, was developed, based on the work from Zhang et al. (2022)[5]. The DES system allows a higher substrate loading than in aqueous medium for the main reaction, and at the same time reduces the Trx-1/TR system allowing the reduction of the NADP+ generated by the ADH or the BVMO (**Fig.1**). The choline chloride-dithiothreitol (ChCl:DTT, 1:2) DES, already generated in a work from Damilano et al. (2022)[6], was prepared and characterized for biotechnological applications. The results from water activity and viscosity analyses, along with the preliminary assessments involving the TR/Trx-1 system, suggest a possible use of this solvent for cofactor regeneration.

[1] Gorke, J. T., Srienc, F., Kazlauskas, R. J., *Chem. Commun.* **2008**, *10*, 1235-1237.

[2] Pätzold, M., Siebenhaller, S., Kara, S., Liese, A., Syldatk, C., Holtmann, D., *Trends Biotechnol.* **2019,** *37*(9), 943-959.

[3] Gotor-Fernández, V., Paul, C. E., J. *Biotechnol.* **2019,** *293*, 24-35.

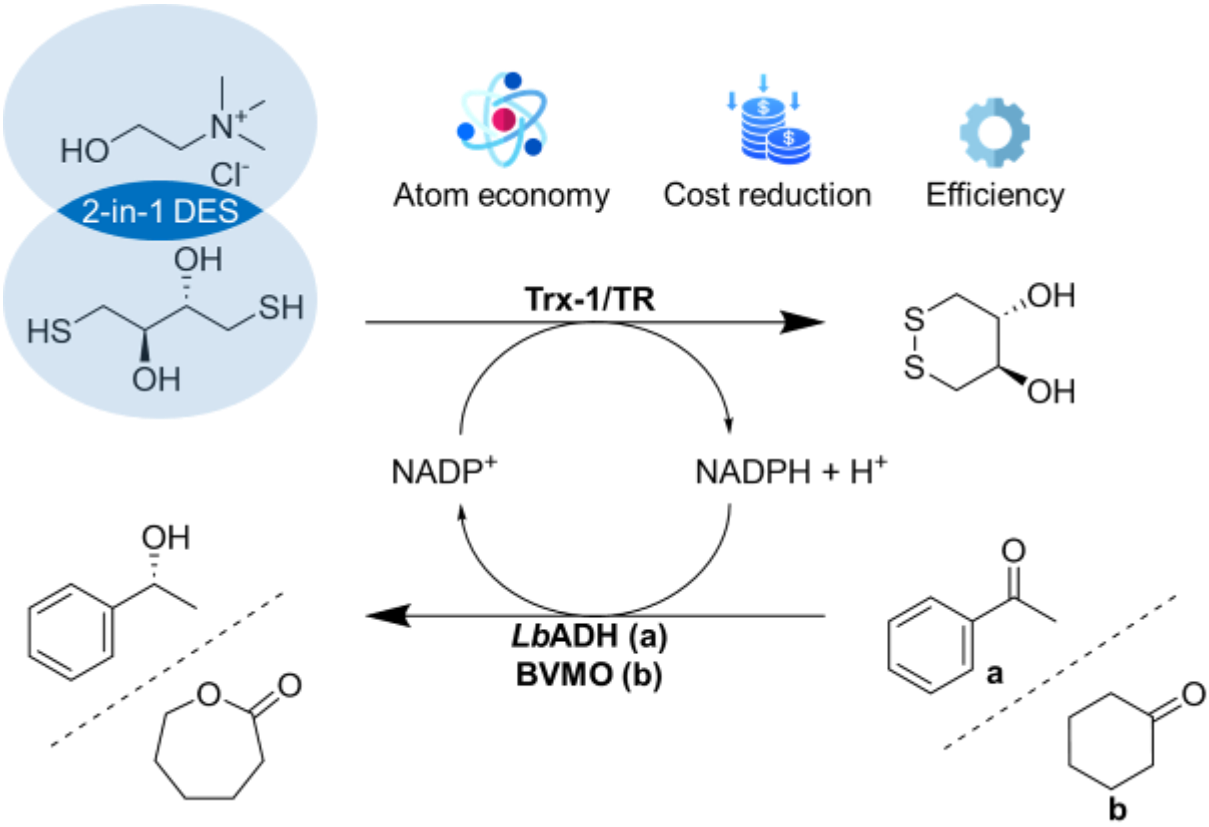
[4] Chanquia, S. N., Huang, L., García Liñares, G., Domínguez de María, P., Kara, S., *Catalysts* **2020,** *10*(9), 1013.

[5] Zhang, N., Müller, B., Kirkeby, T. Ø., Kara, S., Loderer, C., *ChemCatChem* **2022**, *14*(7), e202101625.

[6] Damilano, G., Laitinen, A., Willberg-Keyriläinen, P., Lavonen, T., Häkkinen, R., Dehaen, W., Kuutti L., *RSC Adv.* **2022**, *10*(39), 23484-23490.

Figure 1. Choline chloride-dithiothreitol DES is used in the cofactor regeneration reaction catalyzed by the Trx-1/TR system, yielding NADPH used by the **a**) *Lactobacillus brevis* alcohol dehydrogenase (*Lb*ADH) for the conversion of acetophenone in (*R*)-phenylethanol or **b**) Baeyer-Villiger Monoxygenase (BVMO) to convert cyclohexanone to ε-caprolactone.

Fig. 1


HO
N+
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2-in-1 DES
OH
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Atom economy
Cost reduction
Efficiency
Trx-1/TR
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NADP+
NADPH + H+
OH
O
O
LbADH (a)
BVMO (b)
O
a
O
b

P5-15

Application of immobilized dera enzyme in different types of continuous reactors for the production of statin precursors

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1. Introduction

Statins are a class of pharmacological agents that are used to lower blood cholesterol levels. Constitutively, all statins contain a side chain, (3*R*, 5*R*/*S*)-dihydroxyhexanoate, which is produced on an industrial scale by chemical or biocatalytic methods. The biocatalytic approach, in particular the use of 2-deoxyribose-5-phosphate aldolase (DERA, EC 4.1.2.4) for the synthesis of the statin precursor offers numerous advantages compared to conventional chemical synthesis. However, one of the main challenges with this route is the inactivation of the enzyme. To solve this problem, strategies such as covalent immobilization of the enzyme on a solid support are commonly applied [1].

2. Objectives

The aim of this work was to perform the continuous production of statin side chain precursors catalyzed by the DERA immobilized on two different supports: mesoporous silica (MCF) and magnetic nanoparticles (MNP). The process was carried out at different residence times and in different reactor configurations, including a packed bed reactor for MCF and a fluidized bed reactor with oscillating magnetic field for MNP. MCF was functionalized with (3-aminopropyl)trimethoxysilane (APTMS) and activated with 10% *v/v* succinic anhydride, while MNP were functionalized with (3-aminopropyl)triethoxysilane (APTES) and activated with 15% *v/v* succinic anhydride. Continuous packed bed reactors with volumes of 300 and 500 µL were produced using the fused filament fabrication on a 3D printer. Fluidized bed reactor was made from Teflon tube with volume of 310 µL and was used under the electromagnetic field with H = 1.73 T and frequency of 0.5 Hz. The influence of different flow rates and residence times on the formation of intermediates and reaction products was investigated. Based on the measured values, process indicators, conversion (X) and productivity (Pr) were calculated for different process implementation methods.

3. Results

The highest conversion of X=98.6% was achieved in a 500 µL packed bed reactor (MCF) with a residence time of τ=70 min. This reactor also had the highest conversion rate after 24 h, X=32%. The highest productivity, Pr=1.70 mM min^{-1}, was achieved in a 310 µL fluidized bed reactor (MNP) with a residence time of τ=21 min.

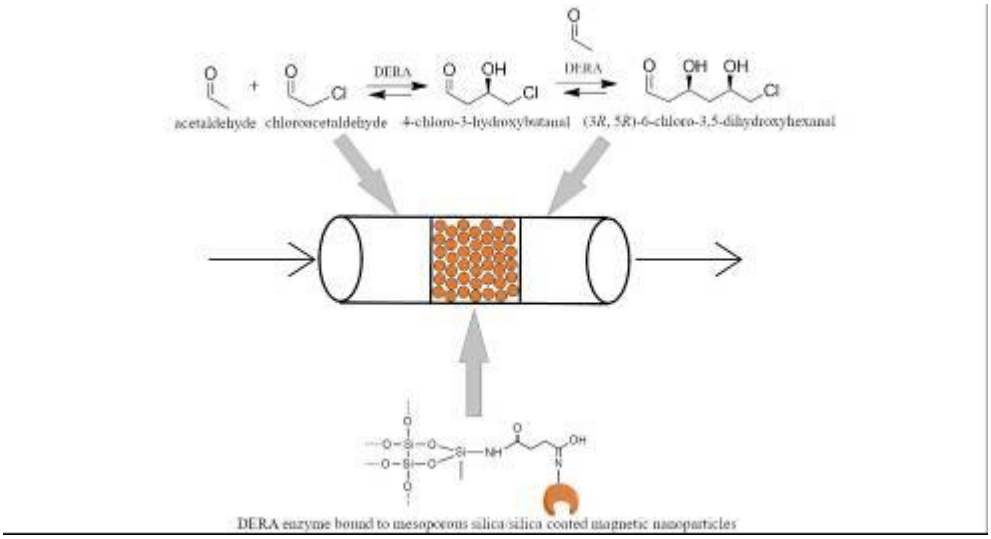
4. Conclusion

When comparing the two reactor types, the packed bed reactor with MCF achieved better results and offers more possibilities for additional modifications. The results of both carriers and reactor types indicate that the accumulation of intermediate was inevitable, leading to lower conversion rates and productivity over time, suggesting that further reaction optimization is necessary.

Figure 1. Synthesis of statin precursor in continuous reactor with DERA immobilized on mesoporous silica/silica coated magnetic nanoparticles.

[1] Patel, J.M. Biocatalytic synthesis of atorvastatin intermediates. *J. Mol. Catal. B. Enzym.* **2009**, *61*, 123–128; DOI: 10.1016/j.molcatb.2009.07.004.

Fig. 1


DERA
DERA
acetaldehyde
chloroacetaldehyde
4-chloro-3-hydroxybutanal
(3R, 5R)-6-chloro-3,5-dihydroxyhexanal
DERA enzyme bound to mesoporous silica/silica coated magnetic nanoparticles

P5-16

A highly efficient immobilization of formate dehydrogenase for biocatalytic applications

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Biocatalysis is a technology that has been key in efficient and sustainable synthesis of chemicals. In industry, enzymes are underutilized due to their stability and complex downstream processes. To counter this and promote reusability, protein immobilisation techniques have been developed. In the process of immobilization, it is important to consider the type of material being used, the dimensions and geometry and the reactive groups of the carriers. These will assist in the optimisation of the heterogenous biocatalyst. Synthetic polymers such as polyacrylamide and polymethacrylate are some examples of carriers used for enzyme immobilization. Inorganic materials such as silica are widely used in industry due to their stability in high temperatures and chemical inertness. They are also low cost, hence favourable as immobilization material.

In this project three immobilisation carriers were compared, that is, IB-HIS-1, IB-HIS-15, and IB-HIS-17. These carriers have a nickel attached via an iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) and are suited for binding enzymes with a His-tag domain. A formate dehydrogenase (FDH) from *Candida boidinii* was used as the heterogenous biocatalyst of choice. We were able to improve FDH production in a fermenter to a 10 L scale. The concentration of the protein collected was 38.71 mg per litre culture. The enzyme was successfully lyophilized and applied to immobilization studies.

To determine how much protein was bound, protein concentration and FDH activity on the supernatant and immobilisation carrier were conducted. Hydride transfer by the FDH from formate to NAD+ forming NADH was measured at 340 nm. Initial protein concentration before immobilisation was 0.306 mg/mL and activity 226.7 U/g. After immobilisation, supernatant measurements showed protein uptake was 90 %, 11 % and 86 % and activity uptake was 98 %, 30 % and 99 % for IB-HIS-1, IB-HIS-15, and IB-HIS-17, respectively. Immobilised enzymes showed measured activity of 0.80 U/g, 2.68 U/g and 3.64 U/g with relative activity recovery of 6 %, 19 % and 26 % for IB-HIS-1, IB-HIS-15, and IB-HIS-17, respectively.

IB-HIS-1 and IB-HIS-17 showed better enzyme uptake as compared to IB-HIS-15. However, taking the activity recovery of the immobilized enzyme into consideration the IB-HIS-17 carrier was most suited and showed no loss in enzyme nor activity and thus is the most suitable to provide FDH carrying beads for biocatalysis.

P5-17

Fatty acid photodecarboxylase for drop in biofuels synthesis in deep eutectic solvents

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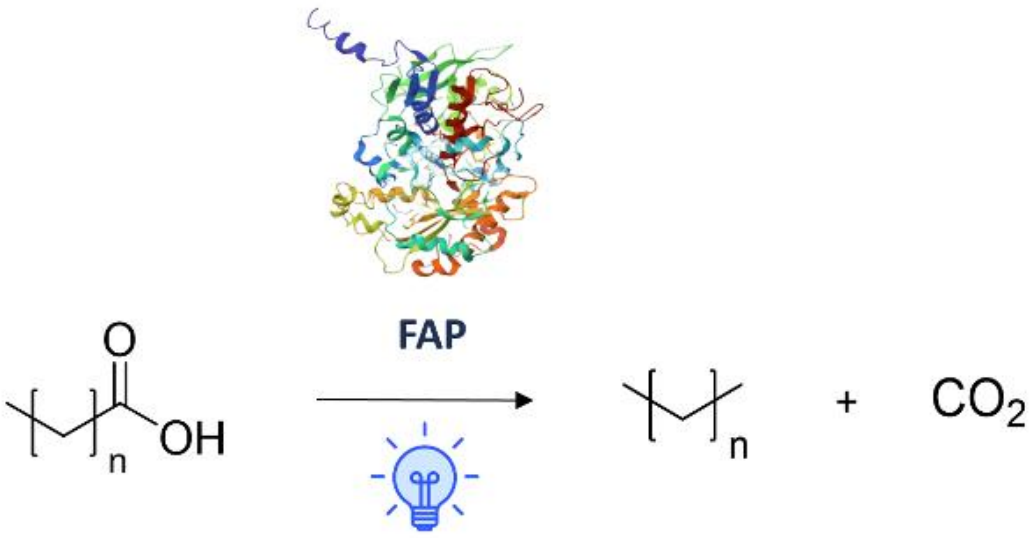
Climate change represents a serious and pressing challenge for our planet. The main cause is the rising levels of greenhouse gases in the atmosphere, such as CO_2. Efforts to decrease CO_2 emissions have escalated, focusing on swiftly transitioning from fossil fuels to low-carbon and carbon-neutral technologies, with biofuels being one proposed solution[1]. "Drop-in biofuels" are biofuels that can be used as a direct substitute for traditional fossil fuels. Enzymes can be employed as a method for synthesizing biofuels[2]. Fatty acid photodecarboxylase (FAP, E.C. 4.1.1.106) is a photoenzyme discovered in 2017[3]. In the presence of blue light, FAP can generate drop-in biofuels from fatty acids (**Figure 1**). The advantages of FAP include the lack of oxygen requirement, there is no overall change in the oxidation state of the reaction and it simply functions by using light. However, addressing the insolubility of FAP's substrates presents a challenge, as most fatty acids do not dissolve well in aqueous solutions. Therefore, alternative, non-conventional media are essential for effective FAP reactions. Biocatalysis is having now transitioning from traditional reaction media to greener solvents. An alternative solution is the deep eutectic solvents (DESs)[4]. A variety of biotransformations have been established with various enzymes in DESs and DES-water mixtures for the synthesis of high-valued chemicals[5]. Several FAP variants were assessed for their activity in DESs with varying water content.

***Figure 1**: Fatty acid photodecarboxylase (FAP)-catalysed synthesis of blended biofuels in presence of blue light (PDB entry: 5NCC). The drop in biofuels that can be synthesized are: Gasoline (C4–C12), Jet fuel (C8–C16), and Diesel (C9–C23) (Image obtained by flaticon.com/free-icons).*

1. Rodionova, M. V., Poudyal, R. S., Tiwari, I., Voloshin, R. A., Zharmukhamedov, S. K., Nam, H. G., & Allakhverdiev, S. I., *International Journal of Hydrogen Energy*, **2017**, 42(12), 8450-8461.
2. Santner, P., Chanquia, S. N., Petrovai, N., Benfeldt, F. V., Kara, S., & Eser, B. E. *EFB Bioeconomy Journal,* **2023**, 3, 100049.
3. Sorigué, D., Légeret, B., Cuiné, S., Blangy, S., Moulin, S., Billon, E., Beisson, F. *Science,* **2017**, 357 (6354), 903-907.
4. Pätzold, M., Siebenhaller, S., Kara, S., Liese, A., Syldatk, C., & Holtmann, D., *Trends in biotechnology,* **2019,** 37(9), 943-959.
5. Zhang, N., Domínguez de María, P., & Kara, S. *Catalysts*, **2024**, 14(1), 84.

Fig. 1



P5-18

3D-printed microreactors for enzyme immobilization: A paradigm towards customized microfluidic screening platforms

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Additive manufacturing, referring to tailor-made designs and fabrication techniques, has been claimed as a game-changer in the construction of reactors and peripheral units in the field of flow chemistry[1]. From one point of view, the inherent nature of this enabling technology allows for freedom of design and adjustment according to the specific needs of each system. Adding to this, the ongoing research on 3D-printable materials is providing a variety of options to explore for the ideal reactor material and/or immobilization matrix to incorporate into the flow system[2]. On the other hand, enzyme immobilization for microbioreactor development has long been proven as a highly efficient approach for biocatalyst retainment and reuse[3]. This work explores both aspects of enzymatic microreactor development: i) the 3D printing technique/3D-printed material, and ii) the immobilization method, to achieve a robust, efficient, and reusable microbioreactor system. The proof-of-concept was demonstrated with an enzyme that has not been elaborated so far for microfluidic applications since there are certain challenges for its immobilization and stability.

The enzyme of choice was unspecific peroxygenase (UPO). Fungal UPOs (EC 1.11.2.1), are heme-thiolate enzymes, displaying characteristic peroxidase activity, but also a unique peroxygenase activity. This way, UPOs are versatile biocatalysts with great importance in synthetic chemistry for an ensemble of highly selective C-H oxyfunctionalizations, while engineered UPO variants are currently under intensive study[4]. After research on the 3D printing technique and material investigation, a surface functionalization protocol was developed, leading to enzyme anchoring on the microreactor"s internal walls. Process parameters optimization was performed in regards to (i) reactor geometry, (ii) flow rates, and (iii) reaction conditions. Ultimately, the developed systems were evaluated for their performance as microfluidic screening tools, to explore the suitability of different enzyme variants for oxyfunctionalization reactions of interest.

Keywords: 3D printing, enzymatic microreactors, enzyme immobilization, oxyfunctionalization

References

(1) Zentel, K. M.; Fassbender, M.; Pauer, W.; Luinstra, G. A. 3D printing as chemical reaction engineering booster. In *Advances in Polymer Reaction Engineering*, Advances in Chemical Engineering, **2020**; pp 97-137.

(2) Gkantzou, E.; Weinhart, M.; Kara, S. 3D printing for flow biocatalysis. *RSC Sustainability* **2023**, *1* (7), 1672-1685.

(3) Bolivar, J. M.; López-Gallego, F. Characterization and evaluation of immobilized enzymes for applications in flow reactors. *Current Opinion in Green and Sustainable Chemistry* **2020**, *25*.

(4) Beltran-Nogal, A.; Sanchez-Moreno, I.; Mendez-Sanchez, D.; Gomez de Santos, P.; Hollmann, F.; Alcalde, M. Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases. *Curr Opin Struct Biol* **2022**, *73*, 102342.

P5-19

Biocatalytic oil cleavage in a Catalytic Membrane Reactor (CMR) using selective lipase variants

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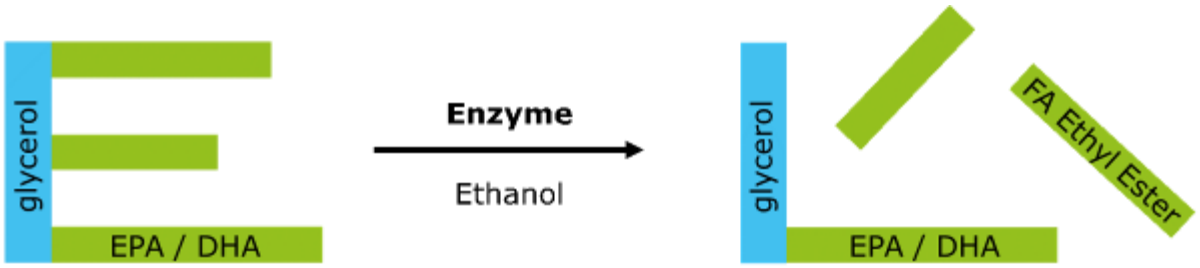
The **MACBETH** (**M**embranes **A**nd **C**atalysts **B**eyond **E**conomic and **T**echnological **H**urdles) consortium provides a breakthrough technology by combining catalytic synthesis reaction with the corresponding separation units in a single highly efficient **C**atalytic **M**embrane **R**eactor (CMR). The revolutionary new reactor design will guarantee substantially smaller and safer production plants and thus reduce operational and investment costs. **MACBETH** is divided into four individual cases. One of them, the **BOC** (**B**io Catalytical **O**il **C**leavage) case deals with the enrichment of the omega-3 fatty acids **EPA** (eicosapentaenoic acid) and **DHA** (docosahexaenoic acid) in fish oil. A lipase as selective catalyst will be used to cleave of the undesired fatty acids as their corresponding ethyl esters. EPA and DHA will still be attached to the glycerol backbone. The removal of the ethyl esters from the omega-3 enriched fish oil will be achieved by a membrane separation process. Here we provide insights into the testing and selection of different variants of the lipase, including the scale-up of the recombinant expression in *Pichia pastoris* and the subsequent immobilization on selected support materials. Two variants showed a better selectivity towards the enrichment of EPA and DHA compared to the *wt*-enzyme.

Fig. 1



P5-20

Application of cross-linked enzyme crystals of halohydrin dehalogenase HheG D114C in microfluidics

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Introduction

Halohydrin dehalogenase HheG from *Ilumatobacter coccineus* shows high industrial potential because of its acceptance of sterically demanding internal epoxides in ring opening reactions [1,2]. However, its industrial application is limited by its low stability with an apparent melting temperature of only 38°C [3]. To increase stability, HheG was previously immobilized as cross-linked enzyme crystals (CLECs) [3,4]. These CLECs were successfully produced in volumes up to 50 mL and enabled the continuous operation of a packed-bed reactor and a fluidized-bed reactor over several weeks [5].

Objectives

Now, downscaling of the crystallisation and cross-linking of the HheG variant D114C in glass microchips with volumes up to 8 µl was intended. In this context, the influence of different channel geometries and surface properties on the crystallisation efficiency was investigated. Furthermore, the catalytic efficiency of these microreactors in epoxide ring opening reactions was studied.

Results

A high loading of the HheG D114C CLECs in the microchannels was achieved. The crystallisation efficiency was influenced by surface properties of the microchannel. Furthermore, the CLECs were catalytically active and a high productivity of the microreactors was achieved in the continuous transformation of cyclohexene oxide with azide. The overall performance of the microreactors depended on the channel geometry.

Conclusions

HheG D114C CLECs can also be generated and applied in glass microchips with volumes up to 8 µl to perform continuous epoxide ring opening reactions with high enzyme loadings and productivities.

Figure 1.

HheG D114C CLECs (stained with fluorescein isothiocyanate) on a glass microchip catalysing the ring opening of cyclohexene oxide with azide as a model reaction.

References

[1] Koopmeiners *et al*., *ACS Catalysis*, **2017**, 7, 6877-6886

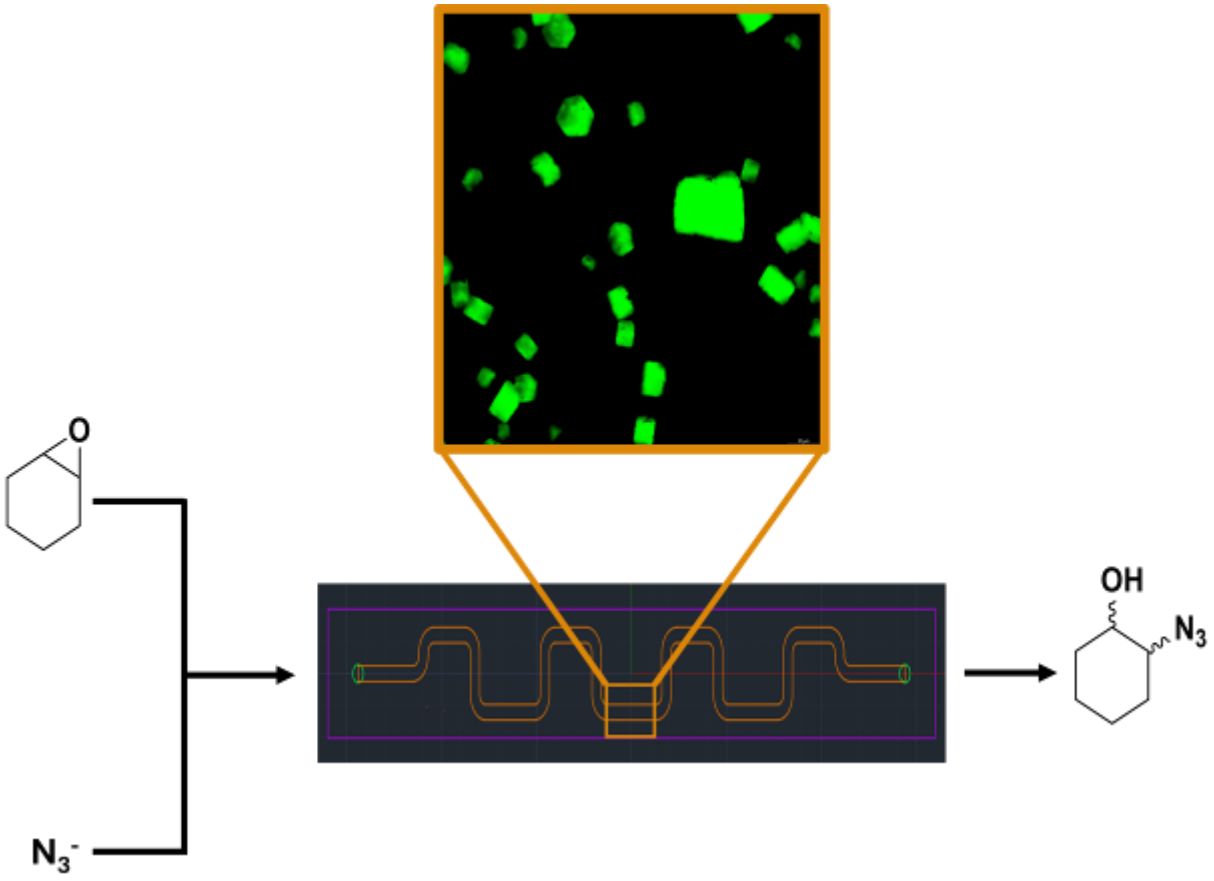
[2] Calderini *et al*., ChemCatChem, **2019**, 11, 2099-2106

[3] Staar *et al*., ChemCatChem, **2022**, e202200145

[4] Staar *et al*., Catalysts, **2022**, 12, 1553-1570

[5] Staar and Schallmey, Biotechnology and Bioengineering, **2023**, 10,3210-3223

Fig. 1


O
N_3^-
OH
N_3

P5-21

Investigation of an Enzyme Cascade Reaction in a Miniplant for Flavor Synthesis

G. Brauckmann[1], F. von Ziegner[2], T. Waluga[2], P. Bubenheim[1]
[1]TUHH, Technical Biocatalysis, Hamburg, Germany
[2]TUHH, Process Systems Engineering, Hamburg, Germany

Biocatalytic production of flavors and fragrances are of great interest, due to a rising demand of "natural" ingredients" for cosmetics and food, produced by eco-friendly processes. Synthesis of these by enzyme cascades with high product purities is possible [1,2], however product isolation is often challenging, increasing the total process costs. Therefore, process integration in biotechnology has been studied increasingly to develop improved processes [1].

In this project a multi-enzyme cascade in a two-phase system is investigated applying a highly integrated reactive extraction centrifuge to further (see Fig. 1, right part) improve the process and the space-time yield respectively [2]. In the organic phase an immobilized lipase (Novozyme 435) in a fixed bed reactor is applied for the synthesis of cinnamyl cinnamate representing a flavor with GRAS (generally regarded as save) status [4].

Figure 1. Reaction system of the 2-phase enzyme cascade.

Cinnamyl alcohol is obtained as an intermediate from the synthesis of cinnamyl cinnamate via a cofactor-coupled reaction of an alcohol dehydrogenase and a formate dehydrogenase in the aqueous phase. Due to the high cost of these enzymes, immobilisation was applied [3]. However, immobilisation resulted in a significant loss of activity. Therefore, the application of free enzyme in a membrane reactor was investigated as an alternative approach (see fig. 1, left part).

For the membrane reactor, suitable membranes had to be identified and the enzymes were characterised independently of each other in lab experiments regarding their kinetics. Based on the kinetic parameters determined, a model for the prediction of the behaviour of the enzyme cascade in the membrane reactor will be established.

References:

[1] P. Žnidaršič-Plazl, Biocatalytic process intensification via efficient biocatalyst

immobilization, miniaturization, and process integration, Current Opinion in Green and Sustainable Chemistry 32 (2021) 100546. https://doi.org/10.1016/j.cogsc.2021.100546

[2] F. Meyer, J. Johannsen, A. Liese, G. Fieg, P. Bubenheim, T. Waluga, Evaluation

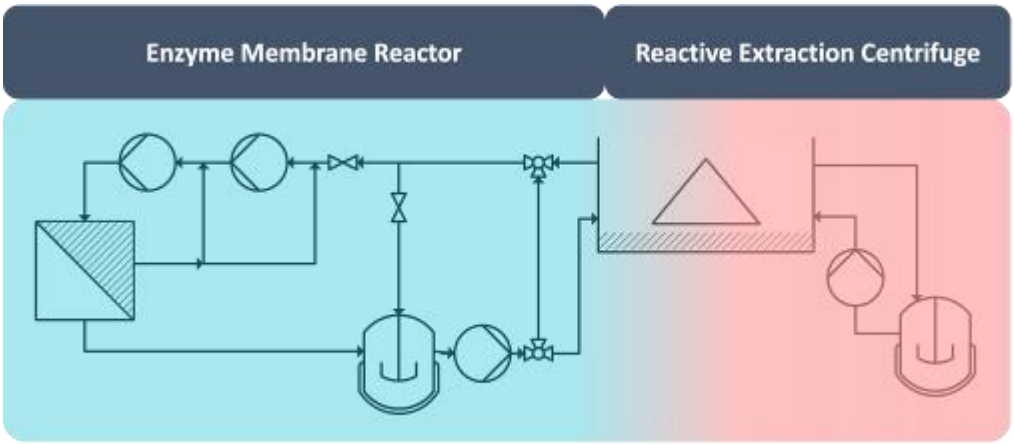
of process integration for the intensification of a biotechnological process, Chemical Engineering and Processing - Process Intensification 167 (2021) 108506. https://doi.org/10.1016/j.cep.2021.108506

[3] Engelmann, C.; Johannsen, J.; Waluga, T.; Fieg, G.; Liese, A.; Bubenheim, P. A

Multi-Enzyme Cascade for the Production of High-Value Aromatic Compounds. *Catalysts* 2020, *10*, 1216. https://doi.org/10.3390/catal10101216

[4] G.A. Burdock, Encyclopedia of food & color additives 1996, 3rd vol., 605

Fig. 1


Enzyme Membrane Reactor
Reactive Extraction Centrifuge

P5-22

Reaction engineering for asymmetric *R*-PAC-synthesis with Ephedrine dehydrogenase in Pickering emulsion

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Pickering emulsions, which are particle-stabilized multiphase systems, offer significant potential for enzyme catalysis in multiphase reaction systems. Recently, the novel enzyme Ephedrine Dehydrogenase (EDH) was discovered for its asymmetric synthesis of a wide range of enantiopure R- configured hydroxyketones. Specifically, it was shown that EDH converts 1-phenyl-1,2-propanedione (PPD) to R-phenylacetylcarbinol (R-PAC), an essential precursor for ephedrine and pseudoephedrine synthesis.

In this study, the production of R-PAC was optimized within a Bioactive Pickering Emulsion system. Ephedrine dehydrogenase was coupled with Formate dehydrogenase for NADH Regeneration, and then encapsulated in a Pickering emulsion. The hydrophilic nature of enzymes, contrasted with the hydrophobic properties of their substrates and products, necessitates their encapsulation within Pickering emulsions. This encapsulation is also crucial to preserve the stability and sustain the activity of enzymes within a biphasic environment. Employing a design of experiments (DOE) approach, we systematically vary parameters such as phase ratio, phosphate buffer and potassium formate concentrations, substrate concentration, and enzyme loading to optimize R-PAC yields. Through Response Surface Methodology, we developed a predictive model for space-time yield and conversion of the emulsion systems. Our findings showcase significant advancements, with optimized R-PAC production achieving a space-time yield of 4.6 g L-1 h-1 and 86% conversion within 55 min. This study underscores the efficacy of employing DOE in optimizing PAC production within Bioactive Pickering Emulsion, offering insights for enhancing enzymatic synthesis in complex systems.

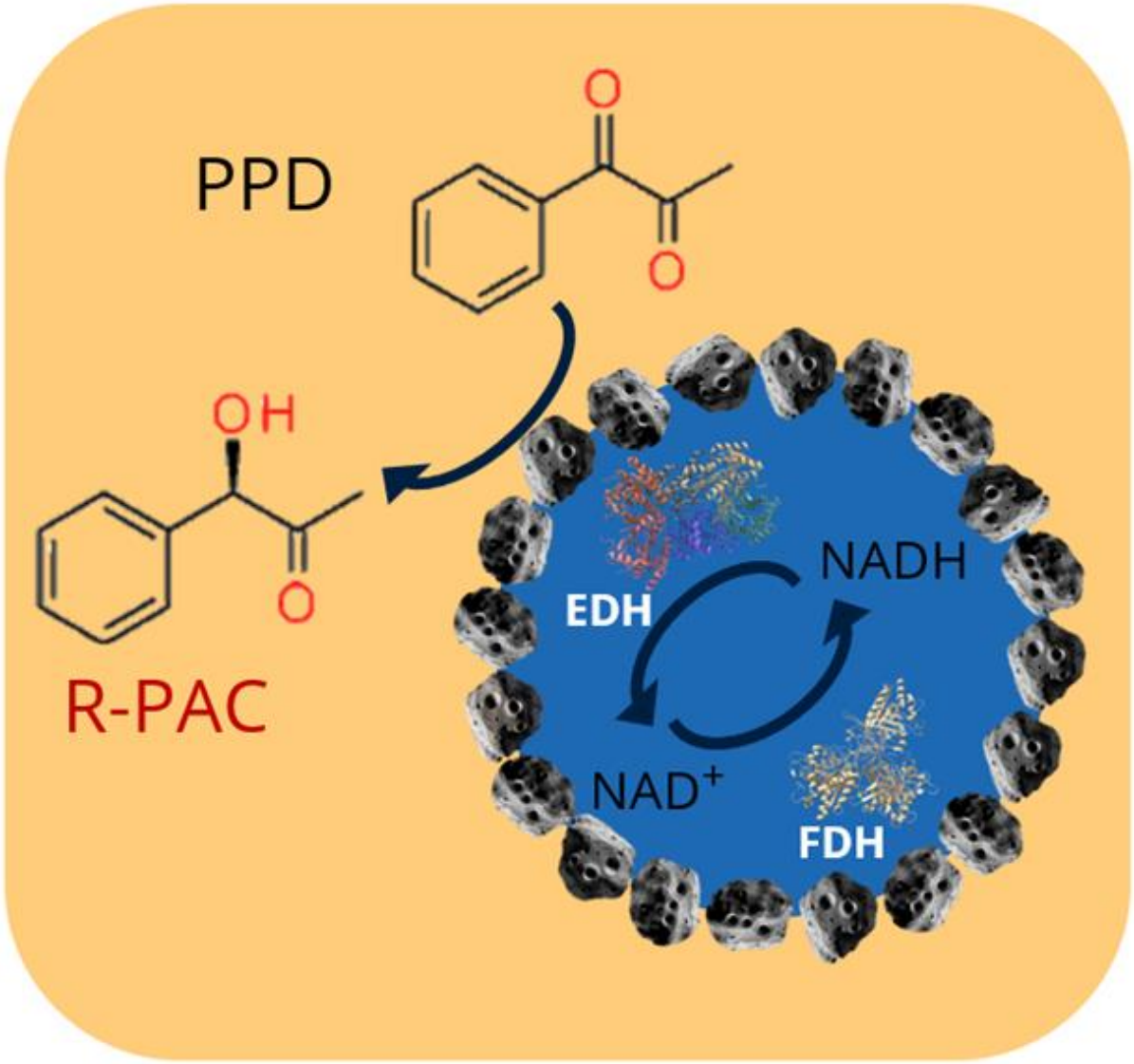
References:

Shanati, T., Lockie, C., Beloti, L., Grogan, G., & Ansorge-Schumacher, M. B. (2019). Two enantiocomplementary ephedrine dehydrogenases from arthrobacter sp. TS-15 with broad substrate specificity. ACS Catalysis, 9(7), 6202–6211. https://doi.org/10.1021/acscatal.9b00621

Ni, L., Yu, C., Wei, Q., Liu, D., & Qiu, J. (2022). Pickering Emulsion Catalysis: Interfacial Chemistry, Catalyst Design, Challenges, and Perspectives. Angewandte Chemie International Edition. https://doi.org/10.1002/anie.202115885

Yuangyai, C., & Nembhard, H. B. (2010). Design of Experiments: A Key to Innovation in Nanotechnology. In Emerging Nanotechnologies for Manufacturing (pp. 207–234). Elsevier Inc. https://doi.org/10.1016/B978-0-8155-1583-8.00008-9

Fig. 1



P5-23

Continuous-flow microreactor-enhanced clean NAD^+ regeneration for biosynthesis of 7-oxo-lithocholic acid

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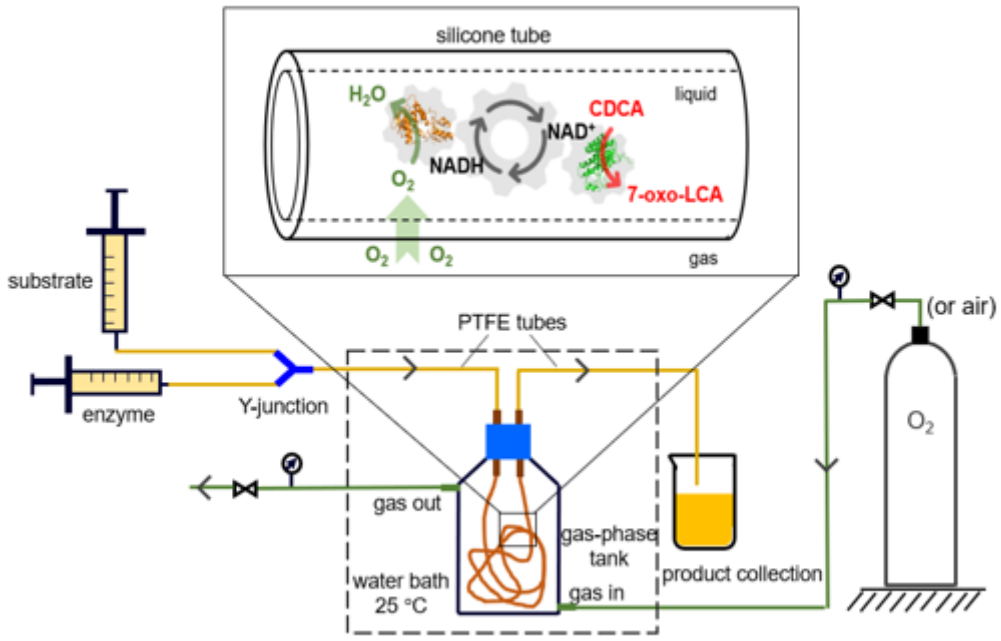
Water-forming NAD(P)H oxidases (NOXs) constitute a clean $NAD(P)^+$ regeneration strategy for $NAD(P)^+$-dependent biotransformations to produce high value-added chemicals because they only consume oxygen and generate water. However, the application of NOXs is still challenging because of limited oxygen transfer in batch reactions. In this work, we design an efficient continuous-flow microreactor (CFMR) to improve the oxygen transfer, which in turn enhances the reaction performance in NAD^+-dependent chenodeoxycholic acid oxidation catalyzed by 7α-hydroxysteroid dehydrogenase (7α-HSDH) for the production of 7-oxo-lithocholic acid, a key precursor of ursodeoxycholic acid. The recycling efficiency of NAD^+ by NOX from *Streptococcus mutans* (*Sm*NOX) was significantly improved in this CFMR. Compared with a conventional batch stirred tank reactor, the space−time yieldof production of 7-oxo-lithocholic acid was increased by 96-fold. Furthermore, the total turnover number of NAD^+ was improved 10-fold, and the enzyme consumption number was decreased 7-fold. Combining the NOX/O_2 system with this microreactor technology provides a general platform that enables various $NAD(P)^+$-dependent biotransformations in a green and sustainable manner, which will be helpful in the design of ecofriendly bioprocesses for production of functional chemicals on a large scale.

Fig. 1



P5-24

Hydrogenase Immobilization in Smart Reactors

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Currently, industrial processes are engineered to manufacture products using raw materials with a consistent quality and composition. However, this design constrains the process to a particular starting material, limiting the use of raw biomass, which exhibits varying compositions based on seasonal and regional factors. To address this limitation, designing a reactor capable of protecting catalysts from contamination, pH and temperature changes is key to using raw biomass as feedstock.

NADH regeneration catalyzed by a soluble hydrogenase (SH) from *Cupravidus necator* H16 (Lenz *et al.*, 2018) using molecular hydrogen (H_2) is used to test the concept of protecting the catalyst via immobilization on smart materials. SH production was performed in *C. necator* and the enzyme was purified using Strep-tag based affinity chromatography. The inherent instability of the enzyme, with a half-life of approximately 5.3 hours at 35 °C and pH 8.0, poses a significant challenge (Herr *et al.*, 2013). The subsequent use of the hydrogenase as cofactor regeneration in an enzymatic cascade requires enzyme protection from glycerol contaminants.

Figure- Microbioreactor prototype. Red area: enzymes are inserted into the reactor. Green area: easy handling.

Drawing inspiration from previous studies, such as Cirillo *et al.*, 2014, which demonstrated improved enzyme stability through immobilization on smart materials, and Goldberg *et al.*, 2007 which showed hydrogenase-coupled NAD(P)H cofactor regeneration, we aim to enhance the stability and control the hydrogenases accessibility in the function of cofactor regeneration. As a first approach, adsorptive enzyme immobilization on poly(*N*-isopropylacrylamide) (PNIPAAm) has been investigated in terms of enzyme load, leaching and activity yield. In this project, we are incorporating the enzyme and smart material into a microreactor as seen in the figure. Additionally, we are developing a spectrometric method to measure NADH levels within the microreactor, enabling real-time monitoring of the enzymatic reaction. This integrated approach facilitates control over reaction conditions, optimizing enzyme performance and process efficiency. By combining enzyme immobilization on smart materials with microreactor technology and spectrometric techniques, we aim to contribute to overcoming the challenges associated with raw biomass.

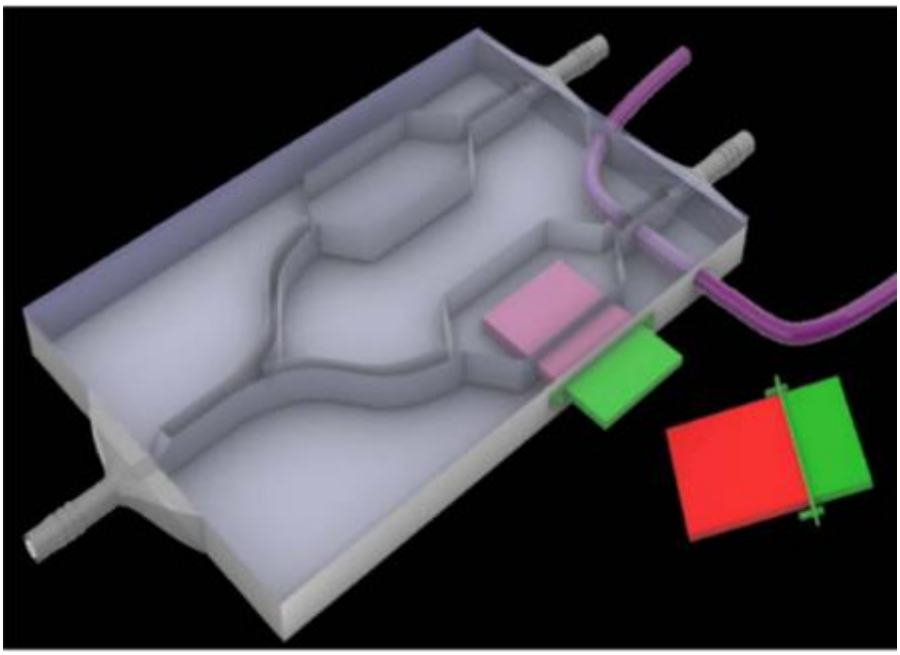
Cirillo, G., Nicoletta, F. P., Curcio, M., Spizzirri, U. G., Picci, N. & Iemma, F. (2014). *Reactive and Functional Polymers*. **83**, 62–69, doi:10.1016/j.reactfunctpolym.2014.07.010.

Goldberg, K., Schroer, K., Lütz, S. & Liese, A. (2007). *Applied microbiology and biotechnology*. **76**, 237–248, doi:10.1007/s00253-007-1002-0.

Herr, N., Ratzka, J., Lauterbach, L., Lenz, O. & Ansorge-Schumacher, M. B. (2013). *Journal of Molecular Catalysis B: Enzymatic*. **97**, 169–174, doi:10.1016/j.molcatb.2013.06.009.

Lenz, O., Lauterbach, L. & Frielingsdorf, S. (2018). *Methods in enzymology*. **613**, 117–151, doi:10.1016/bs.mie.2018.10.008.

Fig. 1



P5-25

Precious-metal free electrocatalysts for CO2 reduction in a combined bio-electrochemical reactor

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For the realization of a sustainable energy economy, it is of great importance to develop CO2 -neutral methods producing multi-carbon organic chemicals used as feedstock as well as carbon-neutral fuels by CO2 capture and conversion. In this work, a bio-electrocatalytical system (BES) consisting of a bioreactor coupled to a CO2 electrolysis cell is established. CO2 is first electrochemically converted to CO in electrolysis cell which is then directly fed to bacteria (acetogens) to further metabolize it to valuable carbon compounds such as acetate. The objective of this study is to develop cost-efficient, biocompatible and high activity electrocatalysts that can selectively convert CO2 to CO.

Porous nitrogen-doped carbons containing atomically dispersed Ni and Co (Ni or Co-N-Cs) are prepared by active-site imprinting approach. First, synthesis of Mg- or Zn-N-C was carried out through pyrolysis of precursors in a salt melt followed by exchange with Ni or Co. N2-sorption porosimetry of the materials reveal a micro-mesoporous structure with high surface areas (> 1000 m2 g-1) and a mass-transport enabling pore system. Extended X-ray absorption fine structure (EXAFS) reveal the existence of single atom sites with no formation of nanoparticles. A variety of Ni-N-Cs and Co-N-Cs were tested for CO2R activity in a rotating disc electrode (RDE) setup, showing high activity (Tafel slopes range from 77 – 130 mV/decade) and selectivity towards CO2R versus the competing HER. Before operation in the BES, first tests were performed in a single cell. Significantly increased current was detected in the CO2R test in CO2-saturated electrolyte vs. in N2 -saturated electrolyte indirectly proving formation of carbon monoxide (CO). Transfer of the electrolysis cell operation into the BES proved to be successful. For biotic operation, the BES was inoculated with *Clostridium ragsdalei*. Partial pressures pCO reached a maximum of 5.7 mbar and pH2 was 2.7 mbar after 30h. The reduction of partial pressures is interpreted as the consumption of the gases by *C.ragsdalei*. We report a specific exponential growth rate of 0.16 h-1, acetate formation rate of 1.8 mg L-1 h-1 and an acetate concetration of 0.103 g L-1 corresponding to acetate formation rate of 0.73 mmol d-1.

In this work we successfully demonstrated an integrated bio-electrocatalytic system (BES) to convert CO2 into value-added chemicals. The usage of a Co- and Ni-N-Cs as the reduction catalyst within the BES allowed production of CO and H2 with relative selectivity for CO resulting in the growth of *Clostridium ragsdalei* and acetate production.

P5-26

Kinetic Intensification of Biocatalytic Oxidation Reactions at High Pressure

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In the era of climate change biocatalysis plays a key role in the development of sustainable and more efficient chemical production processes. Oxidative enzyme catalyzed reactions have gained increasing interest in recent years as a sustainable alternative to chemocatalytic oxidations due to their superiority in terms of regio- and stereoselectivity, side reactions and process conditions. One of the biggest challenges in this field of biocatalysis is the low oxygen solubility in aqueous media, being a limiting factor of the space-time-yield and the catalyst turnover in such reaction systems. Furthermore, there is a complex interdependence between the reaction rate and the mass transfer potential, since a high oxygen solubility would enhance the enzyme activity and at the same time reduce the oxygen transfer rate (OTR) from the gaseous to the liquid phase. The joint optimization of the biocatalyst performance and the process implementation is, thus, compelling.

In our research we investigate the technical applicability of oxygen consuming enzymatic reactions at elevated pressures up to 150 bar. High pressures do not only enhance the gas solubility in the liquid phase, but can also increase the enzymatic activity, stability and selectivity. The solubility of oxygen is enhanced by two orders of magnitude between 1 bar and 150 bar and, therefore, a significant kinetic intensification of the reaction is achievable. For this purpose, a high-pressure laboratory setup is being used that includes an aeration unit for the gas supply and a packed bed bioreactor that are spatially separated from each other. Thereby the enzymes that are placed in the bioreactor are not subjected to the shear stress from bubble bursting in the aeration unit.

The applicability of this concept has been validated by performing the oxidation of glucose to gluconic acid with molecular oxygen at pressures ranging from 1 bar to 150 bar. The reaction is catalyzed by *glucose oxidase*, which is covalently immobilized on epoxy-functionalized carrier particles. The reaction is monitored both *in situ* by oxygen dipping probes as well as offline by HPLC. It could be shown that the enzyme activity is significantly increased with pressure due to the higher oxygen availability. The half-life time of *glucose oxidase* under the tested experimental conditions could also be determined at different pressures. Last but not least, a kinetic analysis at different pressures was performed as well and the results are evaluated and discussed in view of the process intensification potential.

Acknowledgement

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IL6-1

Enzymatic catalysis for the synthesis of biomass-derived materials

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Since its inception in the 1990s, the push to integrate Green Chemistry across the chemical industry has steadily intensified. Sustainability imperatives compel researchers to shift focus from fossil resources towards biomass feedstock. This talk will focus on selected platform molecules which functionality can be exploited using different chemistries therefore enlarging the potential applications of these building blocks. Levoglucosenone (LGO) has emerged as a key platform molecule, synthesized from waste cellulose. Numerous publications now concentrate on enhancing the sustainability of its production. This chiral compound, featuring both a cyclic acetal and an α,β-unsaturated ketone, is increasingly employed as a primary feedstock for the synthesis of solvents, specialty drugs, and polymers [1, 2]. As a second example, itaconic acid received attention since the1950s because of its use as building block for bio-based polymers and additives. This building block is mainly produced via fermentation of the lignocellulosic feedstock as the synthetic route is not efficient enough compared to the fermentative approach. Since a significant amount of IA can be obtained from various sugars and alcohols, the platform chemical was rated as one of the 12 most promising building blocks by the US Department of Energy and can be used to produce functional polymers [3, 4]. The last case will be the use of glycerol, a multifunctional polyol that is obtained from the biodiesel production. This example will showcase the potential of enzymatic catalysis for the selective esterification of the primary hydroxy groups of the molecule while leaving the secondary hydroxy groups available for further post-polymerization functionalization steps [5].

Acknowledgments

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References

[1] Milescu, R. A. et al. 2021, ChemSusChem 14 (16), 3367-3381

[2] Warne, C. M. et al. 2023, Green Chem. Lett. Rev. 16 (1), 2154573

[3] Pellis, A. et al. 2019, Polymer Chemistry 10 (7), 843-851

[4] Schulte, N. et al. *manuscript in preparation*

[5] Raboni, F. et al. 2024, ChemBioChem, 25, e202300839

IL6-2

Enzymatic C1 Gas Conversion for Decarbonization of the Steel Mill Industry

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The steel/iron industry (7% of global CO2 emissions)1 is one of the primary contributors to greenhouse gas emissions in the manufacturing field along with electricity generation and transportation. With the increasing demand for steel and iron predicted to continue, substantial reductions in CO2 emissions are imperative (annually 3 Gt CO2 emission by 2050). A major portion of these emissions originates from the combustion of CO, a byproduct of iron production, releasing 2 tons of CO2 per ton of manufactured steel. Due to CO's toxicity, its current method of disposal through combustion results in considerable CO2 discharge, exacerbating emissions. This reliance on combustion also foregoes the potential to utilize CO as a feedstock for synthesizing alternative fuels and chemicals, thereby missing a critical opportunity to replace fossil resources with more sustainable options. Hence, the capture or transformation of CO into value-added compounds without resorting to combustion emerges as a prospective solution towards industry-wide decarbonization, and by implication, a substantial reduction in CO2 emissions.Here we suggest the enzymatic CO hydration (enCOH), inspired by the biological Wood–Ljungdahl pathway, enabling efficient CO2 fixation. By employing the highly efficient, inhibitor-robust CO dehydrogenase (*Ch*CODH2) and formate dehydrogenase (*Me*FDH1), we achieved spontaneous enCOH, to convert industrial off-gases into formate with 100% selectivity. This process operates seamlessly under mild conditions (room temperature, neutral pH), regardless of varying CO/CO2 ratios. Notably, the direct utilization of flue gas without pretreatment yielded various formate salts, including ammonium formate, at concentrations nearing two molars. Operating the 10-L scale immobilized enzyme reactor feeding live off-gas at the steel mill resulted in the production of high-purity formate powder after facile purification, thus demonstrating the potential for decarbonizing the steel industry.

References

1.Kim, S.M. *et al*. O2-tolerant CO dehydrogenase via tunnel redesign for the removal of CO from industrial flue gas. *Nat. Catal.* **5**, 807–817 (2022).

2. Hwang, H.W. *et al*. Two-stage bioconversion of carbon monoxide to biopolymers via formate as an intermediate. *Chem. Eng. J.* **389**, 124394 (2020).

3. Kim, S.M. *et al*. Identifying a key spot for electron mediator-interaction to tailor CO dehydrogenase"s affinity. *Nat. Commun.* **15**, 2732 (2024).

4. Kim, S.M. *et al*. Tunnel engineering of gas-converting enzymes for inhibitor retardation and substrate acceleration. *Bioresour. Technol.* **394**, 130248 (2024).

5. Kim, S.M. *et a*l. Molar-scale formate production via enzymatic hydration of industrial off-gases, *Nat. Chem. Eng.*, 2024

L6-1

Pet recycling: from enzyme and process optimization to an industrial plant

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Plastics are found everywhere in our daily life due to exceptional properties. The worldwide market reaches 460 million tons. However, they represent a major environmental issue with 125 million tons of generated plastic waste annually. Only 10% of collected plastics are recycled, and, at best, plastic wastes are incinerated but an unacceptable quantity are lost in nature, with 9 million tons ending each year in the oceans.

Carbios (http://www.carbios.com), a young innovative green chemistry company, in collaboration with the laboratory TBI (Toulouse Biotechnology institute; INSA/CNRS/INRAE; http://www.toulouse-biotechnology-institute.fr), developed an enzymatic process to recycle one of the main plastics, PET (~100 million tons per year). A first breakthrough was reached with the optimization of an extraordinary PETase used to break down PET returning to monomers (Nature; Vol. 580 Issue 7802, 9 April 2020). Since then, we continue to optimize this enzyme, to improve kinetics and yields and the performances of our best enzymes will be presented. The scale-up of the process in an industrial demonstrator will be presented with a 20m3 reactor and all the downstream processing to purify both terephthalic acid and ethylene glycol. **The conference will focus on the new results concerning textiles, which account for 70% of the PET market.**

Carbios is building a first industrial unit in France, operational in 2025, which will recycle 50,000 tonnes of PET waste per year and some data will be presented.

L6-2

Enzymatic synthesis and structural modelling of bio-based oligoesters as an approach for the ecodesign of new sustainable bio-based polymers

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The materials and polymers sector is faced with the challenge of integrating the sustainability of both processes and products, including their management after disposal. To minimise the impact of their dispersal in open environments due to specific applications (cosmetics and fishing nets, food packaging, agricultural films), stringent eco-design criteria focusing on biodegradability and ecotoxicity are required. The present integrated study sheds light on the relationship between chemical structure and properties, including marine biodegradability and ecotoxicity. Enzymatic polycondensation was used to prepare a range of polyesters with controlled structures, operating at 50-70°C and under solvent-free conditions. Experimental results show that the aromatic monomers considered (terephthalic acid and 2,5-furandicarboxylic acid) accumulate under the conditions tested (OECD 306), although a slight biodegradation is observed when the inoculum is derived from sites affected by industrial and urban pollution. These biotic catalytic activities are therefore promising for the reduction of plastic pollution.

We computed GRID-derived molecular interaction fields (MIF) to calculate the interactions between the polyester and a chemical probe. The VolSurf computational method then converted the MIFs into quantitative molecular descriptors, which were subjected to PCA analysis to identify clusters in the dataset. In short, stability is related to higher polarity, which is associated with glycine as a monomer, which must be related to the action of an aromatic diacid. Interestingly, the descriptor with the highest impact measures the distance between atoms with the most polar and apolar interactions. The approach presented here is a first example of a rapid method for screening monomers and oligomer structures that meet marine biodegradability criteria. Several bio-based monomers and oligoesters show high biodegradability and low toxicity.

The corresponding bio-based polymers and plastics, which are already produced on an industrial scale, can already be considered as a promising starting point for sustainable polyester-based products. There is room for future research to define clear guidelines for the design of a new generation of chemical and polymer products that do not harm marine ecosystems.

This project has received funding from the European Union"s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 101029444 (RenEcoPol), from ICSC – funded by European Union – NextGenerationEU - PNRR, Missione 4 Componente 2 Investimento 1.4 Grant number CN00000013 and Bruschi R . is grateful to CAFC S.p.A and to NextGenerationEU PNRR (Missione 4, Componente 1, Investimento 3.4 and 4.1).

References:

A. Todea et al., International Journal of Molecular Sciences, 2024, in press.

A. Todea, et al., ChemSusChem, 2022, 15, e202102657.

Fig. 1

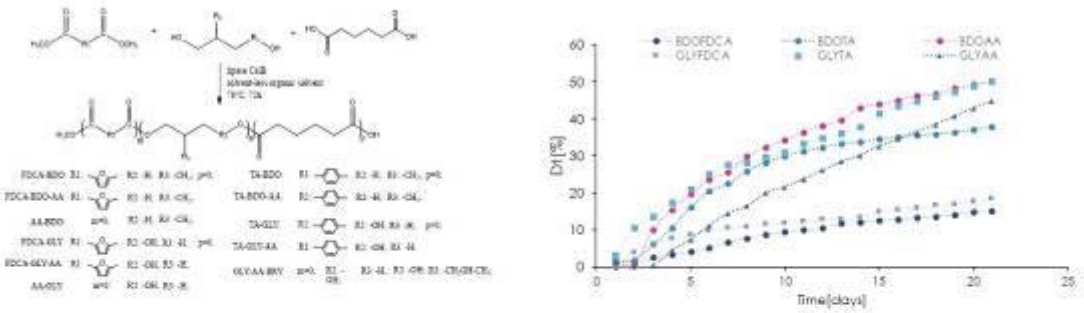

Figure 1. General reaction scheme for the enzymatic synthesis of the considered oligoesters (left) and . Degree of marine biodegradation after 21 days of incubation obtained for the different co-oligomers (right).

L6-3

A hybrid chemical-biological approach can upcycle mixed plastic waste with reduced cost and carbon footprint

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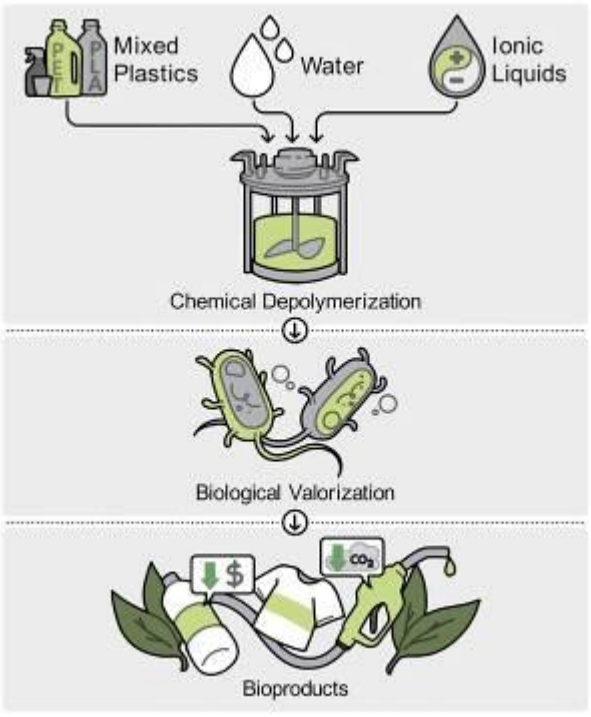
Derived from renewable feedstocks, such as biomass, polylactic acid (PLA) is considered a more environmentally friendly plastic than conventional petroleum-based polyethylene terephthalate (PET). However, PLA must still be recycled, and its growing popularity and mixture with PET plastics at the disposal stage poses a cross-contamination threat in existing recycling facilities and results in low-value and low-quality recycled products. A key challenge in recycling plastics is the commingling of different plastics in the recycling stream. Cross-contamination has significant ramifications, including added burdens to the sorting process, decreased value of the recycled plastics, and compromised properties of recycled polymers. Polyethylene terephthalate (PET) is the most prevalent polyester and ranked as the most recycled plastic in the US. Another polyester, polylactic acid (PLA) is a desirable plastic to consumers because it is bio-based and degradable but still needs to be recycled. With the rapid expansion of the PLA market, there has been an increasing concern that more PLA will be present as contaminants that interfere with the existing PET recycling processes. In particular, the similar appearance, chemicalfunctional groups, and applications of PET and PLA lead to new waste stream separation challenges in plastic recycling facilities, including mechanical recycling of PET. While state-of-the-art sorting technologies (e.g., near-infrared light) can distinguish between polymers such as PLA and PET, some cross-contamination remains unavoidable because of errors in mechanical sortation, especially given the vast volumes of waste processed in modern materials recovery facilities (MRFs). Furthermore, the viability of incorporating a new plastic variant into MRFs is hindered by the expense linked to acquiring dedicated optical sorters and bunkers.Hybrid upcycling has been proposed as a promising sustainable solution for mixed plastic waste, but its techno-economic and life cycle environmental performance remain understudied. Here we propose a hybrid upcycling approach using a biocompatible ionic liquid (IL) to first chemically depolymerize plastics and then convert the depolymerized stream via biological upgrading with no extra separation. We show that over 95% of mixed PET/PLA was depolymerized into the respective monomers, which then served as the sole carbon source for the growth of *Pseudomonas putida*, enabling the conversion of the depolymerized plastics into biodegradable polyhydroxyalkanoates (PHAs). In comparison to conventional commercial PHAs, the estimated optimal production cost and carbon footprint are reduced by 62% and 29%, respectively.

Fig. 1



L6-4

100 years of enzyme immobilization, what's next?

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In 1916 Nelson and Griffin discovered that invertase "exhibited the same activity when absorbed on a solid (charcoal or aluminum hydroxide) at the bottom of the reaction vessel as when uniformly distributed throughout the solution". This discovery was the first of various enzyme immobilization techniques currently available. Besides adsorption, different covalent methods of enzyme immobilization were developed in the 1950s and 1960s.

Up to now thousands of publications and patents have been published on enzyme immobilization techniques. Several hundred enzymes have been immobilized in different forms and approximately a dozen immobilized enzymes, for example penicillin G acylase, lipases, proteases, invertase, etc. have been used as catalysts in various large scale processes.

An increasing range of enzyme carriers with a wide choice of enzymes suiting both batch and continuous processes are commercially available today. Macro- and gigaporous carriers suitable for any enzyme are on the market for large scale usage and guarantee minimized costs. As a result of these developments, the number of industrial applications is still steadily growing and the ongoing progress will ensure continued success in meeting new challenges. Readily available renewable carriers are missing from this range of carriers, however.

To make a 'green process greener' cellulose beads can be used as enzyme carriers to replace non-renewable polluting plastic beads currently used in most industrial biocatalytic processes. Cellulose is a natural biopolymer extracted from wood, a renewable feedstock that can be sourced sustainably. Cellulose is thermally and mechanically stable while at the same time being biodegradable in the presence of microbes (e.g. in wastewater treatment sludge).

In collaboration with Naturbeads and Bath University a new cellulose carrier was developed that can act as a replacement for standard acrylic beads. The cellulose beads have well defined properties such as uniform size, spherical shape and high porosity (>90% pore volume). A series of experiments was run to introduce a variety of functional groups including covalent and ionic binding groups. This functionalization allows for binding of enzymes via different binding modes ensuring high loading, good activity recovery and stable performance.

Various enzymes were immobilized on functionalized cellulose beads and various applications were developed. Equal to even better performance and recyclability compared to the same amount of enzyme immobilized on a standard epoxide acrylic beads was demonstrated. This proofs that an enzyme immobilized on a cellulose bead can be a biodegradable and renewable alternative for plastic beads while at the same time having good or even better economics.

P6-1

Cellulozymes: Cellulose as a renewable carrier for immobilized enzymes

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Enzymes are used in a variety of industries, including pharmaceuticals, cosmetics, biofuel and food applications. The use of enzymes provides an economical solution as often milder conditions and less chemicals and solvents are required, which results in less waste. Immobilization on a suitable carrier increases the recyclability of enzymes and further reduces waste streams because the enzyme can be more easily separated from the product.

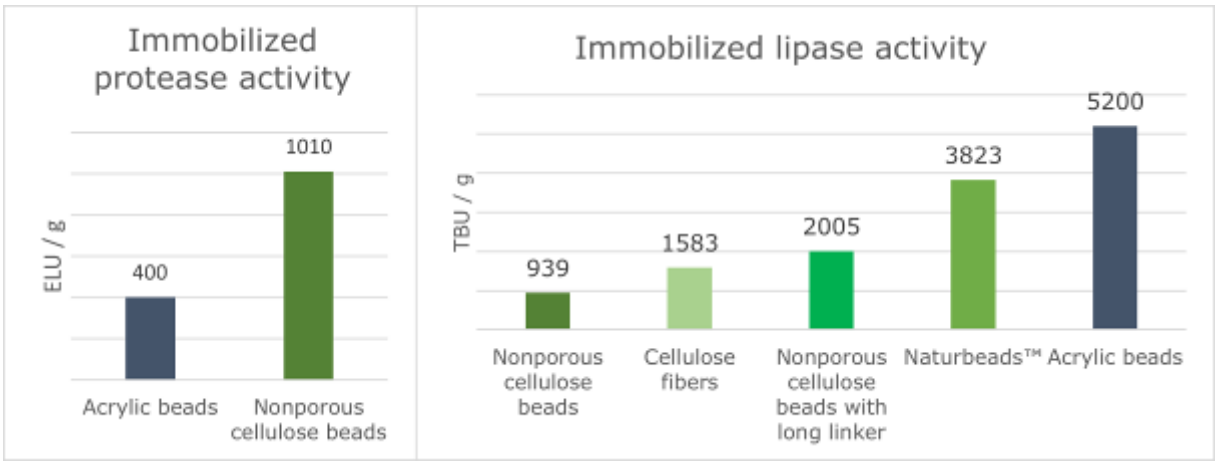
Carriers that are currently used for immobilization are most often based on acrylic resins or polystyrene, both crude oil based plastics that are poorly biodegradable. Ideally another class of carrier is to be developed with similar properties based on a renewable feedstock that can be sourced sustainably. Cellulose could be an excellent candidate as it is cheap, renewable, thermally and mechanically stable while at the same time being biodegradable in presence of microbes (e.g. in wastewater treatment sludge).

Therefore, an investigation into the potential of cellulose as a renewable carrier for enzyme immobilization was started together with Naturbeads, a company which has developed a new type of cellulose bead. These beads have a uniform size, spherical shape and have a very high porosity (>90%). Other than these beads cellulose can be purchased as fibers and nonporous beads. These three types of cellulose were functionalized by introducing a range of binding groups on the surface of the cellulose polymers. These polymers were then used to immobilize enzymes and these were tested in various applications.

In the case of immobilized protease actvity, the best results were obtained with nonporous cellulose beads, which even showed a 2.5 times higher activity than typical acrylic beads. With regards to lipase activity, acrylic beads are still unbeaten thus far, but the giga-porous Naturbeads come very close, being the best of all cellulose beads. The lipase activity of nonporous cellulose beads could be improved by using a long linker. While the investigation is still ongoing, these results do indicate that cellulose carriers could be a viable biodegradable renewable alternative to plastic carriers for enzyme immobilization.

Graph 1: Activities of immobilized protease (left hand side) and immobilized lipase (right hand side) on different carriers. (ELU: hydrolysis of L-ethyl lactate with water to lactic acid. 1 ELU = 1 µmol lactic acid released per minute / g immobilized enzyme at 25 °C, at pH 6.8. TBU: hydrolysis of tributyrin with water to butyric acid and diglycerides. 1 TBU = 1 µmol butyric acid released per minute / g immobilized enzyme at 40 °C, pH 7.5)

Fig. 1



P6-2

Zearalenone (ZEN) degradation using *Aeromicrobium.*

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Zearalenone (ZEN) is a potent estrogenic mycotoxin/metabolite and has a significant negative effect on animal performance. ZEN is produced by several *Fusarium* species that co-infest corn, wheat, barley, and oats. Food and animal feed co-contaminated with ZEN presents a health risk for livestock. Biological degradation is one of many remediation methods for elimination of mycotoxins from animal feed. It has shown promise because it works under mild and environmentally friendly conditions. In this research work, a sample was collected from an environment contaminated with industrial organic residue. The microbe was identified as *Aeromicrobium* genus by NCIMB, UK. This microbe was isolated by an enrichment culture procedure using mineral salts medium and 1 ppm of ZEN as a sole carbon source. *Aeromicrobium sp.* degraded 99,5% of ZEN in a 3-hour period at 30°C, under aerobic conditions. The microbe was able to reduce ZEN at pH levels ranging from 5-9 and temperatures ranging from 30-37°C. It can also degrade 10 ppm of ZEN in 24h. ZEN reduction level was quantified using LC-MS/MS. The final metabolite was identified as hydrolyzed zearalenone, which is at least 50–10,000 times less estrogenic than ZEN. Based on this data *Aeromicrobium sp.* has the potential for use in animal feed to reduce the absorption of ZEN in animals and their carryover from animals to humans.

P6-3

Potato peels as substrate for laccase-catalysed synthesis of phellinsin A

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1. Introduction

The availability of cheap substrates remains a major challenge in enzymatic catalysis. Valorisation of food and agricultural waste can provide a cost-effective substrate source for laccase catalysis. This study evaluated the suitability of potato peels as substrate source for laccase-catalysed modification into value-added product, phellinsin A.

2. Objectives

To investigate the suitability of potato peels as substrate source for laccase-catalysed modification into value-added product, phellinsin A.

3. Materials & methods

Chlorogenic acid (CLA), a caffeic-quinic acid ester, which constitutes 90% of all phenolic compounds in potato peels was extracted from potato peels using extraction parameters optimised by response surface methodology (RSM) using central composite design (CCD). Caffeic acid (CFA), a laccase substrate was then extracted from potato peels by ultrasound assisted alkaline hydrolysis followed by oxidative dimerisation to produce phellinsin A, using the small laccase (SLAC) as catalyst. Comparative analysis of antioxidant capacity and cytotoxicity activity against selected human cell lines was also investigated.

4. Results

The optimal conditions for extracting the highest yield of CLA from potato peels were 300 W (ultrasonication power) and 39.375 min (extraction time). Alkaline hydrolysis of CLA extracted from potato peels produced CFA (yield 76.1%) which was in turn oxidised by laccase to produce a β-β caffeic acid dimer, phellinsin A (yield 32.8%, ~1/3 of CLA). Transformation from CLA to phellinsin A resulted in ~2-fold increase in antioxidant capacity. Phellinsin A exhibited 16.7 and 8.6% cytotoxicity activity against MCF-7 (breast cancer) and HEK-293 (human embryonic kidney) cell lines, respectively.

5. Conclusion

Phellinsin A was successfully produced through a laccase-catalysed oxidation process using caffeic acid obtained from the hydrolysis of potato peels. Based on global potato production statistics, this hydrolytic process can annually recover 1.08e4 tons of CFA from potato peels, subsequently producing 3.53e3 tons of phellinsin A by laccase catalysis. Therefore, potato peel waste is a viable substrate source for laccase-catalysed synthesis of the value-added bioactive compound, phellinsin A. The low cytotoxicity of phellinsin A against normal human cells, coupled with its enhanced antioxidant properties, indicate a good antioxidant ingredient.

P6-4

Enzymatic Upcycling of Textile Wastes for Mycelium Leather Production

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Synthetic fibre such as nylon and polyester form a part of everyday life, with an ever-growing number of textiles produced year on year and global demand expected to increase by 60% from 2015 to 2030, especially considering most are textiles incinerated or landfilled within a few years of manufacture. Unlike their natural fibre alternatives, cotton and wool, synthetic textiles such as polyester (PET) are not readily biodegradable, and therefore contribute to current plastic pollution issues. As such, our work aims to upcycle waste synthetic textiles by enzymatic degradation to produce feedstocks for mycelial leather production, closing the circle in the textile industry. Therefore, we have investigated the metagenomic communities of landfilled textiles with the aim of identifying plastic-degrading enzymes, including PET hydrolases, amidases, oxidases and peroxidases. In parallel we have also isolated organisms via enrichment experiments using nylon and PET, highlighting lytic activity through screens against polyester-polyurethane to isolate and characterise plastic-active enzymes. One isolate from a commercial PET sample underwent further genome sequencing, revealing the presence of a potential PETase, currently being investigated further. With this work, we endeavour to help close the circle in the textile industry through by utilising synthetic textile degradation products to grow and produce mycelium leather.

P6-5

Clearing Oxidoreductases for Take-Off: Biocatalytic Recycling Approaches for Aerospace Epoxy Composites

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Introduction
The aerospace sector relies heavily on epoxy-based carbon fiber reinforced polymer (CFRP) composites for their exceptional strength-to-weight ratio, making aircrafts more fuel efficient and robust[1]. However, the inherent complexity of these materials poses an enormous challenge for recycling and waste management. Current recycling approaches, including mechanical, thermal and chemical methods, are often energy-intensive, environmentally harmful and struggle to effectively separate the carbon fibers from the epoxy matrix[2]. Consequently, there is an urgent need for sustainable and eco-friendly alternatives such as bio-based approaches[3].

Objectives
The present study explores the potential of oxidoreductases such as laccases and peroxidases for the degradation of an aerospace-grade epoxy resin RTM6® by Hexcel. While laccases are lignolytic enzymes that nature employs for the breakdown of lignin, a polymer structurally analogous to the targeted epoxy resin, peroxidases are also known to oxidize recalcitrant structures. By harnessing the unique catalytic capabilities of these oxidoreductases and further coupling them with redox mediatos, the approach aims to enhance the substrate range and accessibility of the catalysts for the complex substrate. The overarching goal is to develop a novel, green and efficient method for recycling CFRP waste from the aerospace industry.

Results
An intitial screening revealed that various fungal laccases as well as horseraddish peroxidase could effectively oxidize the model compound NNBT (N,N-Bis(2-hydroxypropyl)-*p*-toluidine), mimicking aerospace-grade epoxy resins, under mild reaction conditions. Notably, high-redox potential enzymes, such as laccase from *Trametes trogii*, exhibited superior oxidation rates, facilitating the complete conversion of 5 mM NNBT within 1 h. Subsequent GC-MS (gas chromatography-mass spectrometry) analyses identified different degradation products, indicating the potential for backbone scission within the epoxy polymer itself. Incubation of the actual polymeric resin with the enzymatic reaction system in a packed-bed reactor setup revealed a slight oxidation of the resin backbone, indicating the action of the catalyst on the substrate.

Conclusion
The present study demonstrats the promising potential of oxidoreductases for epoxy resin recycling, offering a novel and eco-friendly approach for CFRP waste management. The findings pave the way for further research into enzyme-mediated oxidative degradation methods, contributing to sustainable recycling solutions for the aerospace industry and beyond.

References
[1] Ahrens, A. et al.; *Nature* 2023, 617 (7962), 730–737.
[2] Rahimi, A. et al.; *Nat Rev Chem* 2017, 1 (6), 0046.
[3] Klose, L. et al.; *Polymers* 2023, 15 (12), 2653.

P6-6

Assessing High Pressure Homogenization for Producing Proteolytically Active Spent Yeast Extracts

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Brewers Spent Yeast (BSY) is the second most abundant by-product of the brewing industry, making up about 1.5-2.5 vol-% of the total beer produced [1]. The cells are still viable and considered food-grade when leaving the process. Yet, to date, it is mostly sold as low-cost animal feed or disposed of as landfill [2]. New approaches are investigated to valorize BSY on a large industrial scale. One approach is to extract a proteolytic fraction for use as a food-grade hydrolysis feedstock. This work aims to assess the suitability of using high pressure homogenization as an industrially relevant technology for producing proteolytically active yeast extracts for the food sector.

The model organism chosen for investigation is a bottom-fermenting lager yeast variant of *Saccharomyces pastorianus*. A standardized American Lager recipe was used for all fermentations. Protease activity was determined by a non-specific protease assay suitable to detect exo- and endopeptidase activity as proposed by C. Cupp-Enyard [3].

A commercially available high pressure homogenizer type GEA PandaPLUS was used for all experiments. An inline counter-current heat exchanger operated with water as a cooling medium was used in between each pass to allow for temperature control of the produced extract. Three different homogenization pressures of 400, 600 and 1100 bar with a total of up to ten passes through the homogenization chamber were tested.

Visual analysis via digital imaging software revealed cell disruption efficiencies of 80 – 98 % after max. 10 passes through the homogenizer for all investigated pressure levels. The amino acid content for all homogenate fractions was analyzed and lead to maximum protein extraction yields of 48% by mass in the liquid phase. Residual protease activity in the produced spent yeast extracts was with 2.55 ± 0.02 U/g in the same order of magnitude as a standardized lab-scale glass bead cell disruption method with 2.58 ± 0.26 U/g.

The results highlight the potential to use high pressure homogenization for the production of proteolytically active BSY extracts. This industrially relevant cell disruption method can lead the way to enabling a sustainable processing route for the production of a hydrolysis feedstock for the food sector, potentially reducing operating expenses compared to commercially available enzyme preparations.

Literature

[1] A. Bekatorou, S. Plessas, I. Mantzourani, *Biotechnological exploitation of brewery solid wastes for recovery or production of value-added products*, **2015**, 393-414.

[2] G. V. Marson, R. J. S. de Castro, M.-P. Belleville, M. D. Hubinger, World journal of microbiology & biotechnology **2020**, 36 (7), 95.

[3] C. Cupp-Enyard, *Sigma"s non-specific protease activity assay*, Journal of visualized experiments JoVE, in press, **2008**, 899-901.

P6-7

Modifying the product profile of biocatalytically hydrolyzed PET

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Introduction. Polyethylene terephthalate (PET) is one of the most widely used synthetic polymers for several applications, but it is also highly problematic as it does not decompose naturally and thus tends to accumulate as plastic waste in the environment.[1] Chemical depolymerization techniques of PET have been established that lead to terephthalic acid (TPA) and ethylene glycol (EG), which are then used to re-synthesize PET.[2] Since the discovery of PET-hydrolyzing enzymes, more environmentally friendly biocatalytic approaches are targeted to provide an alternative to these conventional processes.[3]

Objectives. In contrast to the present practice of completely hydrolyzing PET to TPA and EG, this study targets the selective depolymerization to MHET that could be used as an alternative monomer for resynthesis (Fig. 1). Various enzymes and reaction conditions should be studied to produce more MHET than TPA during depolymerization. To avoid enzyme inhibition, MHET should be constantly isolated *in situ*, e.g., by selective crystallization or chromatography.

[Figure 1]

Figure 1: Depolymerization of the model substrate 3(PET). In this study, the selective depolymerization of 3(PET) to MHET or BHET instead of the total hydrolysis to TPA and EG was analyzed.

Results. A total of 13 selected enzymes, which are well described in literature, were investigated in the first screening round for their ability to hydrolyze the model substrate 3(PET). Based on the results, three enzymes were selected that produced either more BHET, MHET or TPA. These enzymes were then analyzed under different reaction conditions and these results were transferred to the more realistic substrate Nano-PET, showing similar results.

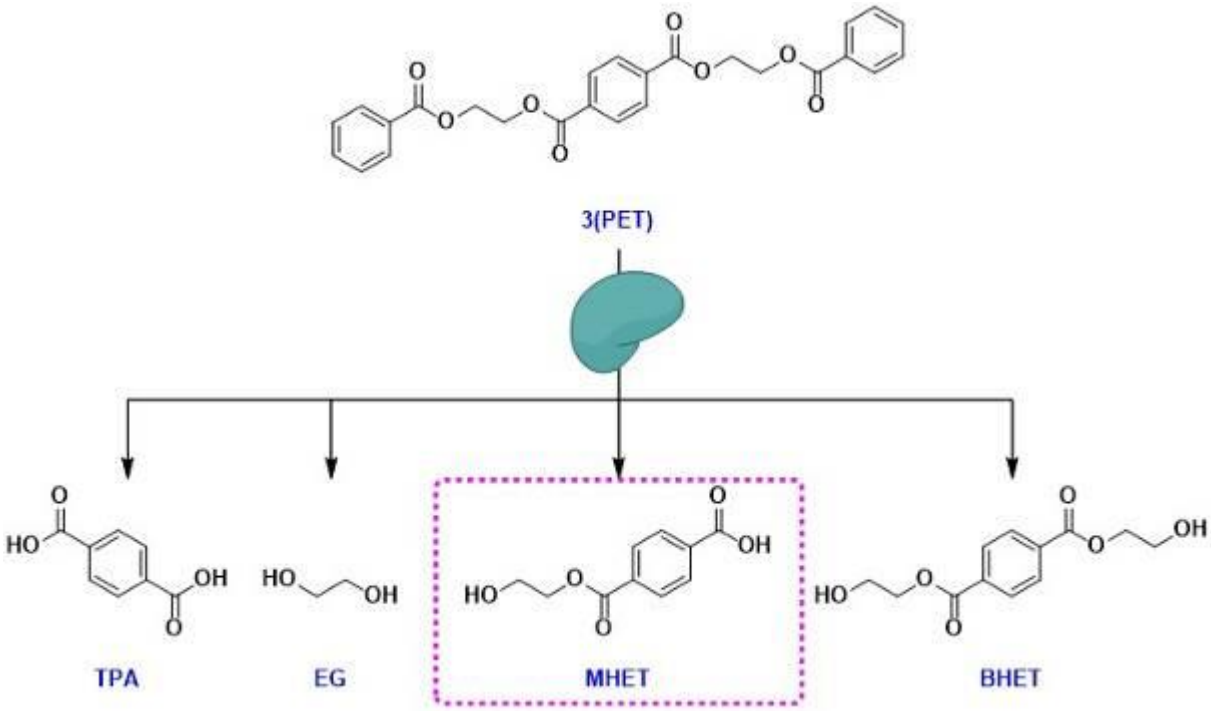
Conclusion. The product profile of biocatalytically hydrolyzed PET is highly dependent on the reaction condition and the enzyme applied, allowing a full control of the desired product.

[1] Tournier V., Duquesne S., Guillamot F., et al. Enzymes" Power for Plastics Degradation. Chem. Rev. 2023; 123 (9), 5612-5701, doi: 10.1021/acs.chemrev.2c00644

[2] Wei, R., Tiso, T., Bertling, J. et al. Possibilities and limitations of biotechnological plastic degradation and recycling. Nat. Catal. 2020; 3, 867–871. doi: https://doi.org/10.1038/s41929-020-00521-w

[3] Arnal G., Anglade J., Gavalda S., et al. Assessment of Four Engineered PET Degrading Enzymes Considering Large-Scale Industrial Applications. ACS Catal. 2023; 13 (20), 13156-13166; doi: 10.1021/acscatal.3c02922

Fig. 1


3(PET)
TPA
EG
MHET
BHET

P6-8

A mycobacterial aminoacylase as a versatile catalyst for the synthesis of *N*-acyl-amino acids

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Biosurfactants with a lipopeptide and acyl-amino acid structure are particularly mild and skin-friendly. However, the chemical synthesis after the Schotten-Baumann reaction is not sustainable and toxic chemicals are required. In order to develop a biocatalytic process for the synthesis of these molecules we cloned the gene of a mycobacterial enzyme that is highly interesting for biocatalysis and 58 % homologous to a streptomycetal enzyme [1, 2]. However, the aminoacylase MsAA was prone to form inclusion bodies (IB). With *E. coli*, soluble protein was only formed by lactose autoinduction. *V. natriegens* could express soluble protein upon IPTG induction. In both organisms, the co-expression of GroEL/S led to an increase in soluble heterologous protein. *E. coli* ArcticExpress (Agilent), which co-expresses a cold-adapted homologue of GroEL/S that is active below 12°C, was best suitable for expression of the enzyme. MsAA was characterized based on hydrolytic properties and synthetic potential. Among long-chain *N*-acyl amino acids especially *N*-lauroyl-L-methionine was synthesized exceptionally well by employing lauric acid and methionine.

References

[1] G. Haeger, J. Wirges, N. Tanzmann, S. Oyen, T. Jolmes, K.-E. Jaeger, U. Schörken, J. Bongaerts, P. Siegert. Chaperone assisted recombinant expression in *Vibrio natriegens* and *Escherichia coli* and characterization of a mycobacterial aminoacylase capable of N-lauroyl-L-amino acid synthesis, *Microb Cell Fact. 2023 Apr 21;22(1):77.*

[2] M.C. Bourkaib, S. Delaunay, X. Framboisier, L. Hôtel, B. Aigle, C. Humeau, Y. Guiavarc'h, I. Chevalot, N-acylation of L-amino acids in aqueous media: Evaluation of the catalytic performances of *Streptomyces ambofaciens* aminoacylases, Enzyme and Microbial Technology 137 (2020) 109536.

P6-9

Functionalised cellulose microspheres as sustainable enzyme carriers

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The use of enzymes as catalysts for chemical transformations continues to accelerate as they are seen as a more effective and sustainable alternative to conventional chemical catalysis. Enzymes are used in various industries, from pharmaceutical manufacturing to biofuels and food production and many others. Despite these successes, more widespread use of enzymes is limited by a lack of long-term stability and challenges in enzyme recovery and re-use. An effective solution to addressing these challenges is the immobilisation or entrapment of enzymes on a carrier or support. However, commercial carriers currently employed for enzyme immobilisation are based on fossil-based and non-degradable plastics, such as polystyrene and PMMA, thereby negating some of the positive features of enzymes over chemical catalysts. With increasing pressure to switch to more environmentally-friendly processes, there is a pressing need in the biocatalysis industry for a sustainable alternative.

Cellulose is a non-toxic, inexpensive, renewable, and biodegradable natural polymer that is available in abundance. *Naturbeads* produces environmentally friendly and biodegradable cellulose microspheres through an innovative and patented process. These cellulose microspheres are highly spherical, uniform in size and possess a high degree of porosity, thus, offering an enhanced surface area resulting in improved enzyme immobilisation efficiencies compared to other cellulose particles (Figure 1).

Figure 1. (A) light microscopy **(B)** SEM micrographs of Naturbeads cellulose microspheres; **(C)** Immobilisation efficiency comparison using a model lipase expressed as a percentage of the starting concentration of enzyme.

To investigate the potential of these microspheres as a sustainable enzyme carrier, *Naturbeads* has started a collaboration with *ChiralVision B.V.*, providing cellulose microspheres of various sizes and porosity produced at pilot scale quantities. The microspheres were subsequently functionalized with a variety of enzyme binding domains to facilitate the immobilization of selected commercially relevant enzymes, showing excellent immobilization efficiencies compared to non-functionalized particles for the tested enzymes (Figure 2A). In terms of enzyme activity, the functionalized microspheres displayed activities that were almost two times higher than the non-functionalized cellulose microspheres using a model lipase enzyme (Figure 2B).

The above results, combined with the particles" spherical shape, which significantly simplifies scale-up of reactors and recovery steps, address outstanding challenges in the biocatalysis industry while at the same time offering a sustainable alternative to the fossil fuel-based supports currently in use.

Figure 2. Bar charts characterising enzyme immobilisation behaviour on functionalised cellulose microspheres produced by Naturbeads: **(A)** Enzyme immobilisation efficiency of different enzymes immobilised on non-functionalised Naturbeads (NB-U) and functionalised Naturbeads (NB-F); **(B)** Enzyme activity comparison of enzyme immobilised on non-functionalised Naturbeads (NB-U) and functionalised Naturbeads (NB-F) using a model lipase enzyme.

Fig. 1

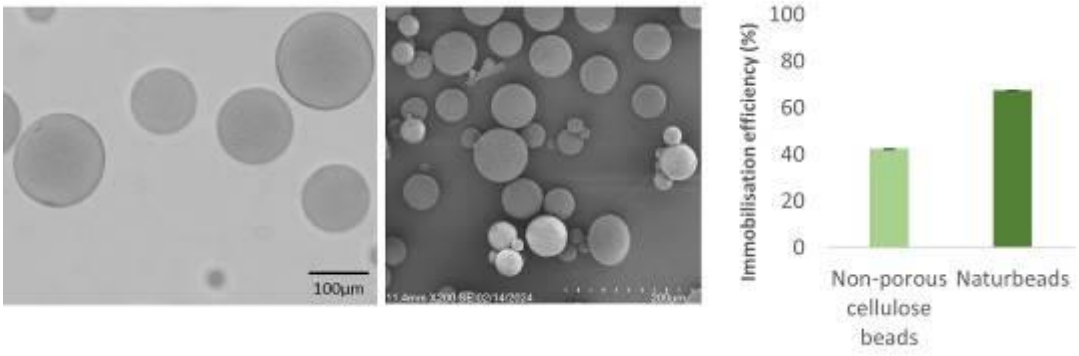
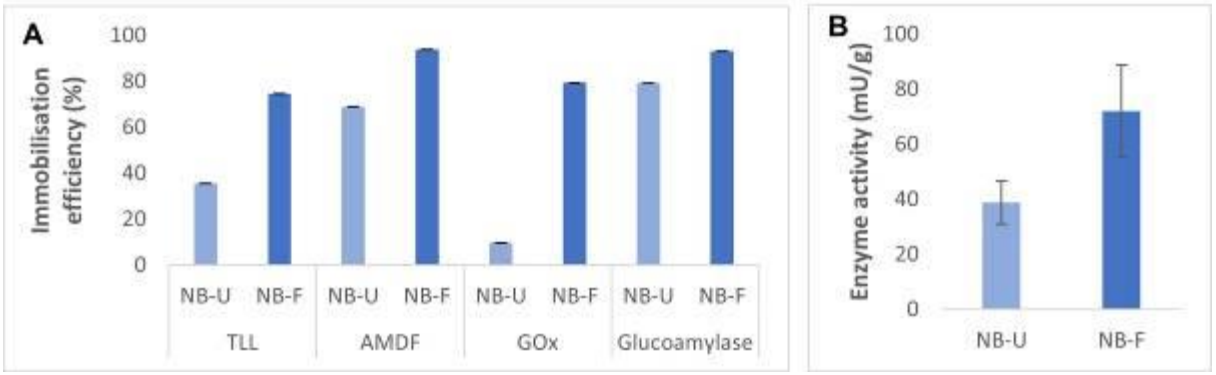


Fig. 2


A
Immobilisation efficiency (%)
100
80
60
40
20
0
NB-U
NB-F
NB-U
NB-F
NB-U
NB-F
NB-U
NB-F
TLL
AMDF
GOx
Glucoamylase
B
Enzyme activity (mU/g)
100
80
60
40
20
0
NB-U
NB-F

P6-10

STEM-Communication Projects Empowering Society to UnderstandResearch on Sustainability and Circular Bioeconomy to Face Climate Change

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What does my life have to do with your research or your company? How can I understand your scientific work to support the decisions needed for biotechnological solutions to face climate change or become a biotechnologist myself? Communicating current Science, Technology, Engineering and Math (STEM) approaches for sustainability to society is detrimental for attaining the public acceptance needed in democratic elections, political decisions and in the minds of all decisionmakers in the global economy. KinderForscher at the TUHH (www.kinderforscher.de) can be a partner in your funding applications and works together with companies to communicate state of the art STEM-research and new career opportunities our circular bioeconomy needs to the public at all ages:

Our free interactive STEM communication platform www.Kniffelix.de reaches out to the public and schools, offering a mix of hands-on experiments with materials that can be obtained in any household or school enriched by a multimedia-educational experience. How does the texture of pizza correlate with biocatalysts in yeast? How are aerogels made and what are their applications? What does the work of bioprocess engineering students look like, when designing new industrial biotechnological processes for future developments to enable a circular bioeconomy? Topics such as "aerogels", "sustainable smoothie production" motivating to become a (bio-)process engineer, as well as "chemical analysis and production methods using HPLC´s" are offered in an edutainment format. No matter what age you are, Kniffelix.de can inspire and empower you to understand what biotechnology and STEM are about!

Innovative company-school cooperations are created by our NachwuchsCampus-team, allowing teenagers to experience industrial applications of biocatalytic and other sustainable processes and interact with employees, to inspire youths to pursue careers needed for our future bioeconomy. Our NachwuchsCampus creates teaching-material to enrich the experience of a company visit for both sides, by enabling visiting teenagers to present the company to its employees and CEOs ("flipped classroom"), giving companies insight to the perspective of the younger generation upon their work. Furthermore, the NachwuchsCampus offers STEM-career information for teenagers on its website www.nachwuchscampus.de and via social media on Instagram @mint_nachwuchscampus.

Hands-on inspiration is possible at all ages with KinderForscher and NachwuchsCampus rental experiment boxes for ages 8-18 years.

Fig. 1

P6-11

Study on recovery of metals from printed circuit board using acidophilic iron-oxidizing bacteria

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In 2019, electrical and electronic equipment waste (e-waste) generated worldwide was 53.6 million tons. Of this, 17.4% was recovered and recycled. Approximately 57 billion dollars worth of precious and rare metals are discarded even though they can be recovered and recycled[1)]. Common methods for recovering metals from printed circuit boards (PCB) include dry and wet smelting methods, but these methods have the problems with air pollution by toxic gases such as dioxin and furans, and the generation of large amounts of acid waste[2)]. This study aimed to recover metals from discarded PCB using the bacterial leaching method with acidophilic iron-oxidizing bacteria.

An iron-oxidizing bacterium isolated from an abandoned mine and having high Fe^{2+} oxidation ability at 25°C, was used in the study. This strain was inoculated into Silverman 9K medium and cultured with shaking (130 rpm, 7 days, 25°C). The culture was centrifuged at 10,500 g, for 10 min, and the precipitates were collected to prepare a 10-fold concentrated bacterium solution, which was used in experiments to leach metals from electronic substrates. Experiments were conducted to compare the elution of metals from PCB in reaction solutions under different conditions. The effects of initial Fe^{2+} concentration, PCB size, and PCB amount on metal leaching were investigated.

The lower initial Fe^{2+} concentration in the solution resulted in the less formation of insoluble iron salts. When large amounts of insoluble iron salts were formed, copper elution from PCB was low, suggesting that insoluble iron salts inhibit copper leaching. In the case of using the smaller size of PCB, the more copper was eluted. This was considered to be due to the larger surface area. Growth and Fe^{2+} oxidation reaction of the bacterium was inhibited in the solution containing large amount of small sized PCB. This inhibition was assumed to be caused by some material in PCB.

References

1) Vanessa Forti *et al*., The Global E-waste Monitor 2020, 2020, 1-120.

2) Y. Xiang *et al*., Journal of Hazardous Materials, 2010, 184 (1-3), 812–818.

P6-12

Immobilization of *Trametes versicolor* laccase for the oxidation of 5-hydroxymethylfurfural to 2,5-furandicarboxylic acid

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Lignocellulosic residues are an interesting alternative to produce furans as 5-hydroxymethylfurfural (HMF), which can be oxidized to produce 2,5-furandicarboxylic acid (FDCA). The complete oxidation of HMF to 2,5-furan dicarboxylic acid (FDCA) is of great interest since FDCA is a precursor for forming polyethylene furanoate (PEF), which can replace petroleum-based PET [1]. There are different alternatives to catalyze this type of reaction, such as applying chemical and biological catalysts, the latter being the most sustainable route [1]. However, a robust biocatalyst is required to conduct the reaction with high activity, reusability, and stability [2]. For this reason, the purpose of this work was the immobilization of the *Trametes versicolor* laccase on the commercial support Immobead 150 P. The immobilization process consisted first of modifying the support with 0.5 M sulfuric acid to hydrolyze the epoxide groups and obtain aldehyde groups. 0.50 g of the modified support was taken and mixed with 5 mL of enzyme solution (20 mg of enzyme) containing 1% glutaraldehyde in 100 mM pH 4.5 citrate phosphate buffer. The activity of the biocatalyst and supernatant was determined by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) at 420 nm. The immobilization yield was 80%, and the activity of the immobilized enzyme was 338 IU/g. In the oxidation reaction of 5-hydroxymethylfurfural to 2,5-furandicarboxylic acid, using 30 mM HMF, 100 mg of biocatalyst, 55 mM TEMPO, 50 mM citrate buffer pH 6 at 30 ºC for 24 hours, 90% conversion was reached with immobilized laccase. These results are like those obtained with the soluble enzyme, making its application in enzymatic bioreactors possible.

P6-13

A combined chemo-enzymatic treatment for the oxidative degradation of epoxy-based CFRPs

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Carbon fiber-reinforced polymers (CFRPs), especially epoxy-based CFRPs, have become indispensable materials in the aerospace, automotive, and construction industries due to their outstanding mechanical properties, high thermal and chemical resistance, durability, and lightweight nature. However, in the case of epoxy-based CFRPs, there is a lack of recycling methods that are environmentally sustainable while also ensuring the recovery of carbon fibers in their original state. Although certain bacterial and fungal strains can colonize epoxy polymers, notable advancements in identifying enzymes capable of degrading these have not been reported. Consequently, there is an urgent need for an efficient, sustainable, and biologically inspired solution for recycling CFRPs. Here, a chemo-enzymatic treatment for the oxidative degradation of epoxy resin has been developed. The recovery of carbon fibers from the epoxy matrix was tested using a combination of various organic acids along with hydrogen peroxide under mild reaction conditions. The findings revealed that the most efficient organic acid candidates for recovering carbon fibers nearly in their native state are acetic and propionic acids. Given their ability to degrade synthetic plastics and natural polymers, and their broad substrate specificity, novel bacterial laccases from the European spruce bark beetle (*Ips typographus*) and horseradish peroxidase were initially screened with three epoxy scaffolds to identify potential enzymes for epoxy polymer degradation. LC-MS analysis revealed that a thermophilic laccase ItL-03, could completely degrade epoxy models within 2 hours. The two-step oxidative method for recycling CFRPs was subsequently validated, demonstrating that the decomposed epoxy matrix converted into a liquid phase was further oxidized by enzymes into smaller compounds. The use of mild reaction conditions renders this process more environmentally friendly and marks the initial stage toward the development of a bio-based recycling strategy for epoxy-based CFRPs.

P6-14

***De Novo* Multienzyme Synthetic Pathways for Lactic Acid Production**

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The utilisation and conversion of one-carbon (C1) feedstocks through biological processes have attracted much interest in building a sustainable carbon cycle economy. However, relevant research on the biosynthetic pathway of lactic acid (LA) from C1 compounds has not been reported. Herein, three de novo multienzyme synthetic pathways for optically pure and racemic LA from methanol were successfully engineered using a modularized construction and optimization approach involving enzyme screening, directed evolution and optimization of reaction conditions. The general synthetic system achieved a productivity of 2.2–2.8 g/L LA with a maximum synthesis rate ranging from 9.6 to 15.6 g/L/day. Both L- and D-LA showed excellent stereoselectivity (>99% *ee*). This work provided an eco-friendly alternative approach for the production of LA, and could contribute to carbon neutrality in the future.

references

[1] Ding, X. W; Rong, J.; Pan, Z. P.; Zhu, X. X.; Zhu, Z. Y.; Chen, Q.; Zhang, Z. J.; Li, C. X.; Zheng, G. W. *Acs. Catal.* 2024, 14: 4665–4674.

Fig. 1

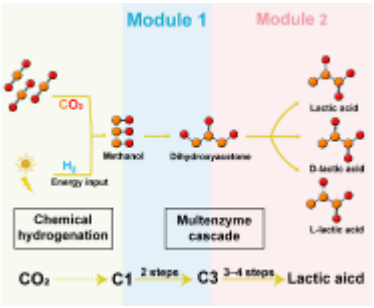
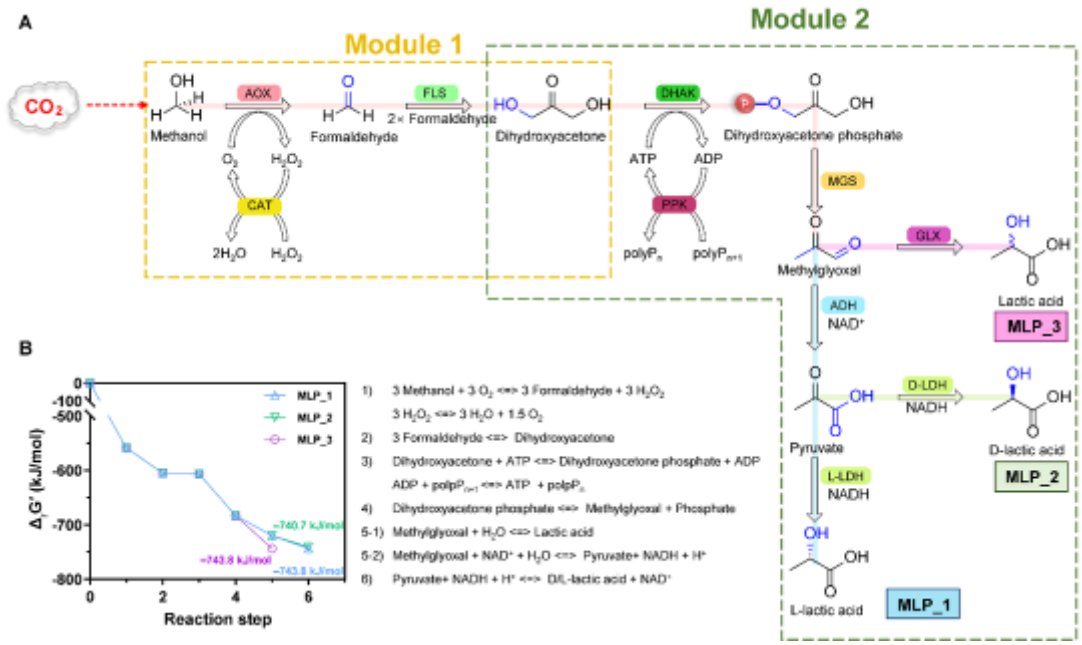


Fig. 2



P6-15

Biodegradable polyacrylates: Assessing the enzymatic and microbial degradation potential for novel polyester-acrylate copolymers

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The environmental pollution with man-made synthetic polymers represents one of the greatest challenges of our time. Over the past decades, plastic wastes have accumulated in nearly all environmental niches [1]. Especially micro- and nano-plastics represent a major problem with hitherto unclear effects on the environment and human health [2]. One emerging idea to handle the accumulation of plastic wastes is the possibility to enzymatically depolymerize them. However, there is still only a limited number of specific enzymes that mostly act on polymers such as PET that do not possess hard to degrade C-C backbones [3]. Therefore, especially already throughout the development process of novel polymers, it is beneficial to implement biological breaking-points in their design and simultaneously assess their biodegradability [1].

In this work, we study the biodegradability of new polyacrylate-based copolymer candidates which are designed to be prone towards enzymatic hydrolysis. As there are no enzymes known to act on these polymer candidates, a functional screening approach is chosen. Here, the identification of microorganisms that are capable to degrade, colonize and / or metabolize the polymers in enrichment cultures is one step towards further sequence-based investigations regarding the phylogenetic diversity and responsible enzymes. Within the framework of microbial decomposition, the evaluation of the principal biodegradability of the candidate polymers is another part of this work. Therefore, the general activity of candidate hydrolases is tested in order to demonstrate the biocatalytic depolymerization of the polymer candidates.

By combining the knowledge gained from functional enzyme-screenings and sequence analysis of enrichment cultures, we further aim to identify enzymes responsible for polymer breakdown within their respective host organisms.

[1] J. Chow, P. Perez-Garcia, R. Dierkes, and W. R. Streit, "Microbial enzymes will offer limited solutions to the global plastic pollution crisis," *Microbial Biotechnology*, vol. 16, no. 2, pp. 195–217, 2023, doi: 10.1111/1751-7915.14135.

[2] S. S. Sana, L. K. Dogiparthi, L. Gangadhar, A. Chakravorty, and N. Abhishek, "Effects of microplastics and nanoplastics on marine environment and human health," *Environ Sci Pollut Res*, vol. 27, no. 36, pp. 44743–44756, Dec. 2020, doi: 10.1007/s11356-020-10573-x.

[3] P. C. F. Buchholz *et al.*, "Plastics degradation by hydrolytic enzymes: The plastics-active enzymes database—PAZy," *Proteins: Structure, Function, and Bioinformatics*, vol. 90, no. 7, pp. 1443–1456, 2022, doi: 10.1002/prot.26325.

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P6-16

Exploring the Diversity of Extreme Habitats for the Degradation of Epoxy Resins

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Man-made polymers pose new challenges to society, as we can see today after decades of use. The extremely inert polymers accumulate in the environment and lead to enormous pollution of important habitats both on land and in water and can also be harmful to health. Epoxy resins have a wide range of applications from household to aircraft, but are among the most polluting polymers. Taking up the circular bioeconomy idea, in our study we tried to achieve a biodegradation of epoxy resins by using classical microbiological and modern metagenomic approaches to produce new products from waste. For this purpose, different samples from extreme habitats in Jordan, Germany, Nagorno-Karabach and Costa Rica were analyzed. The aim was to identify biological systems and enzymes that are active at temperatures above 50 °C and capable of degrading epoxy resins. From these diverse habitats, five promising candidates were isolated. Dataset driven analysis revealed five promising enzyme candidates from *Paenibacillus borealis*, *Paenibacillus* sp., *Paenibacillus lautus* and *Pseudomonas fluorescens* belonging to the class of multicopper (per)oxidases. By means of synthetic gene production and subsequent heterologous expression, the proteins could then be purified and were ready for further activity analysis. Moreover, *Aeribacillus compostii* was identified as another promising candidate. Genome sequencing and annotation showed that 17 oxidase genes are present and further 118 unique genes that need to be subjected to deeper genetic analysis to identify further enzyme candidates to get closer to the goal of epoxy degradation.