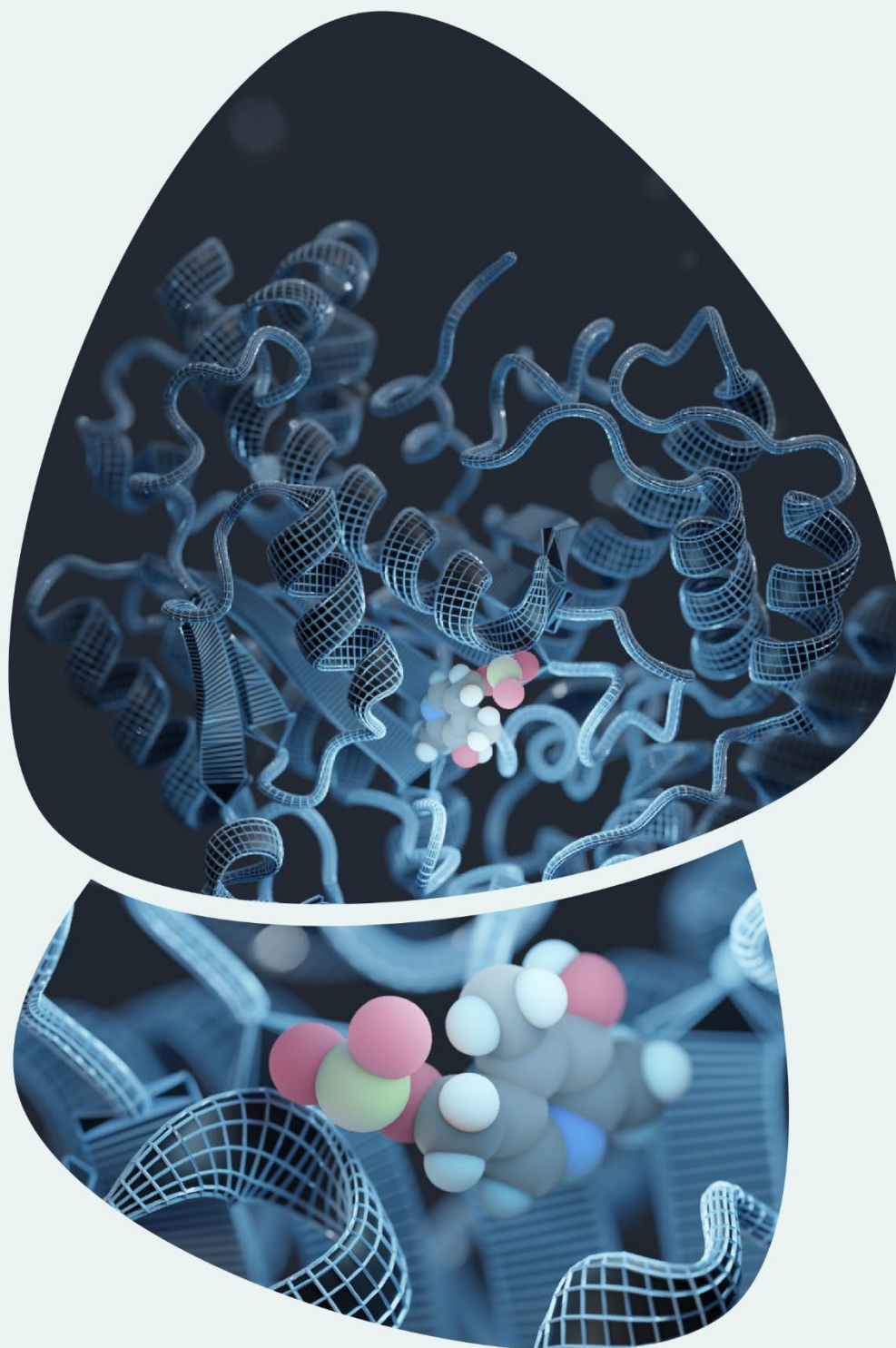




COZYME

BOOK OF ABSTRACTS

Working group meeting | April 24th–25th, 2025 | Athens, Greece

COST ACTION CA21162**Computational Redesign of Enzymes**

Book of abstracts

Working group meeting

April 24th–25th, 2025

Athens, Greece

This publication is based upon work from COST Action COZYME, CA21162, supported by COST (European Cooperation in Science and Technology).

COST (European Cooperation in Science and Technology) is a funding agency for research and innovation networks. Our Actions help connect research initiatives across Europe and enable scientists to grow their ideas by sharing them with their peers. This boosts their research, career and innovation.

Foreword

We are thrilled to extend a heartfelt welcome to you as we gather in Athens, Greece, from April 24th to 25th, 2025, for the Management Committee and Working Group Meeting of Cost Action CA21162 – COZYME.

This gathering represents a pivotal moment for our network, a chance to solidify our collaborative spirit and propel the field of computational enzyme redesign forward. We've designed these two days to facilitate dynamic exchanges, spark new ideas, and lay the groundwork for cutting-edge advancements.

We are certain that the diverse expertise and passion brought by each of you will create an environment for innovation and impactful partnerships. We anticipate a productive and stimulating meeting, where we can collectively shape the future of COZYME.

Within the following pages, you will find a research and collaborative planning undertaken by the network's participants. The content reflects the ongoing efforts to advance the field of computational enzyme redesign through shared knowledge and coordinated action.

This compilation serves as a reference for the activities and progress of the COZYME network, providing a snapshot of the current state of collaborative research within this domain.

We eagerly await your arrival in Athens and are excited for the insightful discussions and collaborations that lie ahead.

The Organizing Committee

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About COZYME COST Action

Enzymes, the catalysts of life's essential chemical reactions, hold immense industrial promise due to their remarkable efficiency and environmental compatibility. Their applications span diverse sectors, from pharmaceuticals and food processing to everyday products like laundry detergents. However, the full potential of enzymes in industrial settings remains largely untapped. A primary obstacle is their sensitivity to the harsh conditions often encountered in industrial processes. To overcome this, enzymes require optimization for stability and activity, a task traditionally reliant on directed evolution. This experimental approach is time-consuming and resource-intensive, involving the screening of vast mutant libraries.

Fortunately, the emergence of computational enzyme engineering offers a transformative solution. By leveraging computer simulations to design and evaluate virtual mutant libraries, or predict beneficial mutations, these tools significantly accelerate and enhance the efficiency of protein redesign. The COZYME (COmputationally assisted design of enZYMES) Action establishes a pan-European collaborative network dedicated to advancing and implementing cutting-edge computational strategies for rapid enzyme improvement. This initiative directly addresses a critical bottleneck in biotechnology: the effective utilization of industrially valuable enzymes.

Specifically, the Action focuses on four topics:

1. Improvement of generic enzyme properties such as stability and solubility;
2. Optimization of catalytic properties e.g. activity and stereoselectivity;
3. Advancement of experimental approaches to generate and evaluate computational predictions;
4. Train young researchers in developing and utilizing computational tools.

Prof. Marco Fraaije
Chair
University of Groningen, The Netherlands

Prof. Andrea Mattevi
Vice-Chair
University of Pavia, Italy

Agenda MC and WG meeting

April 24-25, Athens, Greece

Time	Thur / 24.4	Fri / 25.4
8.30-9.00	Registration	
9.00-9.30		
9.30-10.00	Welcome by the Action Chair and LO	WG2
10.00-10.30	WG1	WG3
10.30-11.00		
11.00-11.30	Coffee break	Coffee break
11.30-12.00	WG1	WG3
12.00-12.30		
12.30-14.00	Lunch	Lunch
14.00-14.30	WG2	STSM session (research done during STSMs)
14.30-15.00		
15.00-15.30		
15.30-16.00	MC meeting	Poster Session
16.00-16.30		
16.30-17.00	Closure (Action Vice Chair and LO)	
	Departure	
17.00-17.30	Meet up for sight-seeing (17.30h)	

Overview of invited lectures

Speaker	Title
WG1 Session	Chair: Dr. Sérgio Marques (WG1 leader)
Dr. Sandra Pucciarelli	<i>Molecular evolution and edaptation strategies in marine ciliates: Insights for enzyme engineering</i>
Dr. Pedro A. Sánchez Murcia	<i>Biasing the mutational landscape in protein engineering using chemical dynamics</i>
Dr. Havva Esra Tütüncü	<i>Computational characterization of unreviewed proteins: A bioinformatics approach for enzyme discovery</i>
Dr. Laura Tiessler	<i>Enhancing enzymes with a state-of-the-art computational pipeline: Targeting distal mutations for improved stability</i>
Dr. Maria Laura Mascotti	<i>Unlocking enzyme mechanisms: Insights from evolution</i>
WG2 Session	Chair: Prof. Aleksandra Maršavelski (WG2 leader)
Dr. Marc Van der Kamp	<i>Efficient multiscale simulations to aid enzyme engineering: methods, tools, and applications</i>
Prof. Nihan Çelebi	<i>Computational identification of promiscuous enzymes for C-C coupling reactions and exploration of their catalytic origins</i>
Dr. Francesco Mutti	<i>Structure-guided engineering of dehydrogenases for the synthesis of chiral amines and amino acids</i>
Dr. Mehdi Davari	<i>Machine learning in enzyme engineering and mining: Lessons learned along the way</i>
Prof. Ioannis Pavlidis	<i>Identification of novel CAZymes for seaweed biorefineries</i>

Speaker	Title
WG3 Session	Chair: Prof. Jürgen Pleiss and Prof. Lígia O. Martins (WG3 leader)
Prof. Anamaria Todea	<i>R-NADES as green solvents for biocatalysis: improving lipases stability and selectivity</i>
Prof. Andrea Mattevi	<i>In vitro construction of the COQ metabolon unveils the molecular determinants of coenzyme Q biosynthesis</i>
Dr. Efstratios Nikolaivits	<i>Protein engineering of a fungal feruloyl esterase enhances both plastic and lignocellulose breakdown</i>
Dr. Yasmina Mekmouche	<i>Tuning heterogeneous chemoenzymatic hybrids conformation toward optimized aerobic oxidation</i>
Dr. Alaric Prins	<i>Outcomes and insights – implementing a Python-based tool, EnzymeML, into a kinetic parameter estimation workflow</i>
STSM Session	Chairs: Dr. Nataša Božić and Prof. Anamaria Todea
Višnja Kosić	<i>Computational engineering of pullulanase from <i>Bacillus paralicheniformis</i> 9945a thermostability and solubility</i>
Hanfeng Cai	<i>Allosteric regulation of L-Lactate dehydrogenase: Insights from molecular dynamic simulation and functional studies</i>
Dr. Io Antonopoulou	<i>Discovery, characterization and improvement of thermostable CAs for application in post combustion CO₂ capture</i>
Pavel Kohout	<i>Investigating machine learning for scalable molecular dynamics and evolution-aware protein design</i>
Konstantinos Grigorakis	<i>Exploring the pH-dependent activity of hemicellulase TtXyn30A</i>
André Taborda	<i>Stabilization of PsG3Ox through a combination of in silico design and random recombination</i>

Overview of poster and software presentations

Presenter		Title
WG1		
01	Laura Tiessler Sala	Enhancing enzymes with a state-of-the-art computational pipeline: targeting distal mutations for improved stability
02	Artur Góra	Discovery and rational design of hydrolases for polyurethane degradation
03	Dajana Vukic	Engineering of β -Galactosidase to Enhance Thermal Stability and Catalytic Efficiency for Industrial Applications and Biosensors
04	Fabrizio Pucci	Predicting enzyme fitness by combining structural and (co)evolutionary methods
05	Nurcan Vardar Yel	Machine learning strategies for identifying cold-adapted α/β hydrolase enzymes
06	Yusuf Sürmeli	The discovery and in silico characterization of Anatolian water buffalo rumen-derived bacterial thermostable xylanases: A sequence-based metagenomic approach
WG2		
07	Aleksandra Maršavelski	Engineering thermostable PLA-degrading enzymes
08	Gizem Tatar Yılmaz	Exploring Tyrosinase Inhibition: Discovery of Potential Inhibitor Candidates for Preventing Hyperpigmentation in the Cosmetic, Pharmaceutical, and Food Industries Using Molecular Modeling Techniques
09	Vladimir Vukic	Rational Design for Enhanced Substrate Affinity of Laccase from <i>Streptomyces cyaneus</i>
10	Ioana-Cristina Benea	A computational and statistical approach in optimizing the enzymatic synthesis of aromatic oligoesteramides derived from ϵ -caprolactam and 5-hydroxyfurancarboxylic acid
11	Yunus Ensari	Identification and Engineering of Novel Enzymes for Industrial Applications
WG3		
12	Amit Pandey	Computational and functional analysis of natural and designed variants in human P450 oxidoreductase
13	Anastasia Zerva	Computational and biochemical study of a new xyloglucanase from the white-rot fungus <i>Abortiporus biennis</i> .
14	Carla García Sanz	Zinc-based Nanobiohybrids as Artificial Metalloenzymes for Efficient Photocatalytic CO ₂ Conversion to Bicarbonate

15	Christina Ferousi	Harnessing synthetic microbial biotechnology for efficient plastic waste upcycling
16	Christos Kosinas	Structural study of an engineered leaf branch compost cutinase (LCC) with PET oligomers
17	Clara Ortega	Metal Nanoparticles-Enzyme Complexes as Multi-Functional Heterogeneous Catalysts
18	Dimitra Zarafeta	Harnessing Extremozymes: A Metagenomic Approach to Sustainable Catalysis
19	Gianina Marian (Toca)	Enzymatic approaches to assess citric acid levels in wine
20	Hasmik Grigoryan	Engineering the substrate specificities of D-hydantoinase
21	Marina Lotti	Glycosyl hydrolases from extremophilic organisms
22	Martin Gand	Discovery and Characterization of Novel O-Methyltransferases from <i>Lentinula edodes</i> for the Sustainable Production of Taste-Active Flavonoids and (Iso-)Vanilloids
23	Max Häussler	EnzymeML Tools: making your biocatalytic data AI-ready
24	Michel Kranendonk	Bacterial cell model for functional evaluation of cytochrome P450 oxidoreductase variants
25	Milica Svetozarević Arsović	Peroxidase extraction from waste and its use for dye biodegradation: application in batch and continuous flow
26	Panagiota Karampa	Structural studies and molecular dynamics simulations of a ferulic acid esterase variant active on PET oligomers
27	Serpil Bayraktar	β -glucocerebrosidase enzyme production: an enzyme replacement therapy using <i>Pichia Pastoris</i> Glycoswitch [®] expression technology
28	Zehra Evla İpekli	Computational identification of promiscuous biocatalysts
29	Zvezdana Findrik Blazevic	The role of reaction engineering in understanding of enzyme catalysed reactions

Software presentation

01	Max Häussler	EnzymeML Tools: making your biocatalytic data AI-ready
02	Sérgio M. Marques	Caver Web 2.0: Analysis of Tunnels and Ligand Transport in Dynamic Ensembles of Proteins

Invited lecture: WG1

Molecular Evolution and Adaptation Strategies in Marine Ciliates: Insights for Enzyme Engineering

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Psychrophiles are organisms that have adapted to thrive in cold environments, overcoming the challenges posed by freezing temperatures. These organisms employ various cold-adaptive strategies, including unique amino acid substitutions that enhance enzyme reaction rates in cold conditions. Enzymes from psychrophiles typically exhibit high activity and reaction rates at low temperatures compared to those from mesophiles and thermophiles. This heightened activity is often due to increased structural flexibility, which can also make these enzymes more susceptible to denaturation with temperature fluctuations.

The molecular thermolability of psychrophilic enzymes has been a significant obstacle to their use in industrial applications. However, advancements in genomic technologies have provided valuable insights into the molecular cold adaptation mechanisms of microbial communities in extreme environments like the Antarctic ecosystem. For instance, genome mining of the psychrophilic Antarctic marine ciliate *Euplotes focardii* and its mesophilic counterpart, *E. crassus*, has revealed unique adaptation strategies specific to the Antarctic ciliate. These closely related eukaryotic microorganisms, adapted to different environments, serve as an ideal system for studying the molecular mechanisms for cold adaptation.

Case studies have demonstrated the role of specific amino acid substitutions in cold adaptation through site-directed mutagenesis. By combining these substitutions with a rational design approach, it is possible to engineer cold-active enzymes that are structurally stable. The differences and mutation sites identified are based on structural and consensus analyses of cold-adapted molecules and their mesophilic counterparts. This knowledge has broad applications, particularly in the rational design of enzymes for industrial use.

The in silico comparison of numerous homologous sequences of hydrolytic enzymes from the two *Euplotes* species, coupled with the estimation of likelihood log odds scores, revealed an increased frequency of small polar amino acids and a decreased frequency of proline residues (Figure 1). These features provide higher molecular flexibility. Unexpectedly, we also observed an increase in the amino acid isoleucine (Ile) in the β strands. The Ile side chain is highly non-reactive and is rarely directly involved in protein function, though it can play a structural role by influencing protein conformation.

Rational enzyme design requires a deep understanding of molecular evolution to identify mutations that enhance desired properties, such as thermostability and catalytic efficiency. Unlike traditional site-directed mutagenesis, which often relies solely on structural analysis and can negatively impact kinetic parameters, the structure-guided consensus approach leverages nature's optimization of protein sequences. This makes adapted proteins more suitable for industrial applications through protein engineering strategies.

Amino acid replacements in *Euplotes focardii* lipase family

likelihood log odd scores (LOS)

$$LOSE_{focardii} = \log \frac{F(X_{E.focardii} \sim Y_{E.cassius})}{F(X_{E.focardii} \sim Y_{E.focardii})}$$

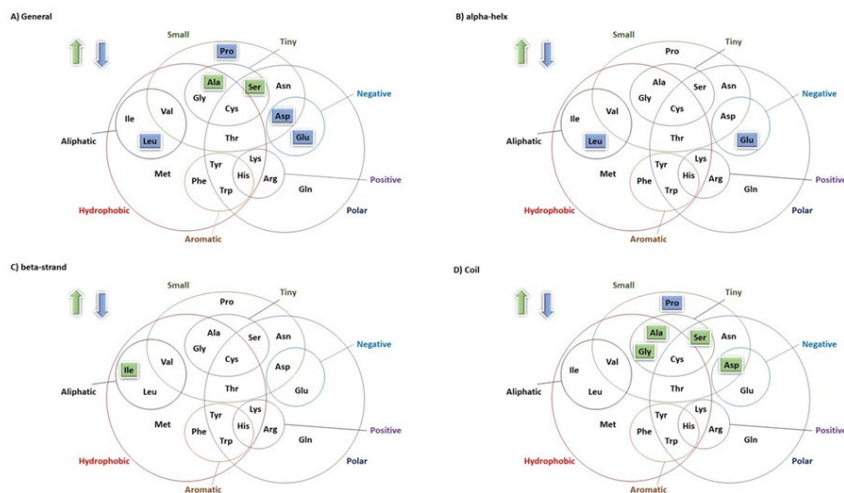


Figure 1. Strategies for molecular cold adaptation in the Antarctic ciliate *Euplotes focardii* lipase family: Estimating amino acid replacements using likelihood log odds scores (LOS).

Acknowledgements: This research was funded by the Italian "Programma Nazionale di Ricerche in Antartide" (PNRA) and by the European Union through the MSCA-RISE and FSE programs. Collaborators: Guang Yang, Matteo Mozzicafreddo, Alberto Vassallo, Angela Piersanti and Cristina Miceli.

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Invited lecture: WG1

Biasing the mutational landscape in protein engineering using chemical dynamics

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The revolution in predictive structural biology has made it possible to generate educated guesses about the structure of any protein or enzyme within a significantly reduced time frame. Even more remarkably, novel protein scaffolds can now be designed in ways never before possible. It is an exciting time to work in structure-based protein engineering. Thus, routines such as improving protein stability by predicting which structural positions to modify have become commonplace. More challenging still is the manipulation of substrate scope, stereospecificity, or catalytic proficiency within a given enzyme scaffold.

In this presentation, I will discuss our recent work utilizing conformational ensembles to explore the impact of remote mutations on enzyme function, including stability, connectivity to the active site, and overall enzyme dynamics.^{1,2} Additionally, I will introduce SmarTSzyme,³ a python-based software tool that analyses quantum mechanics/molecular mechanics (QM/MM) trajectories of the reaction at the active site, to compare key interactions in both substrate and transition-state enzyme complexes. As an outcome, SmarTSzyme delivers a shortlist of positions for protein engineering, aiming to enhance catalytic efficiency.

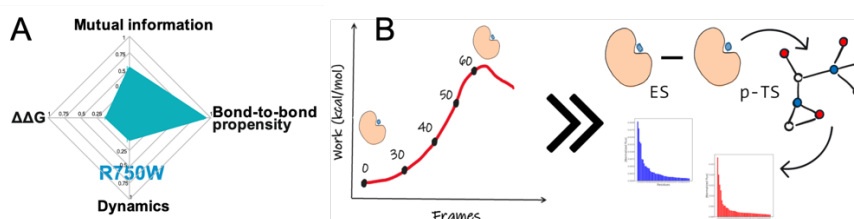


Figure 1. (A) Metrics to evaluate the impact of remote mutations in enzyme function. (B) SmarTSzyme, a new tool to the selection of key amino acids to manipulate catalysis.

Acknowledgements: CETA – CIEMAT (Spain), EU Horizon 2020 (MCSA Agreement No. 847548), FWF doc.fund BioMolStruct program (Austria), Fundación Martínez Escudero (Spain).

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2. B. Di Geronimo, S. Alonso-Gil, B. Zagrovic, G. Reibnegger, P.A. Sánchez-Murcia Digging out the molecular connections between the active site of human lysosomal alpha-mannosidase and its pathophysiology *J. Chem. Inf. Model.* **2025**, ASAP article <https://doi.org/10.1021/acs.jcim.4c02229>
3. SmarTSzyme: <https://github.com/CAMDgraz/SmarTSzyme> (accessed March 1, 2025)

Invited lecture: WG1

Computational Characterization of Unreviewed Proteins: A Bioinformatics Approach for Enzyme Discovery

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Advancements in DNA sequencing have led to a rapid expansion of biological data, making it increasingly difficult to analyse all available sequences experimentally. However, unreviewed protein sequences in databases such as UniProtKB/TrEMBL represent a vast reservoir of unexplored functional potential. Computational tools now allow for the prediction of protein structure and function based on sequence data, providing a powerful alternative to traditional experimental approaches. In this presentation, I will outline the workflow we employ to analyse unreviewed enzyme sequences. Starting with the basic biophysical and biochemical features—such as isoelectric point (pI), predicted melting temperature (T_m), aliphatic index, and instability index—we perform phylogenetic analysis using a top-down approach. From this, we select the most promising candidates for further examination, construct 3D models, perform structural analysis and molecular dynamics simulations. This methodology has been successfully applied to a variety of industrial enzymes, including amylases and lipases, demonstrating the effectiveness of computational techniques in enzyme discovery.^{1,2} Building upon this framework, we have recently launched a project focused on identifying enzymes capable of degrading PET and PUR. The preliminary results of our *in silico* analysis will be presented. This study underscores the significant potential of computational analysis in uncovering novel biocatalysts with important industrial and environmental applications.

Acknowledgements: Part of this study is funded by TÜBİTAK (The Scientific and Technological Research Council of Türkiye) with grant number 124Z423.

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Invited lecture and poster presentation (01): WG1

Enhancing enzymes with a state-of-the-art computational pipeline: targeting distal mutations for improved stability

Laura Tiessler-Sala¹, Lur Alonso-Cotchico¹, Miquel Canyelles-Niño¹, Fabrizio Casilli², Gerard Roelfes², Mario De Simone³, Vânia Brissos³, Lígia O. Martins³, Maria Fatima Lucas¹

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The chemical industry faces the challenge of transitioning to greener and more sustainable processes. Enzymes, with their high specificity, enantioselectivity and broad reaction spectrum, offer a solution to this challenge. However, natural enzymes are often not suited for industrial applications, and may need to be redesigned to tolerate reactor conditions, improve kinetics, or catalyze new reactions. To address this, at Zymvol, we have developed state-of-the-art computational pipelines to identify the most promising enzymes for any target chemical reaction and optimize them for enhanced industrial performance.

Our enzyme engineering pipeline is based on **ZYMEVOLVER**, a multi-approach software that integrates physics- and sequence- based tools to identify the most favorable mutations for a given enzyme property. As part of this technology, **Zymspot** is an algorithm that identifies distal mutations that influence the enzyme dynamics throughout the entire protein sequence. Both technologies have been successfully applied in more than 40 projects, improving activity, selectivity, specificity and thermostability across multiple enzyme families.

To showcase this technology's ability, we present two studies where we optimize enzyme stability by targeting **distal mutations** (>9Å from the active site). In an LmrR-based artificial enzyme, screening of the predicted 73 variants resulted in a recombinant mutant with **14 °C higher thermostability** and 66% higher turnover number.¹ For NOV1 dioxygenase, an enzyme with promising industrial potential for vanillin production, 20 positions were selected using our algorithm, leading to 41 mutated variants. Experimental validation revealed two variants with a 3-fold higher K_{cat} and a **40-fold higher operational stability**.² These findings demonstrate the efficacy of our methods in optimizing both artificial and natural enzymes.

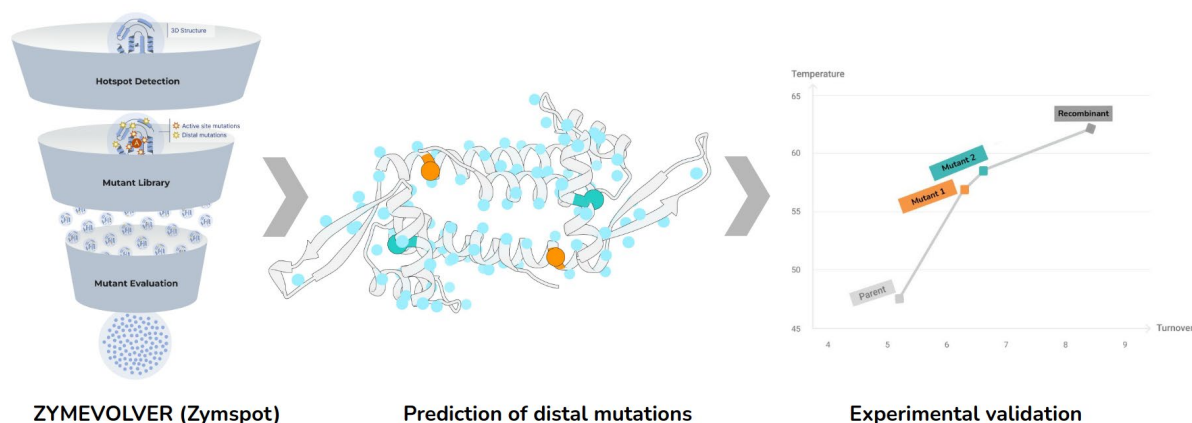


Figure 1: Pipeline based on ZYMEVOLVER for optimization of enzyme stability.

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Invited lecture: WG1

Unlocking Enzyme Mechanisms: Insights from Evolution

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The electron transport chain (ETC) is central to all domains of life, consisting of four canonical protein complexes (I-IV) plus the ATP synthase and auxiliary proteins. In bacteria, NADH:quinone oxidoreductases (NDH-2s) play a crucial role¹. These enzymes catalyze the transfer of electrons from cytoplasmic NADH to the quinone pool in the membrane, similar to Complex I. However, unlike Complex I, NDH-2s do not contribute to the generation of the proton motive force. NDH-2s are absent in animals, making them promising targets for antimicrobial drug design.

In this study, we explored the evolutionary history of bacterial NDH-2s and conducted an ancestral sequence reconstruction campaign to uncover the biochemical features of this enzyme family². Our findings show that substrate specificity and hydride donor identity have been conserved throughout evolution. While much research has focused on reconciling contradictory reports about the catalytic mechanism of NDH-2s³, we found that some NDH-2s follow a ternary mechanism involving the formation of a charge transfer complex, whereas others appear to undergo a ping-pong mechanism. This raises the question: what determines this mechanistic variation? Therefore, by extending our analysis to previously unexplored clades in the NDH-2 phylogeny, we discovered that the formation of the charge transfer complex is primarily restricted to the Firmicutes (Bacillota) phylum. Molecular dynamics (MD) simulations helped rationalize our findings.

Our exploration of NDH-2 evolution has led to the identification of sequence determinants that govern the enzyme's catalytic mechanism, providing insights into the biological causes of this variation. Evolutionary clustering may also serve as a useful proxy for enzyme mechanisms in future ligand/protein design efforts.

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Invited lecture: WG2

Efficient multiscale simulations to aid enzyme engineering: methods, tools and applications

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Enzymes have excellent potential as rapid, selective and efficient biocatalysts for use industry. Obtaining enzyme biocatalysts with both the desired selectivity and activity for non-natural reactions, however, remains a challenge. Atomically-detailed reaction simulations can help evaluate enzyme activity and selectivity, and thus enzyme variant selection. Ideally, simulation protocols require limited computational resource (and thus energy), but maintain sufficient accuracy. Here, the development of tools and methods for '*in silico* enzyme screening' with reaction simulations are discussed, highlighting applications to different enzymes.

We have shown that short QM/MM reaction simulations with semi-empirical QM methods can be used to correctly indicate activity for certain enzymes.¹ When combined with automated protocols to set up simulations, such as *Enlighten2*,² this can result in efficient evaluation of activity *and* selectivity. We demonstrate how this can be used to obtain key insights into the natural Diels-Alderase AbyU,³ a highly stable and promising biocatalyst, as well as other beta-barrel Diels-Alderases, allowing predictions of stereoselectivity.

For highly efficient screening of enzyme activity, such that calculations can be used during enzyme (re)design, further increases in efficiency *and* accuracy are important. Replacing QM by machine-learning (ML) potentials can, in principle, offer QM accuracy at a fraction of the computational cost. However, due to the absence of electrons, ML potentials are generally unable to describe the electrostatic interaction between ML and MM regions, which is crucial for capturing enzyme catalysis. We have developed the "electrostatic ML embedding" (EMLE) scheme that solves this issue, allowing DFT/MM accuracy.⁴ Here, we show that this method can be applied for enzyme reaction simulations to capture key catalytic effects.

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Invited lecture: WG2

Computational Identification of Promiscuous Enzymes for C-C Coupling Reactions and Exploration of Their Catalytic Origins

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Enzymes serve as highly efficient and selective catalysts, enabling essential biochemical reactions at remarkable speeds under mild conditions. Their ability to function sustainably and be produced through environmentally friendly methods has made them attractive catalysts for industrial applications.¹ A key approach in biocatalyst development is leveraging enzyme promiscuity—the capacity to act on multiple substrates (substrate promiscuity) or facilitate different reaction types (catalytic promiscuity) beyond their native function.² Such promiscuous enzymes can be optimized through protein engineering to meet specific industrial needs.³ Identifying these enzymes and understanding their binding sites and catalytic elements is crucial for their successful repurposing. This study presents a computational strategy that integrates advanced modeling techniques to systematically identify promiscuous enzymes capable of catalyzing C-C coupling reactions, which are widely utilized in pharmaceutical synthesis.⁴ Additionally, it aims to uncover the molecular basis of their promiscuous catalytic behavior, providing insights that can guide the rational design of novel biocatalysts.⁵

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Invited lecture: WG2

Structure-guided engineering of dehydrogenases for the synthesis of chiral amines and amino acids

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The synthesis of chiral amines and unnatural amino acids is crucial for the pharmaceutical industry, with enzymes offering advantages in selectivity, safety, and environmental friendliness. In this context, computational-guided enzyme engineering and structurally guided directed evolution can expedite the development of efficient enzymes while minimizing costs and efforts. This oral contribution will present a few examples from my research group.

Amine dehydrogenases (AmDHs) catalyze the conversion of ketones into enantiomerically pure amines using ammonia and a hydride source. Utilizing structural information from computational models, we engineered AmDHs capable of converting pharmaceutically relevant aromatic ketones with high efficiency, achieving quantitative conversions and perfect chemical and optical purities.¹ These enzymes were derived from an unconventional scaffold that does not naturally perform asymmetric transformations. Interestingly, the best variant, LE-AmDH-v1, exhibits a unique substrate-dependent switch in enantioselectivity, producing (*S*)- or (*R*)-configured amine products with enantiomeric excesses up to >99.9%, depending on the substrate. This behavior was explained through *in silico* studies that elucidated the effects of mutations on ligand binding and the resulting enantioselective preferences.

We also evolved an amino acid dehydrogenase for the efficient reductive amination of sterically demanding substituted phenylpyruvic acids. A colorimetric high-throughput assay was developed for the directed evolution of a variant exhibiting an 8-fold enhancement in k_{cat} , and no apparent substrate or product inhibition, all while maintaining stability for a week. *In silico* analysis revealed that the mutation L51F enhances substrate binding by introducing a "flipped" phenylalanine side chain that creates a sandwich π -stacking interaction with neighboring aromatic residues. Bioamination reactions utilizing minimized enzyme and cofactor amounts achieved >99% conversion within 9 hours at a small scale and >98% conversion within 12.5 hours at a pilot scale.

In conclusion, these examples highlight the potential applications of *in-silico* guided enzyme engineering to speed up the development of biocatalysts for the synthesis of chiral molecules such as amines and amino acids, thereby enabling more efficient and environmentally friendly processes in the pharmaceutical industry.

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Invited lecture: WG2

Machine Learning in Enzyme Engineering and Mining: Lessons Learned Along the Way

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Enzyme engineering and mining is crucial for advancements in biotechnology, biomedicine, and industrial applications.¹ However, the vast protein sequence space remains largely unexplored due to experimental limitations in screening throughput. Identifying beneficial amino acid substitutions and their combinatorial effects on function remains challenging.³ Recent developments in machine learning (ML) have significantly improved protein engineering by leveraging large experimental datasets, next-generation sequencing (NGS), high-throughput screening (HTS), and artificial intelligence.^{2a,4} ML models facilitate the discovery and optimization of protein properties by extracting sequence-function relationships and guiding enzyme engineering.

To harness ML in enzyme engineering, we developed PyPEF (Pythonic Protein Engineering Framework), a versatile ML-driven framework for data-driven protein engineering and ML-assisted directed evolution.⁵ PyPEF integrates ML with signal processing and statistical physics to explore sequence space systematically, enabling the identification of high-fitness variants. We evaluated PyPEF's predictive accuracy and performance on four public enzyme datasets, demonstrating its potential for guiding rational protein design. However, applying ML methods often requires large experimental datasets, which can be difficult to obtain. To address this, we introduced MERGE, an ML-based approach that integrates evolutionary information to enhance protein fitness predictions.⁶ By combining statistical modeling with ML-trained sequence representations, MERGE allows efficient sequence exploration even with limited datasets, accelerating directed evolution and rational design.⁷

Beyond enzyme engineering, enzyme mining is essential for discovering novel biocatalysts. Despite their importance, most enzymes remain uncharacterized, limiting their potential for biotechnological applications. Conventional enzyme mining approaches rely on sequence-structure-function relationships but face challenges such as labor-intensive parameter tuning, visualization difficulties, and inaccuracies in clustering due to sequence variability. While protein language models have shown promise in enzyme mining, their complexity often restricts accessibility for non-expert users. To overcome these limitations, we developed SelectZyme (Unpublished), an embedding-based enzyme mining tool for intuitive exploration of large enzyme latent spaces. SelectZyme enables seamless enzyme selection using simple query terms and integrates clustering, dimensionality reduction, and visualization techniques, including protein landscape plots, phylogenetic trees, and minimal spanning trees. Additionally, SelectZyme tracks user selections and outputs annotated tables of relevant sequences. We validated its utility across three datasets addressing key biocatalysis challenges, demonstrating its effectiveness in streamlining enzyme discovery.

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Invited lecture: WG2

Identification of novel CAZymes for seaweed biorefineries

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Marine macroalgae have drawn the attention of the scientific community due to their high annual productivity of biomass and the great variety of applications they present. Seaweeds mostly consist of polysaccharides, with high structural complexity and high chemical and thermal stability, making them challenging for processing and further refinement. An efficient way to gain access to the high value products derived from seaweeds are polysaccharide-degrading enzymes from the marine microbiome. However, the structure of these polysaccharides is different from the plant ones, and thus the identification of new enzymatic tools is of great interest.

Hence, in our group we focus our attention on discovering novel robust biocatalysts, originating from the metagenome of marine microorganisms living in extreme environments, such as the volcanic arc of the Aegean sea. In this work we will present a methodology on identifying polysaccharide-degrading enzymes of industrial interest with different selectivities, such as alginate lyases and laminarisases, as well as the biochemical characterization of these enzymes.

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Invited lecture: WG3

R-NADES as green solvents for biocatalysis: improving lipases stability and selectivity

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Natural deep eutectic solvents (NADESs) have emerged as sustainable, green alternatives for biocatalytic reactions, providing high substrate solubility while improving enzyme stability and efficiency.

In our studies, the design of reactive natural deep eutectic solvents (R-NADESs) and their effect on the esterification activity and thermal stability of free and immobilized lipases in the synthesis of polyol- and carbohydrate-based biosurfactants was investigated. A total of 16 binary and ternary R-NADES systems were synthesized using choline chloride (ChCl) as hydrogen bond acceptor (HBA) and carbohydrate polyols, including mono-, di-, and oligosaccharides, urea, N-methyl urea, and water as hydrogen bond donors (HBDs), in various molar ratios. These systems were characterized based on their physicochemical properties, including viscosity, polarizability, hydrogen bonding, and thermal behaviour.

The results showed that lipases, both free and immobilized, exhibited high stability and catalytic performance in R-NADESs during esterification reactions. In particular, R-NADESs consisting of ChCl and sugar alcohols (D-sorbitol, xylitol, D-arabitol) were efficient in catalyzing the esterification of polyols with fatty acids, with *Candida antarctica* lipase B immobilized on acrylic resins exhibiting exceptional thermal stability. A Box-Behnken factorial design optimized the reaction conditions, resulting in an 80 mol% conversion of lauric acid (LA) to D-arabitol, yielding the product 1,5-dilauryl-D-arabitol with >99.9% purity.

In addition, the esterification of glucose was investigated and only lauroylcholine chloride was obtained due to strong interactions in the H-bond network of a ChCl:Glc:H₂O R-NADES. Molecular dynamics simulations provided insight into these reaction results and explained the observed selectivity and stability of the lipase-catalyzed reactions in R-NADES media.

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Invited lecture: WG3

In vitro construction of the COQ metabolon unveils the molecular determinants of coenzyme Q biosynthesis

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Coenzyme Q, CoQ, is an essential antioxidant ubiquitous in nature. CoQ biosynthesis in humans has thus far been attributed to ten different proteins with many coalescing into a large assembly coined the 'COQ metabolon'. Metabolons are protein assemblies that channel substrates within a metabolic pathway, however, their general aptitude for enzymatic biocatalysis remains poorly understood and several CoQ biosynthetic steps, including C1-decarboxylation and C1-hydroxylation steps, remain enigmatic in our understanding. To elucidate the prerequisites for CoQ biosynthesis and moreover, assess the physicochemical properties of the metabolon, we sought out to construct the entire metabolon in vitro. We employed an innovative evolution-engineering approach rooted by ancestral sequence reconstruction, in addition to spectrophotometric and biophysical assays, and analytical techniques to monitor CoQ biosynthesis. We reconstructed tetrapod ancestors for COQ proteins: COQ3, COQ4, COQ5, COQ6, COQ7, COQ8A, COQ8B, COQ9 and Ferredoxin reductase and Ferredoxin 2, speculated to initiate CoQ biosynthesis.

We observed activity for all ancestral COQ proteins and demonstrated that COQ4 is a Zn-dependent decarboxylase responsible for the C1-decarboxylation and that COQ6 performs the subsequent C1 hydroxylation step. Amalgamating all the proteins together allowed us to construct the entire biosynthetic pathway in vitro. Furthermore, the formation of the COQ metabolon, mediated by an atypical kinase, COQ8B, increases CoQ production and reduces intermediate accumulation. Using a combination of HDX, XL-MS, microscale thermophoresis, and size exclusion chromatography techniques, we have been able to capture several binary interactions including COQ6:COQ3 and COQ7:COQ9 to name a few. Furthermore, using Native PAGE analysis and XL-MS, we have observed the formation of different assemblies with unique crosslinking networks, implying a dynamic and heterogeneous composition of protein interactions.

Our findings suggest that metabolons possess heightened catalytic efficiency, consist of an intricate and dynamic number of protein interactions, and are overseen by chaperoning and organisational protein mitigators.

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Invited lecture: WG3

Protein engineering of a fungal feruloyl esterase enhances both plastic and lignocellulose breakdown

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As plastic waste accumulation continues to rise, the need for sustainable alternatives to conventional waste management has become urgent. Enzymatic degradation has emerged as a promising solution, particularly for polyesters like polyethylene terephthalate (PET), which contain hydrolyzable ester bonds. PET-degrading enzymes (PETases) catalyze polymer's breakdown into water soluble intermediates, mainly mono(2-hydroxyethyl) terephthalate (MHET). MHET hydrolysis, crucial for efficient polymer degradation to its building block terephthalic acid (TPA), is facilitated by MHETases.¹ Structural study of *IsMHETase*, the benchmark MHETase from *Ideonella sakaiensis*, reveals its significant similarities to feruloyl esterases (FAEs), a well-studied enzyme class involved in lignocellulose deconstruction.²

Building on the structural homology between *FoFaeC*, a feruloyl esterase from *Fusarium oxysporum*, and *IsMHETase*, specific single-point mutations (T202E, G122S, I415F) were designed to enhance *FoFaeC*'s MHETase activity.³ Single, double and triple *FoFaeC* variants were expressed in *Pichia pastoris*. Purified enzymes active on MHET, underwent kinetic analysis on PET oligomers/monomers, as well as and methyl esters of hydroxycinnamic acids (ferulate-MFA, caffeate-MCA and coumarate-MpCA). The most efficient variant was further evaluated for its synergistic role in the degradation of both synthetic (PET) and natural (destarched wheat bran-DSWB) polymers.

Experimental results showed that *FoFaeC*-G122S variant exhibited a 4.2-fold increase in catalytic efficiency (k_{cat}/K_M) on MHET and a 2.4-fold higher activity on PET trimer compared to the wild-type enzyme. Regarding natural-substrate mimicking compounds *FoFaeC*-G122S displayed a 2-fold higher k_{cat}/K_M on MpCA, while maintaining a similar catalytic profile for MCA and MFA, as the wild-type enzyme.

In terms of polymer degradation, *FoFaeC*-G122S significantly improved PET breakdown efficiency, when combined with *IsPETase*, by completely (96%) converting the degradation products to terephthalic acid (TPA), compared to a 63% TPA yield achieved by the wild-type enzyme. When it comes to the natural substrate, *FoFaeC*-G122S exhibited a 1.7-fold higher ferulic acid release rate from DSWB, compared to the WT, when combined with a GH10 xylanase.

These findings demonstrate that a single structure-based mutation on *FoFaeC* could greatly enhance its activity on a non-natural substrate (MHET), hence establishing its role as an accessory enzyme for PET degradation, while maintaining its synergistic role in lignocellulose breakdown.

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Invited lecture: WG3

Tuning heterogeneous chemoenzymatic hybrids conformation toward optimized aerobic oxidation

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Chemoenzymatic catalysts that combine enzymes and transition metal complexes are increasingly attractive for selective transformations, especially the oxidation of organic substrates.¹ These hybrids overcome the limitations encountered separately in each field,² offering new reactivities. In addition, the development of material technology has enabled the design and synthesis of a plethora of 3D catalyst supports with tunable pore size and surface functionalization.³ In this endeavour, heterogeneous chemoenzymatic catalysis appears as an emerging field including all possible combinations of enzymes, chemical catalysts and materials.⁴ However, the efficiency of the immobilized catalysts can be altered by their random orientations with respect to their support surface, making this material an integral partner to consider. Therefore, it is crucial to control every level of the catalytic system design, ranging from the choice of enzymes, catalysts and material, and their functionalization, to their orientation, loading and organization. We will present our latest architectures based on the site-directed immobilization of a hybrid catalyst bearing a biquinoline-based-Pd(II) complex ¹ and a robust multicopper oxidase laccase within the cavities of macroporous silica foams to favour veratryl alcohol oxidation.⁵ When operating under continuous flow, we illustrate that it is crucial to control every level of the catalytic system design for optimizing the performance and the sustainability of the catalyst.

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Invited lecture: WG3

Outcomes and insights – implementing a Python-based tool, EnzymeML, into a kinetic parameter estimation workflow

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Multicopper oxidases (MCOs) are a class of oxidative enzymes, including bilirubin oxidases, ceruloplasmin, and laccases. The phylum *Actinomycetota* is one of the largest, diverse groups within the domain *Bacteria*, and omics studies have shown the presence of MCO genes in actinobacteria. As such, these bacteria are a potential source of unexplored MCOs that may be of industrial value.

As observed in several inter- and intra-laboratory studies, the availability of reproducible and reliable data remains challenging.¹ This often leads to experimental data deficient in critical details such as experimental metadata, variables, and crucial protein information.² Therefore, the need exists for analysis pipelines that adhere to STRENDa guidelines to generate data that is Findable, Accessible, Interoperable, and Reusable (FAIR).

Soil from two South African environments was sampled– nutrient-poor soil associated with *Aspalathus linearis*, and an organic-rich soil from a developing peatland. Actinobacterial isolates were screened for extracellular oxidase activity. The genomes of the top two most talented strains exhibiting the highest activity were synthesised and heterologously expressed.

Upon confirmation of the presence of functional enzyme, primary biochemical data was generated by implementing a Python-based workflow, EnzymeML, to yield accurately curated and analysed kinetic data, that adheres to current STRENDa and FAIR guidelines – whilst simultaneously developing a workflow that highlights best-practice technical considerations for wet-lab experimentalists.

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Invited lecture: STSM

Computational engineering of pullulanase from *Bacillus paralicheniformis* 9945a thermostability and solubility

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Pullulanase (EC 3.2.1.41) is a debranching enzyme that hydrolyses α -1,6-glycosidic linkages in branched polysaccharides such as pullulan, amylopectin, and glycogen. Primarily applied in starch modification, pullulanase is valuable in processes like starch saccharification and resistant starch formation due to its ability to hydrolyze α -1,6-glycosidic linkages. Thermostable pullulanase can increase saccharification efficiency, reduce enzyme quantities needed at 50-60 °C (common saccharification temperatures), lower solution viscosity to enhance mass transfer, and decrease microbial contamination during starch hydrolysis¹. However, thermophilic pullulanases remained limited, with most being mesophilic. For instance, pullulanase from *Bacillus acidopullulyticus* (PulA) had an optimum temperature of 60 °C but a half-life of just 34.9 min, while pullulanase from *Bacillus pseudofirmus* 703 (Pul703) deactivated rapidly at 50 °C. By contrast, pullulanase from *Anoxybacillus* sp. SK3-4 (PulASK) retained 50% of activity after 9 hours at 60 °C². Computational tools like FoldX, I-Mutant 3.0, and dDFIRE had improved thermostability, raising the melting temperature T_m by several degrees for variants from *Bacillus thermoleovorans*³. Rational protein design was used to improve thermostability of pullulanase PulB, from *Bacillus naganoensis*⁴. To date, pullulanase engineering has been mainly carried out by structure-based site-directed mutagenesis or domain tailoring based on structure/sequence information. High costs of thermophilic pullulanases are partly due to limited productivity, and most lack optimal properties for industrial applications. The primary interest in this project was to enhance the thermostability of mesophilic pullulanase from *Bacillus paralicheniformis* 9945a (*BliPull*), with a temperature optimum at 40 °C, using tools and methods from the Loschmidt Laboratories, which would benefit the overall stability and catalytic properties of the enzyme. Additionally, tools for improving protein solubility were also explored due to their potential to enhance expression in host cells and simplify downstream purification processes.

Structural modeling was performed using AlphaFold 2⁵ and SWISS-MODEL⁶ to predict the three-dimensional structure of *BliPull*, as no experimental crystal structure was available. Stability-enhancing mutations were identified using FireProt⁷ and PROSS⁸, which predict stabilizing mutations based on energy landscapes. Functional and stability "hot spots" were mapped using HotSpot Wizard⁹, allowing targeted mutagenesis without compromising catalytic activity. Homologous sequences were mined using EnzymeMiner¹⁰ to identify thermophilic variants with improved solubility and stability. Disulfide by Design¹¹ and MAESTROweb¹² were used to introduce disulfide bonds for additional structural stabilization. AggreProt¹³ was employed to identify and reduce aggregation-prone regions. FireProt ASR¹⁴ was used for ancestral sequence reconstruction, identifying evolutionarily stable residues that

could improve enzyme resilience. Multiple single-point mutations were identified and ranked based on their predicted impact on thermostability and solubility. RMSD analysis between AlphaFold 2 and SWISS-MODEL models suggested structural consistency, supporting their reliability for mutation design. Comparative sequence analysis with structurally characterized homologs, including *Bacillus thermoleovorans* pullulanase (PDB ID: 2E8Z), provided insights into key stabilizing interactions. Computational predictions suggest that introducing disulfide bonds and modifying aggregation-prone regions could enhance enzyme stability while maintaining activity. Literature review also indicated that N- and C-terminal truncations could further improve secretion and thermostability, which will be considered in future experimental. This study demonstrates the potential of combining multiple computational approaches to engineer a more thermostable and soluble pullulanase variant. The identified mutations will undergo experimental validation to assess their impact on thermostability, solubility, and enzymatic activity under industrial conditions. If proven successful, these findings will contribute to the development of more efficient biocatalysts for starch processing and other biotechnological applications.

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Invited lecture: STSM

Allosteric Regulation of L-Lactate Dehydrogenase: Insights from Molecular Dynamic Simulation and Functional Studies

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Allosteric regulation is a crucial process through which enzyme activity or structural features are modulated in response to specific metabolites. L-Lactate dehydrogenase from *Geobacillus stearothermophilus* (GsLDH) is an oxidoreductase, catalyzing the conversion between pyruvate and lactic acid with NAD(H). Previous studies showed that its activity is regulated by an allosteric activator fructose 1,6-bisphosphate (FBP). However, these studies lack molecular dynamic (MD)-based analyses which can shed light on oligomeric state transitions, on conformational changes and residue flexibilities, and on specific network interactions governing allosteric regulation.

In this study, we combined MD simulations with biochemical analyses, providing novel and uncharacterized insights into the impact of FBP on GsLDH. MD simulations revealed that FBP stabilizes the tetrameric conformation, reduces residue flexibility, while enhancing interactions between pyruvate and residues in the active pocket albeit a distance of 23Å between the sites. Using MDavocado tool, we identified three dimer-dimer interface regions critical for stability and structural integrity in the presence of FBP. Microcalorimetry titration revealed that NADH binding ($K_d = 1.2 \pm 0.3 \mu\text{M}$) occurs only in the presence of FBP, indicating an enthalpy-driven interaction involving a hydrogen-bond network. Single amino acid replacement, Q189L, maintains GsLDH tetrameric structure without FBP, and enhances the substrate inhibition effect. However, this mutant fails to trigger the allosteric transition toward a conformation with higher affinity for the substrate, resulting in a high K_m value ($3.8 \pm 0.4 \text{ mM}$) and a low k_{cat}/K_m ($25 \pm 4 \text{ s}^{-1}\text{mM}^{-1}$), comparable to that of the WT without FBP. These findings suggest that oligomerization alone does not confer allosteric responsiveness, emphasizing the essential role of specific interactions in allosteric regulation.

Acknowledgements: This work was supported by the ISF grant 1372/24 and COST Action CA21162. H.C. acknowledges the Short-Term Scientific Missions (STSM) grant from COST Action CA21162.

Invited lecture: STSM

Discovery, characterization and improvement of thermostable carbonic anhydrase for application in post combustion CO₂ capture

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The recently released report by the Intergovernmental Panel on Climate Change (IPCC) leaves no doubt about the urgency to dramatically cut greenhouse gas emissions. In order to stay on track to limit earth's warming to 1.5°C, emissions need to be cut by 45% by 2030, compared to 2019 levels. To reach these goals, Carbon Capture and Utilization and Storage (CCUS) is now openly discussed as a solution to decrease net CO₂ emissions, as well as a potential technology to move away from fossil carbon by using CO₂ as an alternative feedstock for renewable chemicals and fuels.¹ A promising and novel alternative to the current state of the art for post-combustion CO₂ capture is the use of the enzyme carbonic anhydrase (CA). CA is one of the fastest enzymes in nature, catalyzing the hydration of CO₂ to bicarbonate ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$) which is the limiting step of CO₂ absorption. CA is a biocatalyst that can be employed alone in a biomimetic approach in aqueous media to capture CO₂, or it can be used as a promoter in traditional chemical absorption processes, such scrubbing with amine or potassium carbonate aqueous solutions, or even the accelerated weathering of alkaline materials that can act as co-sequestering agents.^{2,3} Adding CA could overcome the slow kinetics of common solvents and boost CO₂ removal efficiency to well beyond 95%, solving the efficiency issues that impede the widespread implementation of post combustion carbon capture. However, to develop robust enzyme-accelerated CCUS processes, discovery of novel carbonic anhydrases with ultra thermal and alkali- stability is necessary.

Aim of the presentation is to showcase a successful application of the steps in the biocatalysis cycle for developing an efficient and cost-effective enzyme-accelerated process for post-combustion CO₂ capture. Enzyme mining towards discovery of ultra-stable CAs from harsh environments (i.e. hydrothermal vents) and development of screening techniques to define their realistic range of operations will be presented.⁴ A novel CA, CA-KR1, was discovered from mining of metagenomic data and was characterized for its potential to accelerate the slow kinetics of carbonate solutions in a state of the art pressurized reactor. Finally, examples of the application of computational tools in the improvement of a novel CA will be presented and the potential improvement in the thermostability of the developed mutants will be underlined.

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Invited lecture: STSM

Investigating Machine Learning for Scalable Molecular Dynamics and Evolution-Aware Protein Design

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Despite significant progress in experimental and computational methods, protein design remains a complex task, as only a small fraction of sequences are functional. Machine learning has considerably advanced the field by generating sequences with desired folds, yet many designs fail to match wild-type activity.¹ The intricate interplay between sequence, structure, and dynamics enables even minor changes to significantly alter function. As a result, incorporating protein dynamics into ML-based design is a crucial yet largely unresolved problem. Rich dynamic information is not only difficult to obtain and computationally expensive to generate but also inherently complex to interpret and utilize effectively. The reliability of ML-generated dynamics can be assessed by comparing them to classical, large-scale MD simulations using well-established methods for describing protein dynamical networks, such as the Shortest Path Map (SPM) or Dynamic Flexibility Index (DFI).^{2,3} In this work, we explore recent advances in ML-driven protein dynamics generation within accessible time frames, specifically in relation to their ability to recapitulate SPM and DFI statistics (Figure 1). We further investigate how this information can be leveraged for evaluating novel protein designs. Finally, we examine how DFI can scale dynamic information across datasets and be incorporated into ML-based design, especially the tools based on evolutionary information, such as FireProtASR.⁴ This combination of evolutionary insights with dynamic descriptors can potentially enhance these models for dynamics and evolution-aware protein design.

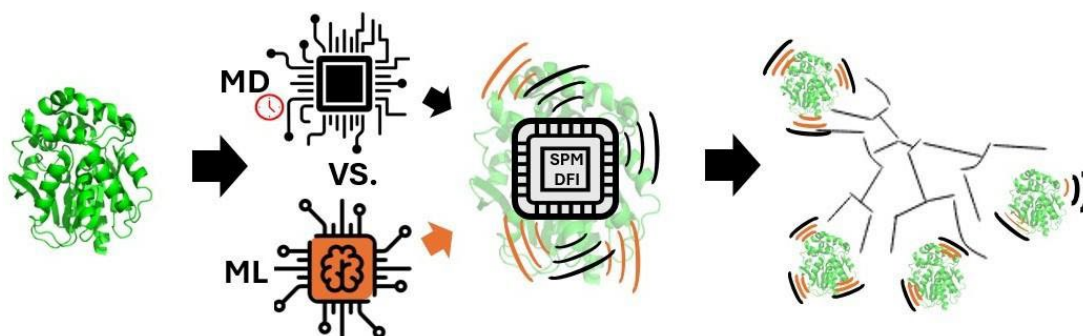


Figure 1. Comparison of ML-generated and classical MD-based protein dynamics, validated using SPM and DFI. Additionally, we investigate DFI dynamic descriptors' integration for dynamics and evolution-aware protein design.

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Invited lecture: STSM

Exploring the pH-dependent activity of hemicellulase *TtXyn30A*

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Xylanases are essential in lignocellulosic biomass degradation, working synergistically with cellulases and other enzymes to enhance the breakdown of plant cell walls. However, their efficiency in enzyme cocktails is often constrained by pH-dependent activity, as mismatches between individual enzyme pH optima can reduce overall catalytic performance. In this study, we employed Constant-pH Molecular Dynamics (CpHMD) simulations to explore the structural and mechanistic basis of pH-dependent activity in *TtXyn30A*, a glycoside hydrolase 30 subfamily 7 (GH30_7) xylanase from *Thermothelomyces thermophila* with both endo- and exo-activity on glucuronoxylan allowing it to release both aldouronic xylooligosaccharides (UXOs) substituted at the penultimate xylose from the reducing end and xylobiose from the nonreducing end respectively, with recent implications in the degradation of the recalcitrant twofold xylan.^{1,2} Several 100 ns CpHMD simulations of the *TtXyn30A* protein with a UXX ligand were carried out at pH values 3, 4, 5, 6, and 7 with 5 replicates each.³ The pK_a values of key residues, including the catalytic E188, E233, and E278, were identified and correlated to experimental findings concerning the retention of activity even in the absence of either one of them. We then investigated potential mutations to shift the enzyme's optimal pH from 4.0 toward neutral conditions, potentially improving compatibility with other lignocellulolytic enzymes and engineering pH activity. These findings contribute to the broader understanding of GH30 xylanase engineering and set the foundation for experimental validation, ultimately advancing the rational design of enzymes for industrial bioconversion processes.

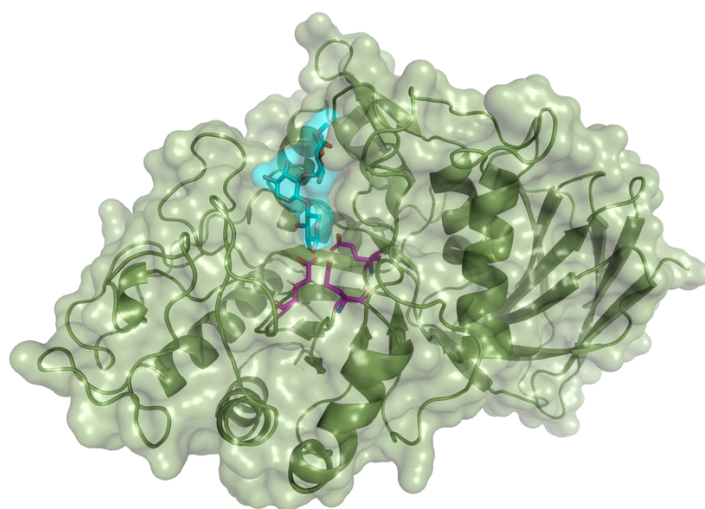


Figure 1. Representation of *TtXyn30A* (green) with an UX ligand (cyan) and the catalytic amino acids E188, E233 and E278 (pink).

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Invited lecture: STSM

Stabilization of PsG3Ox through a combination of *in silico* design and random recombination

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Glycoside 3-oxidases (G3Oxs) are recently identified flavo-oxidases from the glucose-methanol-choline superfamily that oxidize with significantly higher efficiency C-glycosides at C3 than monosaccharides at the C2 position, coupling with O₂ reduction to H₂O₂.^{1,2} G3Oxs have already shown great potential in carbohydrate modification, facilitating the synthesis of rare sugars.³ However, their moderate stability restricts reaction conditions and recyclability, posing challenges to broader industrial applications.

We employed FireProt, an automated computational design to improve its thermal stability. The resulting variant, incorporating 33 mutations, exhibited around threefold increase in protein production, albeit with reduced cofactor incorporation, a 6°C increase in apparent melting temperature (T_m^{App}) and a threefold enhancement in half-life time at 40°C. A random recombination between the wild-type and the variant genes was performed to refine the design by eliminating potential deleterious or neutral mutations. A more stable variant, 17E4, was obtained exhibiting 9°C increase in T_m^{App} compared to the initial design. The contributions of individual mutations to thermal stabilization were reassessed through $\Delta\Delta G$ calculations using multiple computational tools. Mutations were categorized according to their **stabilizing or destabilizing effects**, exposing significant discrepancies among prediction methods. These inconsistencies highlight the **inherent challenges** in accurately modeling protein stability. Ongoing efforts, including X-ray crystallography combined with computational dynamics are undertaken to elucidate key determinants of enzyme stabilization. This study aligns with the objectives of WG1 and WG3 of the COST project, showcasing the effectiveness of computational redesign and experimental validation.

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Poster presentation (02): WG1

Discovery and rational design of hydrolases for polyurethane degradation

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Polyurethanes (PURs) are chemically diverse, synthetic polymers whose resistance to natural degradation has contributed to a growing environmental crisis.¹ This study tackles major challenges in understanding and enhancing the biodegradation of PURs through three complementary objectives: defining PUR substrate diversity, investigating molecular determinants of enzymatic degradation, and discovering novel PUR-degrading enzymes from unique ecological niches. To address the first objective of this study, we developed PUR-GEN, a novel web server designed to automate the generation of PUR fragment libraries.² These libraries are enriched with conformers and property tables, providing a comprehensive view of PUR structures. The use of PUR-GEN in molecular docking of 414 generated oligomers provides an example of the pipeline for initiation of the PUR degrading enzymes discovery. The second aim focused on uncovering the molecular determinants of PUR binding and degradation using the model enzyme *Thermobifida fusca* cutinase (*TfCut2*) and the model substrate Impranil DLN, whose proprietary structure was resolved during the study. Computational approaches were employed to investigate the enzyme's binding modes and key amino acid interactions with PUR, enabling the proposal of mutations to enhance enzymatic activity by improving substrate binding. These computational predictions were experimentally validated by assessing the mutants' thermal stability and initial degradation rates, linking *in silico* findings with practical advancements in enzyme engineering. The third component of the project centered on the discovery of novel PUR-degrading enzymes from the highly polluted Marmara Sea. Using metagenomic sequencing and bioinformatics pipelines, candidate enzymes with potential activity against polyurethane substrates were identified. Selected candidates were further analysed for conserved catalytic motifs and experimentally tested for their degradation activity.

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Poster presentation (03): WG1 and WG2

Engineering of β -Galactosidase to Enhance Thermal Stability and Catalytic Efficiency for Industrial Applications and Biosensors

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Beta-galactosidase (β -galactosidase) is a crucial enzyme extensively utilized in various industries, particularly in the hydrolysis of lactose in dairy products to alleviate lactose intolerance and enhance product quality. Despite its widespread application, challenges such as limited thermal stability and suboptimal catalytic efficiency under industrial conditions impede its broader utilization. Recent advancements in enzyme engineering have employed rational design strategies to overcome these limitations. For instance, site-directed mutagenesis and computational modeling have been utilized to identify and modify key amino acid residues, resulting in β -galactosidase variants with improved thermal stability and catalytic activity. These engineered enzymes not only enhance the efficiency of lactose hydrolysis in food processing, thereby improving digestibility for lactose-intolerant individuals, but also hold potential for innovative applications in biosensor technologies. In biosensors, β -galactosidase can serve as a reporter enzyme, facilitating the detection of specific analytes through its catalytic action. Ongoing research aims to further optimize β -galactosidase from *Bacillus circulans* through advanced design and engineering approaches, expanding its applicability in both food technology and diagnostic devices.



Figure 1. β -galactosidase from *Bacillus circulans*

Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant numbers 451-03-66/2024-03/200134, 451-03-65/2024-03/200134).

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Poster presentation (04): WG1 and WG2

Predicting enzyme fitness by combining structural and (co)evolutionary methods

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Accurately predicting the effects of mutations on enzyme fitness is crucial for designing new enzymes. Indeed, computational predictions can complement experimental approaches by narrowing down the mutations that need to be screened in time-consuming experiments.

In this study, we first present FiTMuSiC,¹ a novel protein fitness predictor that integrates structural, evolutionary, and coevolutionary information. It outputs two scores that independently assess the functional and structural impacts of a variant, offering detailed mechanistic insights into how mutations contribute to fitness changes, whether through enzyme loss/gain of activity and/or stability. Despite its simple underlying model, FiTMuSiC achieves accurate predictions: it was ranked among the top performers for the prediction of variants fitness of the hydroxymethylbilane synthase (HMBS) target in the sixth round of the Critical Assessment of Genome Interpretation challenge (CAGI6)² and matches the performance of more complex deep learning models like AlphaMissense.³

To further validate its reliability, we compared FiTMuSiC's predictions with a new dataset of enzyme deep mutagenesis scanning data collected from the literature, which confirmed FiTMuSiC's accuracy and robustness and demonstrated performance on par with or better than other prediction tools.⁴ To ensure FiTMuSiC accessibility, we make it freely available through an easy-to-use webserver babylone.ulb.ac.be/FiTMuSiC.

Acknowledgements: We acknowledge financial support from the Belgian Fund for Scientific Research (F.R.S.-FNRS) through a PDR project. M.T. benefits from a FNRS-FRIA PhD grant. P.H. benefits from a Win4Doc grant from SPW Recherche of the Walloon Region.

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Poster presentation (05): WG1 and WG2

Machine Learning Strategies for Identifying Cold-Adapted α/β Hydrolase Enzymes

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Psychrophilic enzymes retain high catalytic efficiency at low temperatures primarily by reducing the temperature dependence of the reactions they facilitate.^{1,2} A key factor in their adaptation is the enhanced flexibility of the active site, which allows them to function effectively in cold environments.³⁻⁵ These enzymes have significant industrial applications in various fields, including food processing, environmental bioremediation, pharmaceuticals, textile manufacturing, and detergent formulation.⁶⁻⁸ However, despite their importance, the molecular mechanisms underlying their adaptation to low temperatures remain largely unclear.

This study aims to explore the differences between psychrophilic and non-psychrophilic bacterial α/β hydrolase enzymes. A total of 1026 unreviewed bacterial α/β hydrolase enzymes were retrieved from the UniProt database. Their classification was conducted using various machine learning algorithms, including Decision Tree (DT), Random Forest (RF), eXtreme Gradient Boosting (XGBoost), and Support Vector Machine (SVM). The analysis focused on ten variables, including the content of Ala, Gly, Ser, Thr, charged, aliphatic, aromatic, and hydrophobic amino acids, along with the aliphatic index and the grand average of hydropathy (GRAVY). The findings demonstrate that all machine learning algorithms performed with nearly identical accuracy, achieving a 75-78% accuracy rate through ten-fold cross-validation on the dataset.

This study confirms that some amino acids, especially threonine, are generally involved in the cold adaptation of psychrophilic α/β hydrolase enzymes and may provide an interesting platform from a biotechnological point of view.

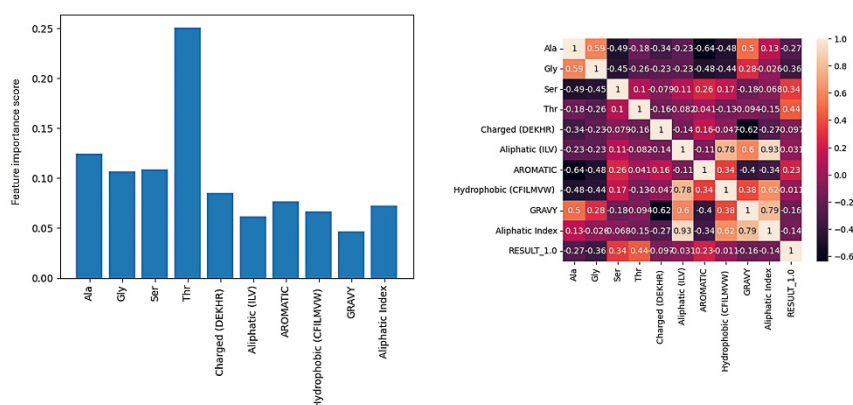


Figure 1. Feature importance analysis from the Random Forest model, illustrating the relative contributions of key variables to model performance (left); Feature correlation matrix illustrating the relationships between variables and their influence on the psychrophilic characteristics of enzymes (right).

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Poster presentation (06): WG1 and WG2

The discovery and *in silico* characterization of Anatolian water buffalo rumen-derived bacterial thermostable xylanases: A sequence-based metagenomic approach

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Xylanases (3.2.1.8) randomly cleave the backbone of the xylan. Their market has steadily grown, they are used in high-temperature applications including food and feed industries. This study analyzed the rumen metagenomes of three Anatolian water buffaloes using shotgun sequencing, exploring the relationship between the microbial community and xylanases. *In silico* analyses of three xylanases (AWBRMetXyn5, AWBRMetXyn10, and AWBRMetXyn19) were conducted at the sequence, structure, and dynamic levels. BLAST, biophysicochemical features, phylogeny, and MSA were analyzed using Blastp, ProtParam, MEGA11, and Clustal Omega, respectively. 3D models were generated with SWISS-MODEL and validated via ProSA, ProCheck, and Verify3D, followed by structural analysis using PyMOL, BANAiT, thermostability predictor, What If, and PIC software. Protein-ligand interactions were studied using docking and MD simulations. Shotgun sequencing and Blastp analyses identified *Clostridium*, *Ruminococcus*, *Prevotella*, and *Butyrivibrio* as dominant genera for xylanase production. Biophysicochemical analysis showed the xylanases had an aliphatic index >80, instability index <40, and $T_m > 65^\circ\text{C}$. Phylogenetic analysis placed the xylanases within the GH10 family, clustered with thermophilic xylanases, and homology modeling identified a thermophilic bacterium as the optimal template. BANAiT analysis revealed lower B'-factor values in the $\beta 3$ - $\alpha 1$ loop/short-helix at the N-terminal site compared to XynAS9. MD simulation showed that the $\beta 9$ - $\eta 5$ loop, containing the catalytic nucleophile glutamic acid, had higher fluctuation in the three xylanases. Distance analysis revealed that AWBRMetXyn5 and AWBRMetXyn10 kept the nucleophile residue closer to the ligand. This study offers insights for protein engineering of xylanase function and thermostability, with AWBRMetXyn5 being a potential candidate for high-temperature industrial applications.

Acknowledgements: The authors would like to thank Berrin Okuyucu, Helin Altan, Neşe Özding, and Ahmet Enes Uray for their technical assistance. Also, the numerical calculations reported in this paper were fully performed at TUBITAK ULAKBIM, High Performance and Grid Computing Center (TRUBA resources). Additionally, this research was funded by the scientific and technological research projects funding program (1001) of the Scientific and technological research council of Türkiye (TÜBİTAK) (Project no: 121Z943).

Poster presentation (07): WG2

Engineering thermostable PLA-degrading enzymes

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Plastic pollution has emerged as a critical environmental and health challenge, underscoring the urgent need for sustainable solutions such as enzyme-driven biodegradation of synthetic polymers like polylactic acid (PLA). In this study, we identified a novel PLA-degrading enzyme, MGY, from a metagenomic database.¹ However, its practical application was hindered by low solubility, poor expression yield, and insufficient thermostability. To overcome these limitations, we employed ancestral sequence reconstruction,² generating three ancestral MGY variants. These variants retained high PLA-degrading activity while exhibiting significantly enhanced solubility, expression levels, and thermostability. Differential scanning calorimetry (DSC) revealed remarkable improvements in thermostability, with one variant achieving a melting temperature (T_m) of 84 °C—the highest reported for any PLA-degrading enzyme to date.³ Notably, one ancestral variant also demonstrated activity against polycaprolactone (PCL), Figure 1. AlphaFold predictions reveal that MGY and its ancestral variants belong to the α/β -hydrolase superfamily, sharing structural homology with the experimentally characterized MGS0156 polyesterase53 (PDB: 5D8M), $T_m = 54$ °C, that shows dual activity towards polylactic acid (PLA) and polycaprolactone (PCL).

This unique combination of stability, solubility, and activity positions MGY and its variants as an excellent model system for understanding enzyme selectivity and versatility in industrial biocatalysis, particularly for the degradation of recalcitrant polyesters.

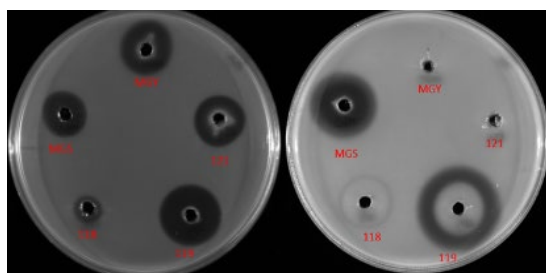


Figure 1. Polyesterase activity against PLA (left) and PCL (right) of purified metagenomic MGS0156 (MGS), newly identified metagenomic MGY enzyme, and its ancestral variants, 121, 119, and 118. 75 μ g of each enzyme was screened against emulsified PLA in agarose after 7 days of incubation. The polyesterase activity is indicated as a clarified zone around the well. The larger the halo effect, the higher activity.

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Poster presentation (08): WG2

Exploring Tyrosinase Inhibition: Discovery of Potential Inhibitor Candidates for Preventing Hyperpigmentation in the Cosmetic, Pharmaceutical, and Food Industries Using Molecular Modeling Techniques

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Tyrosinase, a copper-containing enzyme ubiquitous among various organisms, plays a pivotal role in the enzymatic browning process through melanin synthesis¹. Melanin functions as a photoprotective pigment against UV radiation; however, its uncontrolled production can lead to hyperpigmentation disorders.² Melanin determines skin and hair color in mammals, while in fruits and vegetables, its formation results in undesirable browning that diminishes food quality and nutritional value.^{2,3} Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-DOPA, forming quinones key intermediates in melanin biosynthesis (**Figure 1**). Given these critical functions, tyrosinase inhibitors have garnered significant interest as anti-browning agents in the cosmetic, pharmaceutical, and food industries. This study employed molecular modeling approaches to elucidate the interaction mechanisms between tyrosinase and selected inhibitor molecules. The inhibitors were optimized using quantum mechanical methods, and their pharmacokinetic properties, including absorption, distribution, metabolism, and excretion (ADME), were assessed. Furthermore, molecular docking studies determined the binding affinities between the inhibitors and tyrosinase. The findings provide valuable insights into the effectiveness of tyrosinase inhibitors and highlight their potential applications in managing hyperpigmentation and food quality preservation.

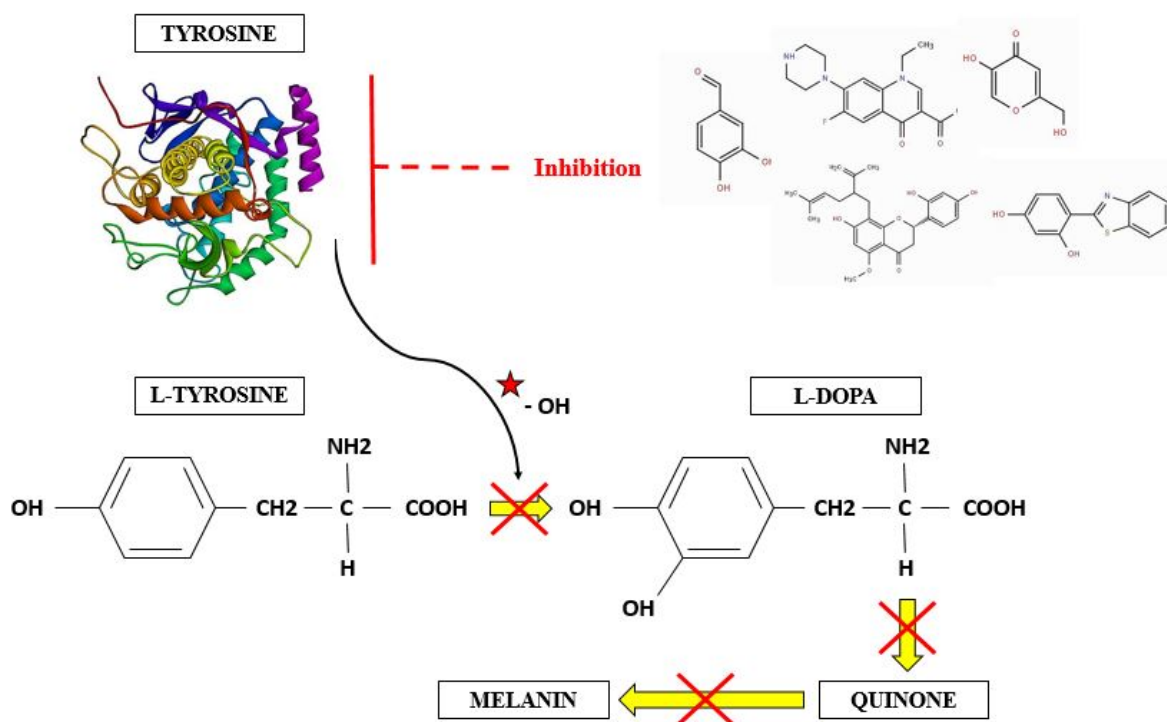


Figure 1. The conversion steps of L-tyrosine into melanin synthesis are shown. This process begins with reactions catalysed by the enzyme tyrosinase.

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Poster presentation (09): WG2

Rational Design for Enhanced Substrate Affinity of Laccase from *Streptomyces cyaneus*

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Laccases are versatile oxidoreductase enzymes with broad substrate specificity.¹ In this study, we constructed a reliable homology model of laccase from *Streptomyces cyaneus* (Uniprot ID: F6L7B5) and identified a potential substrate-binding site through structural analysis and molecular docking. Our docking simulations with tetracycline yielded promising results, suggesting stable binding. Molecular dynamics (MD) simulations confirmed the stability of the enzyme-substrate complex, revealing conformational changes in loop regions near the binding site, which align with literature reports on laccase promiscuity.² Interestingly, the identified binding site is slightly distant from the catalytic histidine-copper center, indicating potential conformational rearrangements during catalysis. To enhance substrate affinity, we designed two mutations, increasing the polarity and electronegativity of the binding site. Simulations of these mutants showed slightly improved binding energies, supporting their potential for experimental validation. Future work will focus on further mutational screening and experimental testing of enzyme activity to optimize laccase performance.

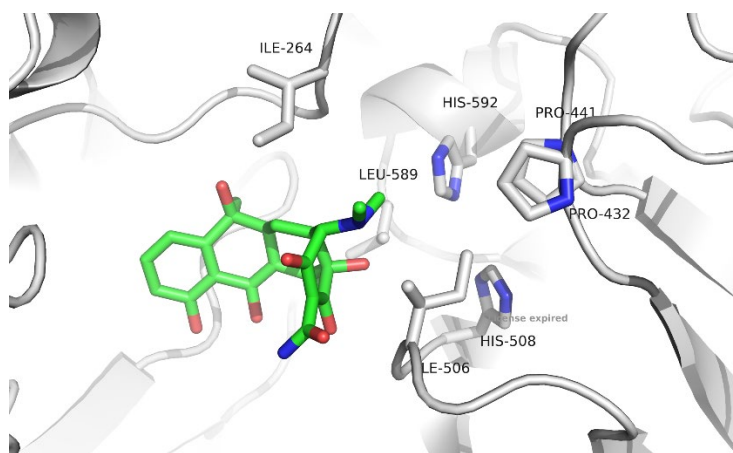


Figure 1. Tetracycline in the active site of laccase from *Streptomyces cyaneus*

Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant numbers 451-03-66/2024-03/200134, 451-03-65/2024-03/200134).

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Poster presentation (10): WG2 and WG3

A computational and statistical approach in optimizing the enzymatic synthesis of aromatic oligoesteramides derived from ϵ -caprolactam and 5-hydroxyfurancarboxylic acidIoana-Cristina Benea¹, Anamaria Todea¹, Francisc Peter¹

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Over the past few decades, enzymatic biocatalysis has been acknowledged as a key method for the sustainable synthesis of polymers and oligomers. Among the various enzymes, lipases, in particular, have demonstrated a notable capacity to catalyze the production of innovative polymeric materials across a diverse array of substrates.¹ Furthermore, from the perspective of polymeric materials, furanic derivatives, such as polyethylene 2,5-furandicarboxylate (PEF), a green substitute for PET, possess remarkable potential as alternatives to fossil-based polymers.² Since furans are becoming one of the most important classes of platform chemicals nowadays, the enzymatic synthesis of aromatic oligoesteramides integrating furan units is a very attractive green route for obtaining advanced and sustainable compounds.³

Considering these aspects, this work explored the synthesis of aromatic oligoesteramides through an enzymatic approach, starting from the furan derivative 5-hydroxymethyl-2-furancarboxylic acid and ϵ -caprolactam as a co-monomer. Several immobilized lipases were tested in the oligomerization reaction, such as lipase B from *Candida antarctica* and lipase from *Pseudomonas stutzeri*, both commercially available and stabilized in our laboratory.

The synthesis of the oligoesteramides was performed in organic and solvent-less medium, at 80 °C for 24 hours. Additionally, this synthesis was optimized utilizing the statistical technique Design of Experiments (DoE), examining factors such as temperature, the ratio of biocatalysts, and the molar ratios of the monomers. The properties of oligoesteramides were further assessed by means of computational methods. This allowed for the fine-tuning of the synthesis process, achieving superior outcomes in the production of oligoesteramides.

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Poster presentation (11): WG2 and WG3

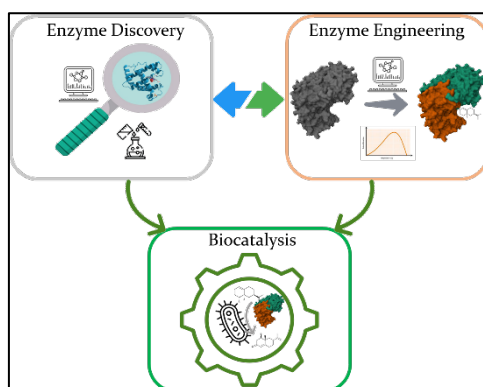
Identification and Engineering of Novel Enzymes for Industrial Applications

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The research focus of the Ensari lab includes; i) identification of novel enzymes, ii) enzyme engineering through directed evolution and semi-rational approach, and iii) biocatalysis involving novel or engineered enzymes.



The utilisation of computational tools in the identification of novel enzymes is a pivotal step in our research. These enzymes are then expressed and characterised through experimental methods. The projects undertaken to date have encompassed the identification and characterisation of novel enzymes for industrial and medical applications. A notable example is the P450 monooxygenase from *Azorhizobium caulinodans*, a thermostable arabinofuranosidase from *Alicyclobacillus acidocaldarius*, α -galactosidase and α -glucuronidase enzymes from thermophilic *Geobacillus kaustophilus*, and asparaginases from human gut microbiome for the treatment of ALL using virtual screening.

The current studies focus on P450 monooxygenases, asparaginases and carbohydrate-active enzymes engineering employing directed evolution and computer-aided enzyme engineering. In the context of biocatalysis, a recent proposal was funded by TÜSEB (Ministry of Health) with the aim of constructing a whole cell bacterium for the biotechnological production of retinol by combining up to 10 different enzymes and transporter proteins. Furthermore, a project funded by the EU's CetP has been accepted, with the aim of engineering carbonic anhydrase and formate dehydrogenase.

These studies have been conducted in collaboration with partners who are members of COZYME from Turkey and abroad, including Mehdi D. Davari from IBP Halle-Germany. A CETPartnership proposal has been submitted with Barış Binay from Turkey.

Poster presentation (12): WG3

Computational and functional analysis of natural and designed variants in human P450 oxidoreductaseMaria Natalia Rojas Velazquez^{1,2,3}, Isabel Sousa Barata^{1,2,3} and Amit V. Pandey^{1,2}

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Cytochrome P450 oxidoreductase (POR) is an essential redox partner for steroid and drug-metabolizing cytochromes P450 located in the endoplasmic reticulum.¹ Mutations in POR lead to metabolic disorders, including congenital adrenal hyperplasia, and affect the metabolism of steroids, drugs, and xenobiotics. We have studied a large number of natural and designed variations in POR protein to study their impact on protein function. In this study, we examined approximately 450 missense variants of the POR gene listed in the Genome Aggregation Database (gnomAD) using eleven different in silico prediction tools. We then selected variants that could potentially change the stability of P450 reductase protein. To validate our findings, we conducted functional analysis and selected two variations in POR for further investigation. The human POR wild type, along with the R268W and L577P variants, were expressed in bacteria and subjected to enzyme kinetic assays using model substrates. We also examined the activities of several cytochrome P450 proteins in the presence of POR (WT or variants) by combining P450 and reductase proteins in liposomes. We observed a decrease in enzymatic activities (ranging from 35% to 85%) of key drug metabolizing enzymes, supported by POR variants R288W and L577P compared to WT-POR. These results validate our approach of curating a vast amount of data from genome projects and provide an updated and reliable reference dataset. Identification and characterization of POR variants that could enhance the activity of redox partner proteins like cytochrome P450, may serve as valuable tools for studying the relationship between structural and functional changes in these proteins and offer insights for biotechnological applications that would benefit from increase in protein stability and activity for commercial production of biopharmaceuticals.

Acknowledgements: This study is funded by the Swiss National Science Foundation (SNF) grant 204518 and SNF-COST Projects in Switzerland 229294 to Amit V Pandey

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Poster presentation (13): WG3

Computational and biochemical study of a new xyloglucanase from the white-rot fungus *Abortiporus biennis*.

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Lignocellulosic biomass is a composite material consisting of cellulose, hemicellulose and lignin. Xyloglucan is a complex, highly substituted plant biomass hemicellulose, covering cellulose fibrils. Xyloglucanases, the enzymes responsible for its degradation, can be incorporated in enzyme cocktails that target cellulose-containing substrates¹. In order to shed light on the enzymatic degradation of xyloglucan, a novel xyloglucanase from the basidiomycete *Abortiporus biennis* (*AbiXega*), with unknown structure was studied. Initially, the structure of xyloglucanase was predicted by AlphaFold 2.0² starting from the residue sequence. The modelled structure was subjected to 4µs molecular dynamics (MD) simulations in the GROMACS MD engine and representative conformations were obtained after k-means clustering. Ligands with a combination of XXXG, XLXG, XXLG and XLLG units were docked to the representative xyloglucanase structures and the best pose for each one was subjected to additional MD simulations (2.5 µs each). The ligand binding free energy was calculated using the MMPBSA method. The enzyme was heterologously produced in *P. pastoris* as the host and purified to determine its biochemical and catalytic properties. *AbiXega* was found to be a glycoprotein with relatively strict substrate specificity, since it is only active in xyloglucan and glucomann, yielding XXX and XXXG, XLXG/XXLG, XLLG as the main hydrolysis products, while galactose substituents were found to be tolerated only in specific subsites. Overall, the experimental and computational results provide valuable insights on the structure-function relationships of glucoside hydrolases, and illustrate the importance of computational methods in the study of enzymes with limited structural information.

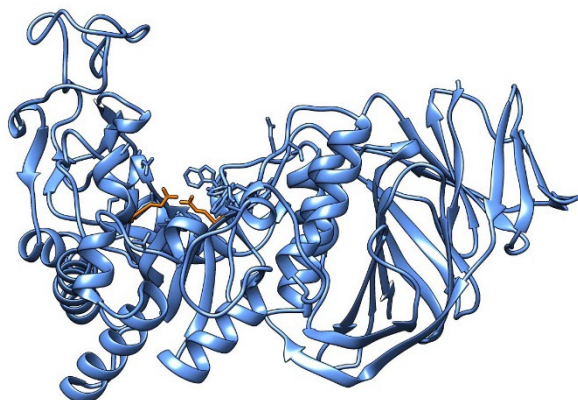


Figure 1. AlphaFold model structure of AbiXega. The proposed catalytic residues are highlighted in orange.

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Poster presentation (14): WG3

Zinc-based Nanobiohybrids as Artificial Metalloenzymes for Efficient Photocatalytic CO₂ Conversion to BicarbonateCarla García-Sanz¹ and Jose M. Palomo¹

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Given the rising global CO₂ emissions and the urgent need to address climate change, developing efficient and sustainable CO₂ conversion strategies is crucial. This study presents, for the first time, the synthesis of photocatalytic zinc nanobiohybrids—materials combining enzymes and zinc nanostructures—for CO₂ conversion under sustainable conditions (aqueous medium, room temperature). Various proteins were tested as scaffolds to mimic enzyme activities involved in CO₂ transformation, forming distinct ZnO nanocloud-like structures that influenced catalytic performance. These hybrids exhibited esterase-like activity and mimicked carbonic anhydrase by hydrolyzing CO₂ to bicarbonate. Additionally, they showed photocatalytic properties, with Zn-CALB achieving the highest efficiency: under UV light, bicarbonate conversion doubled (61 ppm/h) compared to natural light (33 ppm/h), and Xe light increased the rate 2.5 times (83 ppm/h).

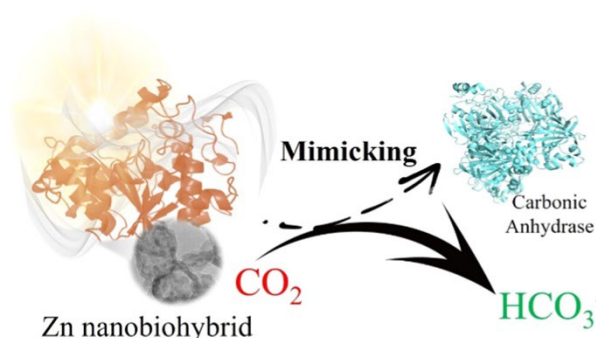


Figure 1. Photocatalytic conversion of CO₂ to bicarbonate using Zn nanobiohybrids.

Acknowledgements: The authors thank the support of the Spanish National Research Council (CSIC) and Ministry of Science.

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Poster presentation (15): WG3

Harnessing synthetic microbial biotechnology for efficient plastic waste upcycling

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Plastics have become indispensable in various industries today, but their extensive use has resulted in a severe environmental crisis—plastic waste accumulation. Traditional methods of plastic waste management are insufficient, facing persistent pitfalls, such as overall material downgrading and release of hazardous microplastics and chemicals into the environment. Plastic upcycling, on the contrary, is gaining prominence in converting plastic waste into valuable chemicals and materials in an eco-friendly manner. The current project presents an innovative approach to plastic waste upcycling: employing green bio/mechano/chemical technologies for the hydrolytic degradation of plastic polymers into oligomers and their constituent building blocks, is followed by the creation of microbial cell factories capable of producing industrially relevant compounds from these plastic-derived feedstocks. Successful microbial upcycling hinges on efficient carbon assimilation from plastic feedstocks and the presence of functional intracellular biosynthetic pathways to generate valuable bioproducts. To achieve this, a wide variety of microorganisms with natural capabilities for these processes are identified through bioprospecting, and synthetic biology is employed to engineer or enhance the other necessary functions. Alternatively, some well-established microbial hosts including *Bacillus* spp., *Pseudomonas* spp. or *Yarrowia* spp. serve as suitable chassis. By integrating these advanced methodologies, we aim to create sustainable and efficient microbial systems for plastic waste valorization. Our ultimate goal is to establish a research and innovation hub in Synthetic Microbial Biotechnology that would advance scientific knowledge, raise public awareness, promote environmental protection, and boost economic development, all the while minimizing humanity's reliance on finite fossil fuel resources.



Figure 1. Conceptual representation of the twinning innovation hub for microbial platforms in plastic upcycling.

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Poster presentation (16): WG3

Structural study of an engineered leaf branch compost cutinase (LCC) with PET oligomers

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The identification of the leaf branch compost cutinase (LCC) as a promising enzyme to tackle poly(ethylene terephthalate) (PET) depolymerization has attracted much attention to PET degradation community.¹ Several engineering attempts have been reported in the past decade, among which the work by Tournier *et al*, that led to an efficient quadruple variant (LCC^{ICCG}, UniProtKB – G9BY57) exhibiting increased thermal stability by 10 °C.² Since then, LCC^{ICCG} variant has been used as a new template for engineering attempts with enhanced catalytic characteristics.³

Currently, there are only two crystal structures of the LCC^{ICCG} variant in complex with plastic oligomers (PDB ID 8JMO, 8JMP), available in the PDB. Our study aims to determine complex structures of the variant with PET oligomers.⁴ Thus, LCC^{ICCG} was expressed in *Escherichia coli*, purified and submitted to crystallization trials. Its crystal structure, in apo form, was determined to 1.83 Å resolution. Attempts to obtain complex structures with PET oligomers and Monohydroxyethyl terephthalic acid amide (MHETA) are currently underway. As an alternative to protein crystallography, docking simulations of the aforementioned substrates with the enzyme will be also presented. The determination of complex structures can reveal the role of residues that could be targeted in the future towards enzyme engineering attempts.

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Poster presentation (17): WG3

Metal Nanoparticles-Enzyme Complexes as Multi-Functional Heterogeneous Catalysts

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One-pot multi-step transformations present a significant chemical challenge today. Advancing this field requires the development of new catalytic systems capable of efficiently performing bio- and chemical transformations in a sustainable manner.

To address this, we have designed and synthesized enzyme-metal hybrid nanomaterials that show great potential as multiactive heterogeneous catalysts. Various nanostructures were developed by inducing the *in situ* formation of metal nanoparticles (MeNPs) on a preformed *Candida antarctica* lipase-graphene (G@CALB) complex. In these systems, metal nanoparticles were synthesized exclusively under enzyme induction and uniformly dispersed across the enzyme scaffold.

This biosynthesis, carried out in aqueous media under mild conditions, enables the creation of bifunctional systems where enzymatic activity is preserved while the metallic activity is enhanced, resulting in synergistic effects. We evaluated different metals, including palladium, copper or silver, testing their performance in various one-pot cascade reactions of synthetic interest. Furthermore, we explored the generation of a bimetallic-enzyme system, leading to the development of a trifunctional catalyst with significantly enhanced metallic activity due to synergistic interactions.

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Poster presentation (18): WG3

Harnessing Extremozymes: A Metagenomic Approach to Sustainable Catalysis

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Enzymes are essential in industrial processes, acting as biocatalysts that enable precise and environmentally friendly transformations. Their adaptability benefits a wide range of industries, from food production to bioremediation, enhancing both process efficiency and sustainability. However, traditional enzymes often struggle under the harsh conditions present in industrial settings, such as high temperatures. To address this, extremozymes—enzymes that thrive in extreme environments—are employed. These robust enzymes are typically found in microorganisms residing in extreme habitats, although 99% of them cannot be cultivated using standard microbiology techniques. To overcome this challenge, we utilize metagenomic analysis, which examines the collective genetic material of all microorganisms in an environment, probing the untapped protein potential of extremophiles. Our in-house developed tool, *ProteoSeeker*¹ conducts extensive bioinformatics analysis on large metagenomic datasets to identify promising biocatalysts for industrial use. Designed for non-expert users, *ProteoSeeker* is a publicly accessible pipeline tailored for metagenomic exploration and protein discovery. Through recombinant production and biochemical characterization of the newly discovered extremozymes, we uncover innovative biocatalysts. These biocatalysts support industrial biotransformation in a sustainable, cost-effective, and environmentally conscious way, promoting product accessibility while fostering a bio-based economy. Here, we illustrate the power of our approach by presenting the discovery and biochemical characterization of novel extremozymes including a novel carbonic anhydrase CA-KR1², which hold significant promise for biomimetic CO₂ capture.

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Poster presentation (19): WG3

Enzymatic approaches to assess citric acid levels in wineGianina Marian (Țoca)¹ and Gabriela Râpeanu¹

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Citric acid is a mono hydroxy tricarboxylic acid found in grapes, must, and wine. It is formed in the roots of the vine, from where it migrates into the grapes and only a small part is also formed in the leaves. The amounts of citric acid that accumulate in grapes are lower (0.1-0.8 g/L), compared to the other acids, tartaric and malic. Botrytized grapes contain the highest amount of citric acid, which sometimes exceeds 1.5 g/L.¹ During the ripening period of the grapes, the citric acid content remains stable, or is registering a slight increase. From this reason is considered to be the most stable acid from the wine. Nowadays, a correction of deficient wines with low acidity by citric acid addition is accepted by the OIV and the EU. In the national rules, the correction by adding citric acid is allowed, within the maximum limit of 2.5 g/L the total acidity expressed in tartaric acid of the wines, but citric acid content should not be higher than 1 g/L. In wines, a higher amount of citric acid indicates wine adulteration (Ivanova-Petropulos et al., 2020). Citric acid can also be converted by lactic acid bacteria in acetic acid. The result is the increasing of volatile acidity of wine with a negative impact on sensorial properties.³

The main objective of this study was to develop an enzymatic method for citric acid in wines able to identify possible frauds in the collected wine samples. The enzymatic method was based on the transformation of citric acid (citrate) into oxaloacetate and acetate by a reaction catalyzed by a specific enzyme - citrate lyase (CL). The results were evaluated by comparison with a classical method of citric acid determination based on the reaction with acetic anhydride in a basic medium that forms a product whose maximum absorbance was read at 363 nm.

The results revealed after analysis of 27 wine samples (white, red and rose) that the difference between the citric acid values found by both methods was less than 1%. Only for 2 samples of wines the citric acid content was higher than recommended limit of 1 g/L.

Acknowledgements: The Integrated Center for Research, Expertise, and Technological Transfer in the Food Industry is acknowledged for providing technical support.

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Poster presentation (20): WG3

Engineering the substrate specificities of D-hydantoinase and D-carbamoylase

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The hydantoinase process, a cascade reaction catalyzed by D-hydantoinase and D-carbamoylase enzymes, is one of the methods for the synthesis of D-amino acids.

The aim of the research was to create an enzymatic cascade containing D-hydantoinase and D-carbamoylase enzymes for the production of D-amino acid derivatives. The cloning of genes for D-specific hydantoinase and carbamoylase enzymes was carried out by using *E. coli* expression systems. The following substrates were synthesized for the carrying out of biotransformation process: (D,L)-5-(4-hydroxyphenyl)-hydantoin, (D,L)-5-(2-hydroxy-4-bromophenyl)-hydantoin, (D,L)-5-(2-hydroxy-4-chlorophenyl)-hydantoin, (D,L)-5-(2-hydroxy-4-fluorophenyl)-hydantoin, (D,L)-5-(4-methoxyphenyl)-hydantoin, (D,L)-5-(4-methylphenyl)-hydantoin.

Substrate specificity tests showed that only (D,L)-5-(4-hydroxyphenyl)-hydantoin was preferred substrate by the enzymes, leading to the synthesis of D-4-hydroxyphenylglycine. The reaction was optimized for temperature, pH, duration, and mixing, followed by purification and identification of the target amino acid.

We considered the interaction with (D,L)-5-(4-hydroxyphenyl)-hydantoin for D-hydantoinase, and the interaction with N-carbamoyl-4-hydroxy-D-phenylglycine for D-carbamoylase as a prototype and designed mutations accordingly based on molecular docking results. After the identification of binding clefts and different interactions, we identify target amino acids for mutagenesis. The mutated variants of the enzymes have been investigated using computational methods including molecular dynamics and dockings to evaluate how interactions with substrates change. 5 mutations for D-hydantoinase enzyme have been generated and tested for their substrate specificity but none of them have shown activity for above mentioned substrates.

The study established an enzymatic cascade for the production of D-4-hydroxyphenylglycine, however, engineered mutations did not enhance activity towards the tested substrates, highlighting the need for further optimization.

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*Poster presentation (21): WG3***Glycosyl hydrolases from extremophilic organisms**Marco Mangiagalli, Marco Orlando, Stefania Digiovanni, Marina Lotti

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Glycoside hydrolases (GHs) catalyse the hydrolysis of glycosidic bonds, playing a crucial role in the breakdown of oligo- and polysaccharides. In recent decades, advanced DNA sequencing campaigns and targeted experimental screening efforts have led to a significant increase in the number of identified GH families. This has provided new opportunities for the discovery of enzymes with novel functions, enriching the known repertoire of microbial polysaccharide catabolic systems. We are studying GHs from different enzyme families (GH1, GH42, GH3, GH5, GH50, GH26, GH11, GH30) with particular interest towards the degradation of marine polysaccharides.

We target in particular enzymes from extremophilic organisms, mainly psychrophilic and halophilic. This last topic is carried out in collaboration with teams of the university of Crete (Prof. Pavlidis and Prof. Sarris).

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Poster presentation (22): WG3

Discovery and Characterization of Novel O-Methyltransferases from *Lentinula edodes* for the Sustainable Production of Taste-Active Flavonoids and (Iso-)Vanilloids

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With the increasing demand for natural food ingredients, enzyme-catalyzed transformations of flavonoids offer a sustainable approach in line with Green Chemistry principles. In this study, a novel O-methyltransferase (OMT) activity was identified in the mycelium of *Lentinula edodes* (syn. Shiitake) and applied for the biosynthesis of taste-active flavonoids, including hesperetin, hesperetin dihydrochalcone, homoeriodictyol, and homoeriodictyol dihydrochalcone. Additionally, this mycelium-mediated OMT activity facilitated the conversion of various catecholic substrates into their respective (iso-)vanilloids, whereas monohydroxylated compounds remained unaltered (Fig. 1). To elucidate the underlying enzymes, a bottom-up proteomics approach led to the identification of three putative OMTs in *L. edodes*. The corresponding genes were synthetically optimized, heterologously expressed in *Escherichia coli*, and purified to confirm their O-methylation activity against targeted flavonoids containing catechol motifs. The newly discovered fungal OMTs exhibit significant potential for the biotechnological production of valuable flavor-active compounds, providing an eco-friendly alternative to conventional chemical synthesis.

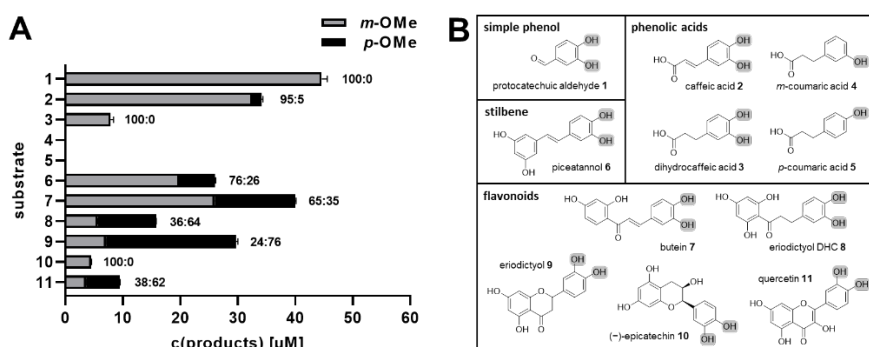


Figure 1. Product concentrations and regioisomeric ratios (% m-OMe:p-OMe) from biotransformations of phenolic compounds (B) by O-methylation activity from mycelium.¹

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Poster (23) and software presentation (01): WG3

EnzymeML Tools: making your biocatalytic data AI-ready

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The digitalization of biocatalysis is crucial for enabling data-driven research and unlocking the potential of machine learning (ML) in catalyst design, kinetic modeling, and reaction optimization.¹ However, a significant challenge remains the availability of well-structured measurement data, particularly with comprehensive reaction conditions. Without standardized data, ML applications in biocatalysis remain limited.

To address this, EnzymeML provides a standardized data exchange format for structuring and sharing biocatalysis data, ensuring machine readability and interoperability for seamless integration into computational workflows.^{2,3} Complementing this framework, Python tools have been developed to ingest raw experimental data from plate readers and chromatographic instruments, enriching it with reaction conditions and reactant information to create a structured dataset for each assayed reaction.

By organizing experimental data in a standardized, machine-readable format, these tools enable direct use of experimental data in machine learning applications, providing feature-rich training data for modeling, kinetic analysis, reaction optimization, and enzyme property prediction. By bridging the gap between experimental and computational scientists, they open new opportunities for data-driven research and accelerate the development of optimized enzymes.

The development of EnzymeML tools thrives on collaboration, and we welcome your contributions. Whether you are an experimentalist integrating computational tools or a data scientist, we invite you to join us in shaping the future of data-driven enzyme research.

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Poster presentation (24): WG3

Bacterial cell model for functional evaluation of cytochrome P450 oxidoreductase variantsMichel Kranendonk¹

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In the past, we developed different bacterial (*E. coli*) cell models, incorporating a bi-plasmid system for the co-expression of human cytochrome P450 oxidoreductase (CPR) alongside its redox partner (CYP). The bi-plasmid system enables the separate yet coordinated expression of CPR and CYPs, allowing precise fine-tuning of their relative expression levels.^{1,2} Compared to single-plasmid or *in vitro* reconstitution approaches, our system more accurately replicates the electron transfer process, facilitating the analysis of CYP-CPR interaction efficiency across different combinations. Moreover, the cost effectiveness and ease of application characterize these models. Through various studies, we have demonstrated their versatility in investigating the impact of genetic polymorphisms, mutations, and engineered modifications on enzyme function (e.g., for CYP1A2³), particular those of CPR.⁴⁻⁷ This approach is not limited to studying single mutants^{8,9} but also extends to the screening of random CPR mutant libraries,¹⁰ using our medium- to high through-put whole cell activity assays.¹¹ In summary, our bacterial models serve as adaptable tools for studying CPR, facilitating structure-function analyses, particularly valuable for pharmacogenetics, enzyme engineering, and toxicology research.

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*Poster presentation (25): WG3***Peroxidase extraction from waste and its use for dye biodegradation: application in batch and continuous flow**

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Wastewater from textile industry contains considerable amount of dissolved dye that can trigger various environmental issues if it is not treated properly and efficiently. Variety of processes have been developed to safely and utterly degrade recalcitrant pollutants. Processes that include enzymes are advantageous because less side-reactions and waste by-products are generated. Peroxidase was isolated from agro-industrial waste and used for biodegradation of dyed effluents. In that way, waste material is used for the treatment of another waste, increasing the overall cost-effectiveness and sustainability of the process. The enzyme was extracted from different waste materials and selection of the most suitable one was done. As a means to a more advanced, efficient and greener process, the enzyme was immobilized. Thus, peroxidase obtained from waste material can be reused several times. Afterwards, cross-linking of peroxides by oxidized pectin was performed in a batch system and in a continuous flow. For the continuous flow, the peroxidase was cross-linked onto the inner walls of the PTFE tube microreactor. Scanning electron microscopy analysis confirmed the successful outcome of the immobilization. Next, the optimization of the biodegradation batch process was carried out. Furthermore, the enzyme and cross-linker concentration were optimized, together with reusability assessment of the immobilized enzyme microreactor. Contact time, pH, enzyme, hydrogen peroxide and dye concentration, temperature, were optimized as well for both systems.

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Poster presentation (26): WG3

Structural studies and molecular dynamics simulations of a ferulic acid esterase variant active on PET oligomers

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Enzymatic degradation represents a promising approach to address the continuous accumulation of synthetic plastics in the environment.¹ Since 2000, numerous polyethylene terephthalate (PET) hydrolases, known as PETases, have been discovered. These enzymes target the polymer's ester bond, producing mono-(2-hydroxyethyl) terephthalate (MHET), as the primary degradation product.² MHET esterases (MHETases) then cleave the ester bonds of MHET, yielding terephthalic acid (TPA) and ethylene glycol as end products, enabling their utilization as feedstocks. On the other hand, ferulic acid esterases (FAEs) are enzymes of biotechnological interest, that cleave the ester bonds between hydroxycinnamic acids and arabinose in the plant cell wall. A FAE from *Fusarium oxysporum* (FoFaeC; PDB ID: 6FAT),³ belonging to tannase-like family, is a structural homolog of a well-studied bacterial MHETase from *Ideonella sakaiensis* (PDB ID: 6JTT).^{4,5} This enzyme shows activity on PET oligomers and demonstrates synergistic effect for PET degradation when combined with PETases.⁶ The present work focuses on an FoFaeC variant, G122S, that was created by structure-guided mutagenesis, in an effort to mimic the MHETase active site. G122S exhibits increased catalytic activity against MHET compared to wild-type FoFaeC. The crystal structure of G122S, in apo and benzoic acid bound form, were determined (PDB codes 9I50 and 9HUN, respectively), to 1.90 and 1.71Å resolution, respectively. These were subsequently used for molecular dynamics and docking simulations, in order to gain a deeper understanding of the biochemical findings. Analysis of the experimental and computational results sheds light on the structural determinants of PET active enzymes.

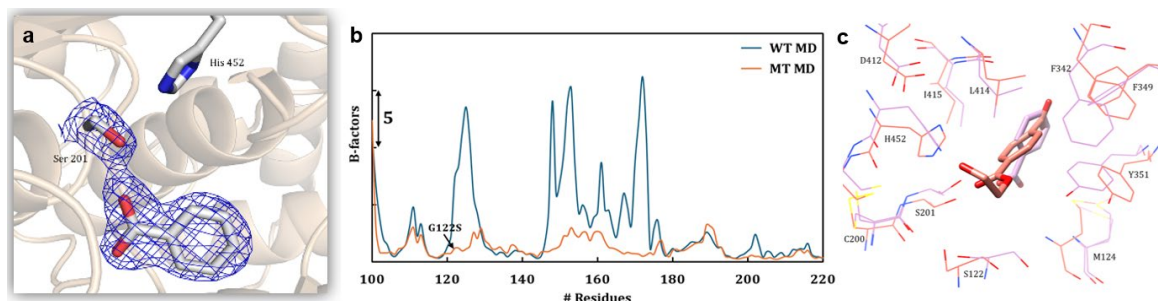


Figure 1. a) Catalytic center of G122S in complex with benzoic acid, b) Comparison between B-factors of wild-type FoFaeC and G122S focusing on the region carrying the mutated residue after 0.5 μ s MD simulation and c) Superposition between crystal structure of G122S in

complex with benzoic acid and G122S MD structure in complex with MHET after docking simulations.

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Poster presentation (27): WG3

β -glucocerebrosidase enzyme production: an enzyme replacement therapy using *Pichia Pastoris* Glycoswitch® expression technology

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Glucocerebrosidase (acid β -glucosidase, glucosylceramidase, GCase, EC.3.2.1.45) is a lysosomal glycoside hydrolase that hydrolyzes the β -glucosidic bond of glucosylceramide, yielding glucose and ceramide; thus, it is crucial for the breakdown of complex lipids and the cycle of cellular membranes.^{1,2} Recently, rare diseases have emerged as critical public health concerns owing to their prolonged and expensive treatment regimens.³ Gaucher disease, albeit uncommon, is the predominant lysosomal storage disorder. Gaucher disease results in the detrimental buildup of certain lipids, particularly a glycolipid called glucocerebroside, in essential organs such as the spleen, liver, lungs, bone marrow, and brain.⁴ This occurs owing to a deficiency of the enzyme β -glucocerebrosidase.⁵ This study introduces a novel, efficient, and economical method for examining the GCase enzyme in the *Pichia pastoris* Glycoswitch® strain with the synthetic substrate p-Nitrophenyl- β -D-glucopyranoside (PNPG). The activation parameters (optimal pH, T_m, K_M, and V_{max}) were characterized and modelled GCase compared to the crystal structure of recombinant GBA. The results indicate that the ideal pH and temperature for GCase enzyme activity with PNPG are 7.0 and 50°C, respectively. The K_M and V_{max} values are 2440 $\pm$ 0.2 μ M and 323 $\pm$ 0.1 U/mg, respectively. This study outlines the preliminary purification and characterisation of the human-derived β -glucocerebrosidase enzyme utilizing the *P. pastoris* GlycoSwitch® strain.

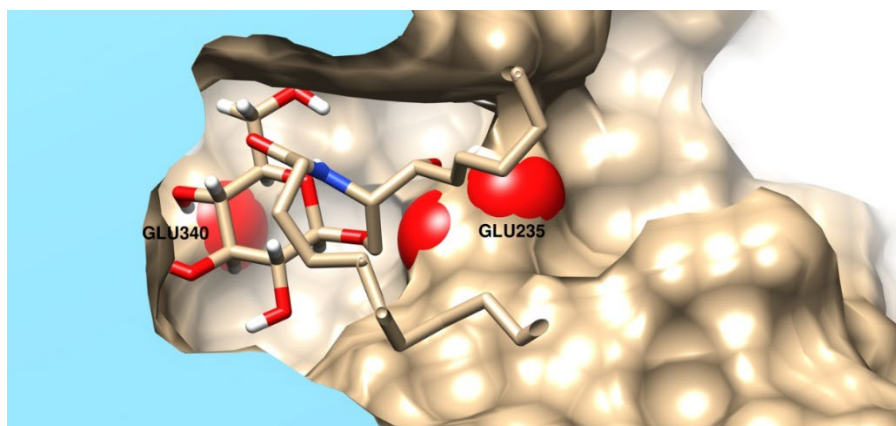


Figure 1. Modelled β -glucocerebrosidase compared to the crystal structure of recombinant GBA.

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Poster presentation (28): WG3

Computational Identification of Promiscuous Biocatalysts

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Catalytic promiscuity holds significant importance for understanding the evolutionary pathways of enzymes,¹ as well as for advancing industrial applications of biocatalysis.² The improvement of the activities of promiscuous enzymes through protein engineering paves the way for new potential applications in the field of biocatalytic synthesis.³ In this work, we computationally explore proteins involving the required catalytic machinery in optimal three-dimensional arrangement for the catalysis of three important target reactions: (1) Scytalonedehydration, a fundamental reaction in melanin biosynthesis, (2) Diels-Alder Reaction, which plays a role in the biosynthesis of terpenes and in the synthesis of complex natural products, and (3) Hydrolysis of Sarine nerve agent. Nerve agents inhibit acetylcholinesterase (AChE) by forming a covalent bond with the serine residue in the active site leading to overstimulation of the nervous system, followed by respiratory failure and death [4]. It is important to develop green catalyst that will bind nerve agents stronger than AChE and break them down through an environmentally friendly decomposition pathway. For this purpose, quantum mechanical active site models called theozymes, were used to generate catalytic atom maps, which were screened in protein database to identify proteins with potential promiscuous activity for the three target reactions.

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Poster presentation (29): WG3

The role of reaction engineering in understanding of enzyme catalysed reactions

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Biocatalysis is gaining attention in industry as it enables new routes to novel and existing compounds.¹ However, to reach the stage of industrial application, a lot of effort is needed. Primarily, different hurdles need to be overpassed in reaction development on lab scale, and the research timeline shortened. From the viewpoint of chemical engineer, a lot can be done by investigating reaction's kinetics of enzymatic systems. Also, viability assessment can be done for the purpose of decision-making regarding upscaling.² Kinetic investigation implies conducting small-scale lab experiments that give an insight on important features of the system, such as inhibitions or enzyme instability making it possible to draw conclusions on the reactor setup as well as process conditions to perform the reaction. This kind of research is followed hand in hand by *in silico* experiments based on simulations of mathematical models.³ In this talk features of the approach will be presented based on the results collected within several projects on the topics like the synthesis of analogues of *N*-acetylneuraminic acid, as well as the synthesis of N-N bond containing compound.

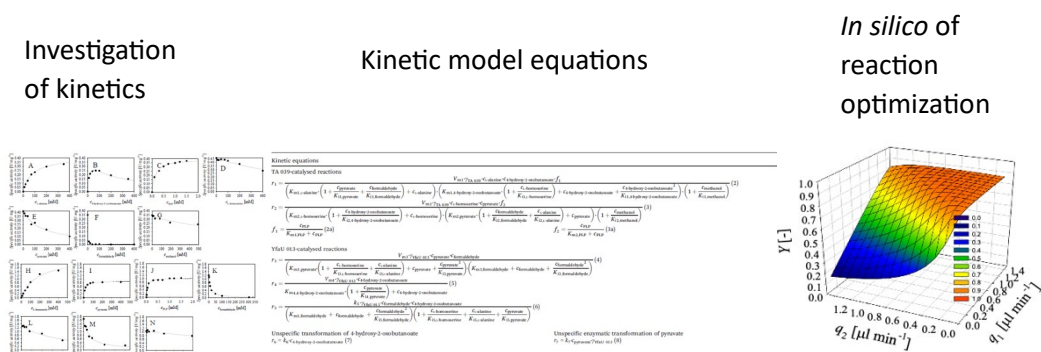


Figure 1. Methodology for process development via chemical engineering approach.

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*Software presentation (02): WG1***Caver Web 2.0: Analysis of Tunnels and Ligand Transport in Dynamic Ensembles of Proteins**

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Enzymes with buried active sites use molecular tunnels to exchange substrates, products, and solvent molecules with the surface, while channel proteins selectively transport ions or small molecules through their channels. These features are crucial for molecular transport and influence multiple properties. Understanding their structure-function relationships can be important for drug discovery and biocatalyst engineering. Since all proteins are intrinsically dynamic, their tunnels/channels also vary. Caver Web 2.0 is a user-friendly interactive web server that retains the original functionalities of Caver Web 1.0 and expands it with the possibility of running MD simulations and performing different analyses on the resulting dynamic ensemble, providing biologically more relevant information. Caver Web 2.0 allows assessing protein flexibility, identifying tunnels with CAVER, and predicting the trajectory and energy profile of a ligand passing through those tunnels with CaverDock. These features are available for single structures and ensembles. The screening of drugs and other ligands is available for the complete FDA/EMA-approved drugs or any user-specified datasets, and can be useful to identify strong inhibitors or substrates binding to the active site or tunnels. The server is freely available at <https://loschmidt.chemi.muni.cz/caverweb>.

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