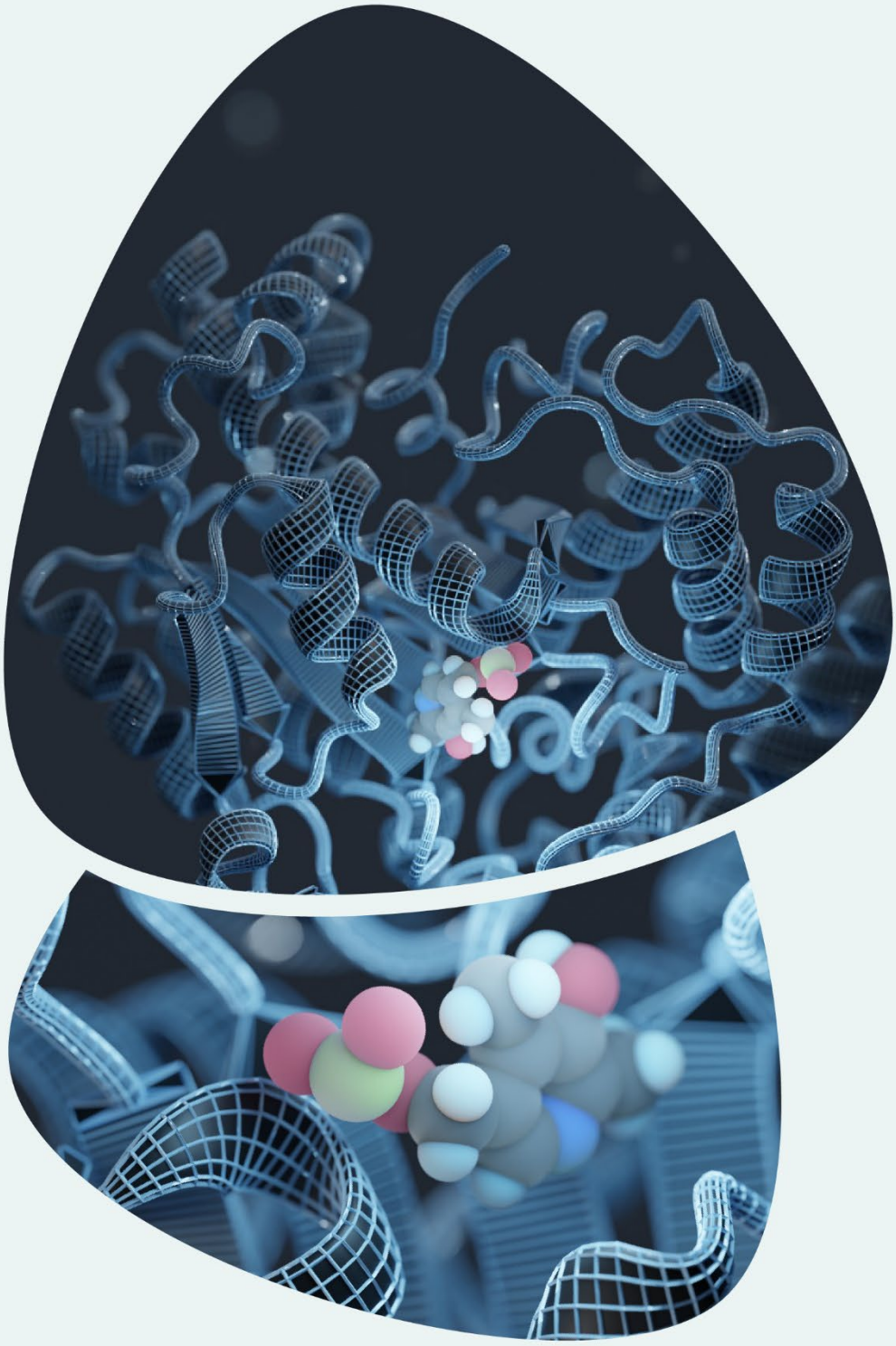


# COZYME

## BOOK OF ABSTRACTS

Working group meeting | April 16th–17th, 2026 | Belgrade, Serbia

**COST ACTION CA21162**

**Computational Redesign of Enzymes**

# Book of abstracts

Working group meeting

April 16th–17th, 2026

Belgrade, Serbia

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.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

# Foreword

We are thrilled to extend a heartfelt welcome to you as we gather in Belgrade, Serbia from April 16th to 17th, 2026, 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 Belgrade and are excited for the insightful discussions and collaborations that lie ahead.

*The Organizing Committee*

**Table of Contents**

**Foreword** ..... **2**
**About COZYME COST Action** ..... **6**
**Agenda MC and WG meeting** ..... **7**
**Overview of invited lectures** ..... **8**
**Overview of poster presentations** ..... **10**
**IL1, Bruno Victor:** Assessing Methodological Dependencies in Transmembrane Protein Simulations ..... 12
**IL2, Alfonso Gautieri:** Engineering of HyperStable and Acid-tolerant PETase Enzymes for Plastic Biodepolymerization ..... 14
**IL3, Marianne Rooman:** Integrating statistical potentials, (co)evolutionary information, and language models to boost protein stability change predictions ..... 16
**IL4, Peter Stockinger:** Ensemble-Aware Design of Thermostable Enzymes ..... 17
**IL5, Marc Garcia-Borràs:** Harnessing Reactive Intermediates: Computationally Guided Enzyme Design for Abiological Transformations ..... 18
**IL6, Miguel Machuqueiro:** Computational Methods to Capture pH Effects in Proteins ..... 19
**IL7, Aleksandra Maršavelski:** Engineering thermostability in metagenomic polyesterase ..... 20
**IL8, Gemma Kinsella:** Activation of CALB by alkylammonium ions: a new paradigm for activity enhancement of enzymes ..... 22
**IL9, Jan Deska:** Synthetic applications and de novo design of haloperoxidases ..... 23
**IL10, Pedro Sánchez Murcia:** Use of conformational ensembles of enzymes to assign functional effects ..... 24
**IL11, Robert Kourist:** Mechanism-inspired enzyme engineering of hydrophobic pockets for improved enzyme productivity – an experimentalist's perspective ..... 25
**IL12, Erica Elisa Ferrandi:** Functional Characterization and Structure-Guided Engineering of a Thermostable (S)-Selective Amine Transaminase from *Streptomyces* sp. BV333 ..... 26
**IL13, Io Antonopoulou:** Discovery and improvement of novel carbonic anhydrases towards accelerating post-combustion carbon capture ..... 27
**IL14, Ayelet Fishman:** Development of an enhanced enzymatic degradation system of polylactic acid ..... 28
**IL15, Lígia Martins:** Network Dynamics as Fingerprints of Thermostability in an In Silico-Engineered DyP-type Peroxidase ..... 29
**IL16, Konstantinos Grigorakis:** Mapping the catalytic free-energy landscape of an engineered MHET hydrolase via QM/MM Simulations ..... 31
**IL17, Tomás Frazão:** Mechanistic Determinants of Substrate Specificity in Bacterial Glycoside 3-Oxidase ..... 33
**IL18, Armen Sargsyan:** Increasing the substrate specifies of N-carbamoyl-β-alanine amidohydrolase by computational modelling ..... 34

**IL19, Sady Roberto Rodriguez:** Rational Design and Computational Analysis of *Candida boidinii* Formate Dehydrogenase for Improved $CO_2$-Reducing Activity ..... 36

**IL20, Rui P. P. Neves:** Deep-learning the committor function for the accurate characterization of the mechanism of sulphide oxidation by the bacterial flavin-dependent sulphide:quinone reductase ..... 38

**IL21, Garazi Ortiz-Orruño:** High-throughput screening for enhanced HMFO expression ... 40

**PP1, Sladek Vladimir:** Some computational perspectives on residue roles in dynamic protein complexes ..... 42

**PP2, Vardar-Yel Nurcan:** Dipeptide-Based Modeling of Protein Melting Temperature Using Linear and Nonlinear Machine Learning Approaches ..... 43

**PP3, Batista Marta:** pH-Driven Conformational and Protonation Changes in LipT6 Revealed by CpHMD Simulations ..... 45

**PP4, Das Nayanika:** Computational analysis of the free energy profile of the first step of Glutathione Peroxidase 6 (GPX6) catalysis: insights into the role of selenocysteine and species-specific variations ..... 46

**PP5, Krammer Lisa-Marie:** Bayesian optimization of ancestral arylmalonate decarboxylase for the asymmetric synthesis of sterically hindered profen derivatives ..... 48

**PP6, Pavlović Marija:** Structure-guided analysis of a pectin lyase from *Bacillus velezensis* ..... 49

**PP7, Pires D. S. Inês:** *In silico* study of the pH-dependent Au nanoparticle growth mechanism of a novel artificial metalloenzyme ..... 50

**PP8, Raczyńska Agata:** Unraveling the Molecular Basis of Polyester-Polyurethane Hydrolysis: Structural Insights and Engineering of TfCut2 ..... 52

**PP9, Stefanović Marija:** Biochemical and Structural Characterization of a Novel Gluten-Hydrolyzing protease from *Bacillus velezensis* 16B ..... 54

**PP10, Tomić Antonija, Matić Antonia:** Regulating the Antioxidative Response by Designing Kelch Domain Mutants to Inhibit DPP III Activity in the Keap1–Nrf2 Signaling Pathway ..... 56

**PP11, Wiley Brian:** Mechanistic Insights into Dioxygen Diffusion in the PHD2 Oxygenase from Long-Timescale Simulations ..... 57

**PP12, Borges Patricia:** Structural Loop Dynamics Drive Enhanced Mn(II) Oxidation in *Pp*DyP ..... 59

**PP13, Gand Martin:** Identification and application of oxidative enzymes from *Cerrena zonata* unraveled by a multi-omics and *in silico* approach ..... 61

**PP14, Gardossi Lucia:** Enzymes for synthesis and degradation of biobased polyesters: Automatic *in silico* screening and experimental validation ..... 62

**PP15, Häussler Max:** Data Processing and Kinetic Modelling of HPLC Reaction Time Courses with EnzymeML Tools ..... 63

**PP16, Kosić Višnja:** Assessment of Pullulanase Variant Stability under Operational Conditions ..... 64

**PP17, Kranendonk Michel:** A Bacterial Cell Platform for Functional Evaluation of Cytochrome P450 Oxidoreductase Variants ..... 65

**PP18, Makryniotis Konstantinos:** Stabilizing the thermophilic *Dm*PETase to improve PET degradation by computational protein engineering ..... 67

**PP19, Marian (Toca) Gianina:** Evaluation of reducing sugar levels in wine using enzymatic methods ..... 70

**PP20, Mekmouche Yasmina:** Tailoring Oxidative Enzymes and Catalytic Cascades for Efficient Biocatalysis ..... 71

**PP21, Nikolaivits Efstratios:**Tuning the polyesterase promiscuity of Antarctic MoPE: Biochemical and docking insights into substrate-specificity shifts ..... 73

**PP22, Sedlák Erik:** Yeast surface display of the human kappa opioid receptor to enhance solubility ..... 74

**PP23, Timková Ivana:** Directed Evolution by Ribosome Display Optimizes Haloalkane Dehalogenases for Bioremediation ..... 76

**PP24, Wanz Aron:** Defluorination by Design: ML guided Enzyme Engineering ..... 78

**PP25, Zerva Anastasia:** Biochemical and computational tools for the study and valorization of white-rot fungal xyloglucanase *Abi*XegA ..... 79

**PP25, Zuson Jasmin:** Uncovering the Evolutionary Origin of Enantioselectivity in Borneol-type Dehydrogenases ..... 81

EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

# 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 16-17, Belgrade, Serbia

| Time | Thur / 16.4 | Fri / 17.4 |
|---|---|---|
| 8.30-9.00 | Registration | |
| 9.00-9.30 | | WG2 |
| 9.30-10.00 | Welcome by the Action Chair and LO | |
| 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 | MC meeting | Poster Session |
| 15.30-16.00 | | |
| 16.00-16.30 | | Closure (Action Vice Chair and LO) |
| 16.30-17.00 | | |
| 17.00-18.30 | | |
| 18.30-20.30 | Meet up for sight-seeing at 18.15 h | Cocktail in Botanical garden |
| 20.30 | Dinner | |

# Overview of invited lectures

| Speaker | Title |
|---|---|
| **WG1 Session** | Chair: **Dr. Sérgio Marques** (WG1 leader) |
| **Dr. Bruno Victor** | *Assessing methodological dependencies in transmembrane protein simulations* |
| **Dr. Alfonso Gautieri** | *Engineering of hyperstable and acid-tolerant PETase enzymes for plastic biodepolymerization* |
| **Prof. Marianne Rooman** | *Integrating statistical potentials, (co)evolutionary information, and language models to boost protein stability change predictions* |
| **Dr. Peter Stockinger** | *Ensemble-aware design of thermostable enzymes* |
| **Dr. Marc Garcia-Borràs** | *Harnessing reactive intermediates: Computationally guided enzyme design for abiological transformations* |
| **WG2 Session** | Chairs: **Prof. Lucia Gardossi and Prof. Aleksandra Maršavelski** (WG2 leaders) |
| **Prof. Miguel Machuqueiro** | *Computational Methods to Capture pH Effects in Proteins* |
| **Prof. Aleksandra Maršavelski** | *Engineering thermostability in metagenomic polyesterase* |
| **Dr. Gemma Kinsella** | *Activation of CALB by alkylammonium ions: a new paradigm for activity enhancement of enzymes* |
| **Prof. Jan Deska** | *Synthetic applications and de novo design of haloperoxidases* |
| **Dr. Pedro Sánchez Murcia** | *Use of conformational ensembles of enzymes to assign functional effects* |

| Speaker | Title |
|---|---|
| **WG3 Session** | Chairs: **Prof. Lígia O. Martins and Prof. Jürgen Pleiss** (WG3 leaders) |
| **Prof. Robert Kourist** | *Mechanism-inspired enzyme engineering of hydrophobic pockets for improved enzyme activity* |
| **Dr. Erica Elisa Ferrandi** | *Functional characterization and structure-guided engineering of a thermostable (S)-selective amine transaminase from Streptomyces sp. BV333* |
| **Dr. Io Antonopoulou** | *Discovery and improvement of novel carbonic anhydrases towards accelerating post-combustion carbon capture* |
| **Prof. Ayelet Fishman** | *Development of an enhanced enzymatic degradation system of polylactic acid* |
| **Prof. Lígia Martins** | *Network dynamics as fingerprints of thermostability in an In Silico-engineered DyP-type peroxidase* |
| **STSM Session** | Chairs: **Dr. Nataša Božić and Prof. Anamaria Todea** |
| **Konstantinos Grigorakis** | *Mapping the catalytic free-energy landscape of an engineered MHET hydrolase via QM/MM Simulations* |
| **Tomás Frazão** | *Mechanistic determinants of substrate specificity in bacterial glycoside 3-oxidase* |
| **Armen Sargsyan** | *Increasing the substrate specificities of N-carbamoyl-β-alanine amidohydrolase by computational modelling* |
| **Sady Roberto Rodriguez** | *Rational design and computational analysis of Candida boidinii formate dehydrogenase for improved $CO_2$-reducing activity* |
| **Rui P. P. Neves** | *Deep-learning the committor function for the accurate characterization of the mechanism of sulphide oxidation by the bacterial flavin-dependent sulphide:quinone reductase* |
| **Garazi Ortiz-Orruño** | *High-throughput screening for enhanced HMFO expression* |

# Overview of poster presentations

| Presenter | | Title |
|---|---|---|
| **WG1** | | |
| **01** | Sladek Vladimir | Some computational perspectives on residue roles in dynamic protein complexes |
| **02** | Vardar-Yel Nurcan | Dipeptide-Based Modeling of Protein Melting Temperature Using Linear and Nonlinear Machine Learning Approaches |
| **WG2** | | |
| **03** | Batista Marta | pH Driven Conformational and Protonation Changes in LipT6 Revealed by CpHMD Simulations |
| **04** | Das Nayanika | Computational analysis of the free energy profile of the first step of Glutathione Peroxidase 6 (GPX6) catalysis: insights into the role of selenocysteine and species-specific variations |
| **05** | Krammer Lisa-Marie | Bayesian optimization of ancestral arylmalonate decarboxylase for the asymmetric synthesis of sterically hindered profen derivatives |
| **06** | Pavlović Marija | Structure-guided analysis of a pectin lyase from *Bacillus velezensis* |
| **07** | Pires D. S. Inês | *In silico* study of the pH-dependent Au nanoparticle growth mechanism of a novel artificial metalloenzyme |
| **08** | Raczyńska Agata | Unraveling the Molecular Basis of Polyester-Polyurethane Hydrolysis: Structural Insights and Engineering of TfCut2 |
| **09** | Stefanović Marija | Biochemical and Structural Characterization of a Novel Gluten-Hydrolyzing protease from *Bacillus velezensis* 16B |
| **10** | Tomić Antonija, Matić Antonia | Regulating the Antioxidative Response by Designing Kelch Domain Mutants to Inhibit DPP III Activity in the Keap1–Nrf2 Signaling Pathway |
| **11** | Wiley Brian | Mechanistic Insights into Dioxygen Diffusion in the PHD2 Oxygenase from Long-Timescale Simulations |
| **WG3** | | |
| **12** | Borges Patricia | Structural Loop Dynamics Drive Enhanced Mn(II) Oxidation in *Pp*DyP |
| **13** | Gand Martin | Identification and application of oxidative enzymes from *Cerrena zonata* unraveled by a multi-omics and *in silico* approach |
| **14** | Gardossi Lucia | Enzymes for synthesis and degradation of bio-based polyesters: Automatic *in silico* screening and experimental validation |

| | | |
|---|---|---|
| **15** | Häussler Max | Data Processing and Kinetic Modelling of HPLC Reaction Time Courses with EnzymeML Tools |
| **16** | Kosić Višnja | Assessment of Pullulanase Variant Stability under Operational Conditions |
| **17** | Kranendonk Michel | A Bacterial Cell Platform for Functional Evaluation of Cytochrome P450 Oxidoreductase Variants |
| **18** | Makryniotis Konstantinos | Stabilizing the thermophilic *Dm*PETase to improve PET degradation by computational protein engineering |
| **19** | Marian (Toca) Gianina | Evaluation of reducing sugar levels in wine using enzymatic methods |
| **20** | Mekmouche Yasmina | Tailoring oxidative enzymes and catalytic cascades for efficient biocatalysis |
| **21** | Nikolaivits Efstratios | Tuning the polyesterase promiscuity of Antarctic MoPE: Biochemical and docking insights into substrate-specificity shifts |
| **22** | Sedlák Erik | Yeast surface display of the human kappa opioid receptor to enhance solubility |
| **23** | Timková Ivana | Directed Evolution by Ribosome Display Optimizes Haloalkane Dehalogenases for Bioremediation |
| **24** | Wanz Aron | Defluorination by Design: ML guided Enzyme Engineering |
| **25** | Zerva Anastasia | Biochemical and computational tools for the study and valorization of white-rot fungal xyloglucanase *Abi*XegA |
| **26** | Zuson Jasmin | Uncovering the Evolutionary Origin of Enantioselectivity in Borneol-type Dehydrogenases |

*Invited lecture: WG1*

# Assessing Methodological Dependencies in Transmembrane Protein Simulations

Marta S.P. Batista[1], Miguel Machuqueiro[1], Bruno L. Victor [1,2,3]

bruno.victor@ipluso.pt

[1] BioISI – Instituto de Biossistemas e Ciências Integrativas, Faculdade de Ciências, Universidade de Lisboa, 1749-016, Lisboa, Portugal
[2] Accelbio: Collaborative Laboratory to Foster Translation and Drug Discovery, 3030-197 Cantanhede, Portugal
[3] EET - Escola Superior de Engenharia e Tecnologias, IPLuso - Instituto Politécnico da Lusofonia, 1950-396 Lisboa, Portugal

Membrane proteins and membrane-associated enzymes are indispensable to numerous biological processes. However, their rational engineering and optimization remain severely bottlenecked by their structural complexity and extreme sensitivity to the surrounding lipidic environment. Aquaporins (AQPs), a ubiquitous family of transmembrane channels responsible for the transport of water and small solutes serve as paradigmatic, highly demanding systems for computational modeling and protein redesign. Their core function hinges on highly conserved structural motifs, dynamic pore conformations, and intricate lipid-protein interactions, positioning them as ideal benchmarks for the development and validation of computational protocols targeting improved protein stability and functionality.

Recent studies combining computational and experimental methodologies have provided high-resolution atomistic insights into AQP function and modulation, including the identification of small-molecule inhibitors capable of interfering with glycerol permeation in aquaglyceroporins. Concurrently, computational analyses have demonstrated that simulation outcomes for AQPs are highly sensitive to methodological variables, specifically force-field parameterization, membrane mimetic representation, and system scale. This extreme sensitivity underscores the critical need for systematic benchmarking and the standardization of robust modeling workflows (1).

Directly aligned with the objectives of Working Group 1 of the COST Action COZYME, this work showcases the systematic development and rigorous evaluation of a computational pipeline designed to enhance the predictive modeling and optimization of complex proteins. By utilizing AQPs as representative membrane systems, this study identifies key methodological parameters that dictate simulation reliability and predictive accuracy (2). Ultimately, this work defines the computational resources and standardized protocols necessary to establish robust, reproducible platforms. Such frameworks are critical for driving rational protein redesign efforts, specifically focusing on the optimization of global macroscopic properties within complex biomolecular environments.

**Acknowledgements:** Fundação para a Ciência e a Tecnologia through grant 2023.03251.BD and projects 2023.10710.CPCA.A2, 2025.00038.CPCA.A3, and 2025.07857.CPCA.A3.

**References:**

1. Pacetti-Alves, I., et al. “Unraveling the aquaporin-3 inhibitory effect of rottlerin by experimental and computational approaches”, *Int. J. Mol. Sci.,* **2023**, 365, *24, 6, 6004*
2. Batista et al, 2026, *submitted*.

*Invited lecture: WG1*

# Engineering of HyperStable and Acid-tolerant PETase Enzymes for Plastic Biodepolymerization

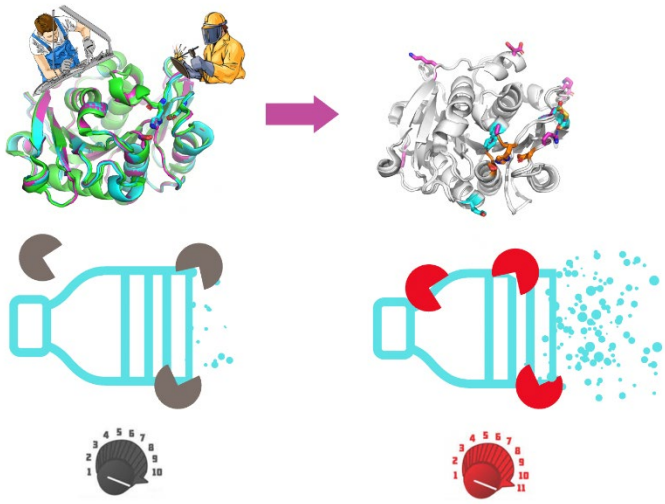
Alfonso Gautieri[1]

alfonso.gautieri@polimi.it

[1]Biomolecular Engineering Lab, Dipartimento di Elettronica, Informazione e Bioingegneria, Politecnico di Milano, 20133 Milan, Italy

Polyethylene terephthalate (PET), representing about 6% of global plastic production, is a major contributor to solid waste and environmental pollution. Enzymatic PET recycling has emerged as a promising route for circular-plastic valorization, yet most PET-hydrolyzing enzymes still face barriers to industrial deployment, including limited thermostability, modest catalytic efficiency, product inhibition, and loss of activity under acidic conditions. Building on the current benchmark enzyme LCC-ICCG [1] and its thermostable variant C09 [2], this work focuses on improving performance in acidic environments while preserving substrate binding and catalytic activity.

An initial strategy was based on introducing positive charges near the catalytic histidine to lower its pKa, which showed to enhance acid tolerance but proved to also reduce activity through unfavorable electrostatics. To systematically assess new designs, we developed a computational protocol combining molecular docking and molecular dynamics to evaluate ligand affinity and binding stability in candidate mutants, and introduced an additional screening criterion to flag mutations likely to raise the reaction energy barrier near the catalytic triad. Using complementary approaches such as homology modeling guided by known acid-active PETases, and a genetic algorithm to explore combinatorial mutants across 52 catalytic-neighborhood positions, we identified promising variants and key sequence positions for pKa modulation. These results provide actionable starting points for experimental validation and further optimization of PETases for robust recycling under industrially relevant conditions.



EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

**Acknowledgements:** The author wish to thank the European Union's HORIZONWIDERA-2023-ACCESS-04 program for financial support under grant agreement 101159534 (WIDEnzymes), the COST Action COZYME for financial support via STSM funding, and CINECA under the ISCRA initiative for the availability of high-performance computing resources. The author wish to thank collaborators Emilio Parisini, Rossella Castagna, Shapla Bhattacharya, Alessio Perazzoli, Sabrina Guerini Rocco, Sabrina Frigerio.

**References:**

3. Tournier, V. *et al.* An engineered PET depolymerase to break down and recycle plastic bottles. *Nature* **580**, 216–219 (2020).
4. Bhattacharya, S. et al. Development of a highly active engineered PETase enzyme for polyester degradation. *FEBS J.* **293**, 443–455 (2025).

*Invited lecture: WG1*

# Integrating statistical potentials, (co)evolutionary information, and language models to boost protein stability change predictions

P. Hermans[1], M. Tsishy[1], F. Pucci[1], M. Rooman[1]

pauline.hermans@ulb.be, matsvei.tsishyn@ulb.be, fabrizio.pucci@ulb.be, marianne.rooman@ulb.be

[1]Computational Biology and Bioinformatics, Université Libre de Bruxelles, Belgium

Determining the impact of mutations on protein stability and other biophysical properties is essential for a wide range of applications including rational protein design and genetic variant interpretation. We have contributed to this field through the development of several computational tools. The earliest method, PoPMuSiC [1], predicts how point mutations affect protein stability by leveraging structural information and statistical potentials. It combines high speed with good prediction accuracy and remained competitive over the years. To enhance its performance, we are developing a new version of PoPMuSiC that incorporates additional types of information. In this context, we first developed a simple evolutionary model, RSALOR [2], which is based on the log-odds ratio of wild-type and mutated residue frequencies in evolutionarily related proteins, scaled by each residue's relative solvent accessibility. This model performs as well as, or slightly better than, most benchmarked predictors, many of which are considerably more complex. To account for epistatic effects, we have developed a co-evolutionary model, StructureDCA [3], which employs a direct coupling analysis approach and incorporates residue–residue distances and solvent accessibility. Finally, we tested several protein language models designed to capture complex sequence patterns and evolutionary relationships for predicting mutational impacts. The new version of PoPMuSiC [4] is built on these analyses and integrates a broad range of complementary sequence, structural, evolutionary, and co-evolutionary information to provide a robust predictor of mutation effects on protein stability.

**Acknowledgements:** The FNRS scientific research fund is acknowledged for financial support.

**References:**

1. Y. Dehouck, A. Grosfils, B. Folch, D. Gilis, Ph. Bogaerts, M. Rooman. *Bioinformatics* **2009**, *25*, 19, 2537-2543.
2. M. Tsishyn, P. Hermans, M. Rooman, F. Pucci. *Bioinformatics* **2025**. 41, 6, btaf322.
3. M. Tsishyn, P. Hermans, M. Rooman, F. Pucci. **2026**, submitted.
4. P. Hermans, M. Tsishyn, M. Rooman, F. Pucci. **2026**, in preparation.

*Invited lecture: WG1*

# Ensemble-Aware Design of Thermostable Enzymes

Peter Stockinger [1,2]

peter.stockinger@unibe.ch

[1] University of Bern, Department of Chemistry, Biochemistry and Pharmaceutical Sciences
[2] Swiss Institute of Bioinformatics, Interfaculty Bioinformatics Unit

Engineering enzyme thermostability remains a major challenge. Most computational approaches rely on single static structures, implicitly assuming that rigidification enhances stability. However, most proteins populate dynamic conformational ensembles whose temperature-dependent behavior shapes both thermal adaptation and function. As a result, stabilization strategies based on static structures are highly sensitive to the chosen input model. Consistent with this view, our analysis of previously published PETase variants showed that thermostable enzymes can rigidify in a counterintuitive manner at elevated temperatures, rather than simply becoming globally stiffer. This finding underscores the importance of explicitly accounting for conformational ensembles when designing for thermostability. Adding to this complexity, we observed that established stability predictors perform poorly for multiple point variants, suggesting that neglecting effects on conformational dynamics limits their predictive power. To address this limitation, we developed an ensemble-aware stability scoring framework that estimates energies from molecular dynamics–derived conformational ensembles, thereby supporting the computational design of enzymes that are both thermostable and active.

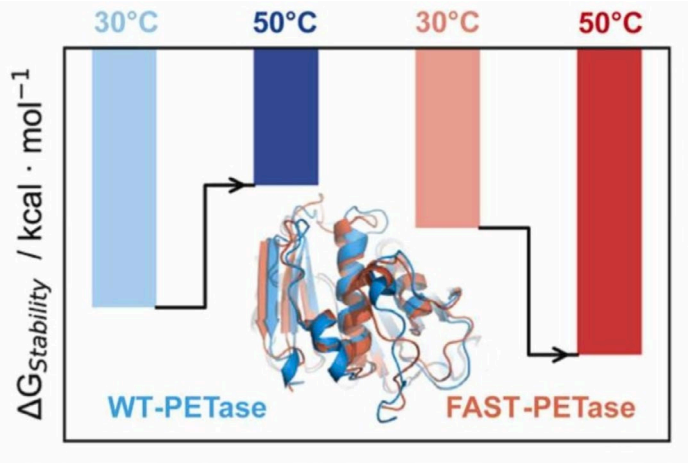



*Figure 1. Temperature-induced stabilization of FAST-PETase*

**Acknowledgements**: COZYME, NCCR Catalysis, SIB, Prof. Dr. Rebecca Buller, Prof. Dr. Sebastien Farnaud, Dr. Sean Hüppi and Cornel Niederhauser.

**References:**

1. M. Scherer, *JACS*, **2025**, 147, 1, 1911-1918.
2. P. Stockinger, *CSBJ*, **2025**, 27, 1, 969-977.

*Invited lecture: WG1*

# Harnessing Reactive Intermediates: Computationally Guided Enzyme Design for Abiological Transformations

Marc Garcia-Borràs[1,2]

marc.garcia@udg.edu

[1]Institut de Química Computacional i Catàlisi (IQCC) and Departament de Química, Facultat de Ciències, Universitat de Girona (UdG), Carrer M. Aurèlia Capmany 69, 17003 Girona, Spain
[2]ICREA, Pg. Lluís Companys 23, 08010 Barcelona, Spain

Enzymes catalyze complex chemical reactions with high specificity, efficiency, and selectivity. Many enzymes require cofactors that participate in biocatalytic cycles involving the formation of organometallic, ionic, or radical reactive intermediates. These reactive intermediates can follow divergent reaction pathways, leading to enzymatic promiscuity and providing opportunities for designing new challenging enzyme-catalyzed transformations. Experimental characterization of these "*fleeting*" reactive intermediates is challenging due to limitations in structural and spectroscopic techniques.[1] However, computational methods offer a powerful alternative for describing these intermediates with atomic-level and real-time precision.[2]

Our research program aims to develop and apply new multiscale computational protocols to study, characterize, and rationally improve the formation and stabilization of highly reactive intermediates in enzyme active sites, directing them towards desired reaction pathways.[1] This can be achieved through designing precision confinement and specific polar environment within the protein's active site. The ultimate goal is to implement new synthetically useful biocatalytic reactions. Herein, we present recent successful cases in computationally guided enzyme engineering, showcasing how precision confinement and tailored active-site electrostatics can be leveraged to direct enzymatic transformations toward synthetically valuable outcomes. These strategies have led to the successful implementation of metalloenzyme-catalyzed non-natural oxidative processes, carbene and nitrene transfers, and radical-mediated C–H functionalization.[3,4,5] Our findings provide atomistic descriptions and fundamental insights into enzymatic catalysis and offer a blueprint for designing enzymes capable of performing novel transformations with potential applications in green chemistry, pharmaceutical synthesis, and sustainable catalysis.

**References:**

1. M. Reetz, M. Garcia-Borràs, *J. Am. Chem. Soc.*, **2021**, 143, 14939.
2. A. Romero-Rivera, M. Garcia-Borràs, S. Osuna, *Chem. Commun.*, **2017**, 53, 284.
3. (a) M. Garcia-Borràs, S. B. J. Kan, R. D. Lewis, A. Tang, G. Jimenez-Osés, F. H. Arnold, K. N. Houk, *J. Am. Chem. Soc.*, **2021**, 143, 7114. ; C. Calvó-Tusell, Z. Liu, K. Chen, F. H. Arnold, M. Garcia-Borràs, *Angew Chem Int Ed,* **2023***, 62, 35, e202303879.*
4. (a) Soler, J.; Gergel, S.; Hammer, S.; Garcia-Borràs, M. *J. Am. Chem. Soc.*, **2022**, 144, 35, 15954. DOI: 10.1021/jacs.2c02567 ; (b) Gergel, S.; Soler, J.; Klein, A.; Schülke, K.; Hauer, B.; Garcia-Borràs, M.; Hammer, S., *Nat Catal,* **2023,** *6, 7,* 606-617.
5. Rui, J.; Zhao, Q.; Huls, A. J.; Soler, J.; Paris, J. C.; Chen, Z.; Reshetnikov, V.; Yang, Y.; Guo, Y.; Garcia Borràs, M.; Huang, X. *Science*, **2022**, 376, 6595, p.869-874.

*Invited lecture: WG2*

# Computational Methods to Capture pH Effects in Proteins

Marta Batista, Inês Pires, Miguel Machuqueiro

machuque@ciencias.ulisboa.pt

BioISI: Biosystems and Integrative Sciences Institute, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

Constant-pH molecular dynamics (CpHMD) simulations are powerful computational tools that couple biomolecular conformational space to pH, allowing the protonation states of ionizable sites to change dynamically in response to their local environment [1]. This is a significant improvement over traditional MD simulations, which use fixed protonation states, because it provides a more realistic description of pH-dependent effects on molecular structure and function. Recent advances have focused on enhancing the accuracy and efficiency of these methods, thereby extending their applicability to larger and more complex systems.

In the meeting, we will present our most recent advances using CpHMD simulations of gold nanoparticles (AuNPs) modified with *Geobacillus thermocatenulatus* (GTL), in collaboration with the Palomo group in Madrid. We built two variant systems (S114C and A193C), in which AuNP growth occurs either at the protein surface or at the active site, coordinated by the mutated cysteine. Our *in silico* models help to understand how pH can alter the nanoparticle size and shape [3].

In another project, we joined forces with the Fishman group in Haifa to study the lipase *Geobacillus stearothermophilus* T6 (LipT6). This promising biocatalyst converts waste oils into fatty acid methyl esters (biodiesel) [4]. Our computational model aims to understand the molecular basis for the decrease in enzymatic activity under acidic conditions.

**Acknowledgements:** This work was supported by the European Cooperation in Science and Technology (CA21162 COST Action) and FCT, Portugal (UID/04046/2025).

**References:**

1. Gomes, A. M. M., Costa, P. J., Machuqueiro, M. *BBA Advances*, **2023**, 4, 100099.
2. Yousef, M. J. M., Oliveira, N. F. B., Vitorino, J. N. M., Reis, P. B. P. S., Draczkowski, P., Maj, M., Jozwiak, K., Machuqueiro, M. *J. Chem. Theory Comput.*, **2025**, 21, 2655-2667.
3. C. Garcia-Sanz, B. De Las Rivas,J. M. Palomo. *Nanoscale* **2024** 16, 14, 6999-7010.
4. A. Dror, E. Shemesh, N. Dayan, A. Fishman. *Appl. Environ. Microbiol.*, **2014**, 80, 1515-1527

*Invited lecture: WG2*

# Engineering thermostability in metagenomic polyesterase

Aleksandra Maršavelski,[1] Ivana Kekez,[1] Lucija Brtan,[1] Anđela Pustak,[2] Andrea Mattevi,[3] Margaux Aubel,[4] Erich Bornberg-Bauer[4]

amarsavelski.chem@pmf.hr

[1] University of Zagreb, Faculty of Science, Department of Chemistry, Horvatovac 102a, 10000 Zagreb, Croatia
[2] Ruđer Bošković Institute, Division of Materials Chemistry, Bijenička cesta 54, 10000 Zagreb, Croatia
[3] Department of Biology and Biotechnology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy
[4] Institute for Evolution & Biodiversity, University of Münster, Hüfferstraße 1, Münster, Germany

Plastic pollution poses an escalating threat to environmental and public health, underscoring the urgent need for efficient biocatalytic strategies to degrade synthetic polymers, among which polyesters[1] represent a major fraction. In this study, we report the engineering of a novel polyesterase, MGY, identified from a metagenomic dataset and exhibiting poly(lactic acid) (PLA)–degrading activity. While MGY showed promising catalytic potential, its low solubility, poor expression yield, and limited thermostability constrained its practical applicability. To overcome these limitations, we employed ancestral sequence reconstruction[2] to generate ancestral variants of MGY. All reconstructed variants retained substantial PLA-degrading activity, and one variant additionally exhibited activity toward polycaprolactone (PCL). Differential scanning calorimetry revealed a melting temperature ($T_m$) of 84 °C for the most stable variant, representing the highest $T_m$ reported to date for a PLA-degrading enzyme. Furthermore, thermal characterization of PLA and PCL substrates[3] enabled the identification of optimal temperature ranges for efficient enzymatic degradation. Structural and computational analyses indicate that enhanced thermostability arises primarily from loop rigidification,[4] achieved through direct modifications of a short loop and through remote core mutations stabilizing a longer loop-helix motif (Figure 1). Despite similar numbers of noncovalent interactions—including hydrogen bonds, persistent native contacts, and hydrophobic interactions—between the longer loop-helix motif in both the mesophilic wild-type MGY and the thermophilic ancestral variant and the rest of protein structure, the latter exhibits a substantially higher average number of simultaneous hydrophobic contacts. Notably, 52 remote mutations distinguishing the thermophilic ancestral variants from the mesophilic wild-type MGY were sufficient to stabilize the flexible longer loop-helix region without altering its primary sequence. Collectively, these findings provide mechanistic insights into enzyme thermostability and demonstrate ancestral sequence reconstruction as a powerful strategy for engineering robust polyester-degrading biocatalysts.

EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

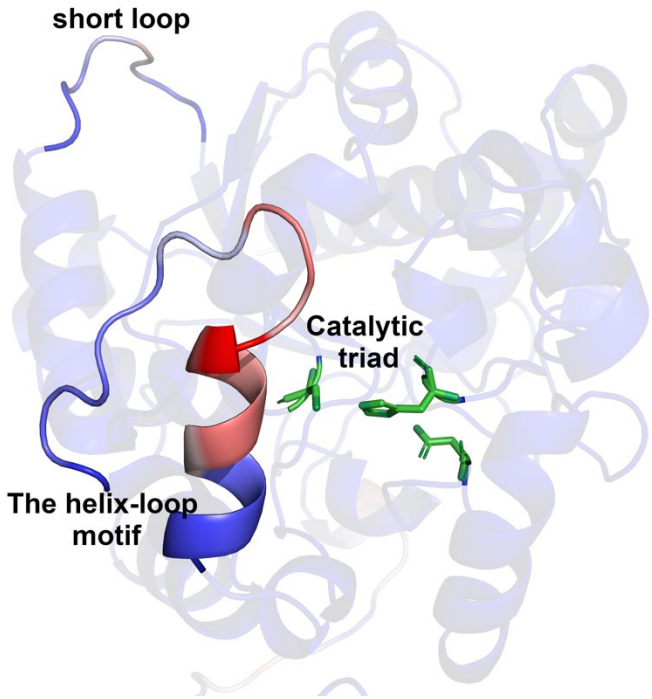



*Figure 1. B-factor–mapped flexibility of loop regions in MGY.*

*Ribbon representation of MGY colored according to B-factors, with blue indicating low mobility and red indicating high mobility. The short loop and the helix–loop motif adjacent to the active site are highlighted, revealing elevated flexibility relative to the protein core. The catalytic triad residues are shown as green sticks. The remainder of the protein is displayed as a semi-transparent cartoon to provide structural context.*

**Acknowledgements:** This research was funded through National Recovery and Resilience Programme, The Development Research Support (NextGenerationEU), project Enzyme engineering for sustainable recycling of bioplastics (NPOO.C3.2.R2-I1.06.0041). We also acknowledge the Short-Term Scientific Missions (STSM) grant from COST Action CA21162.

**References:**

1. Y. Wang, R. J. van Putten, A. Tietema, J. R. Parsons, G. M. Gruter. *Green Chem.*, **2024**, 26, 7, 3698–3716.
2. M. A. Spence, J. A. Kaczmarski, J. W. Saunders, C. J. Jackson. *Curr. Opin. Struct.* Biol., **2021**, 69, 131–141.
3. N. G. Khouri, J. O. Bahú, C. Blanco-Llamero, P. Severino, et al. *J. Mol. Struct.*, **2024**, 1309, 138243–138259.
4. H. Yu, H. Huang. *Biotechnol. Adv.*, **2014**, 32, 2, 308–315.

*Invited lecture: WG2*

# Activation of CALB by alkylammonium ions: a new paradigm for activity enhancement of enzymes

Rangasamy Savitha[1], Ebin K. Baby[1], Kieran Nolan[2], Barry J. Ryan[1], Gemma K. Kinsella[1], Gary T. M. Henehan[1]

Gemma.kinsella@tudublin.ie; gary.henehan@tudublin.ie

[1] School of Food Science and Environmental Health, Technological University Dublin, Grangegorman Lower, Dublin 7, D07 E244, Ireland.
[2] School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9, D09 V209, Ireland.

*Candida antarctica* lipase B (CALB) is widely used in biocatalysis with applications in plastics degradation and chemical synthesis. CALB is activated by hydrophobic matrices and shows striking activation in polar, choline-based, Deep Eutectic Solvents (DES). CALB activation and stabilisation by tetraalkylammonium (TAA) is caused by binding to choline's TAA moiety. Several related TAA salts also caused CALB activation which was proportional to the hydrophobicity of their alkyl substituents. Notably, tetraoctylammonium bromide showed activation of ~500% even at low micromolar levels. These TAA salts represent a new class of enzyme activator. Molecular modelling identified the possible alkylammonium binding location as a hydrophobic patch centred around Asp-145 of CALB and binding at this site could explain lipase activation in polar DES solvents.

CALB, like many lipases, is activated by calcium. Intriguingly, mixed soluble activator experiments showed that calcium and choline bind to different CALB sites, suggesting a two-site model for CALB activation. These observations, along with previous findings, show that TAA activation is a wider property of enzymes and constitutes a novel and potent means to enhance enzyme turnover and stability.

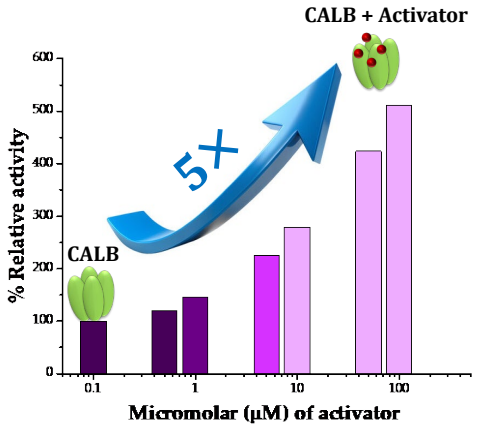



*Figure 1. Activation of CALB by alkylammonium ions.*

**Acknowledgements:** This research was funded under a Science Foundation Ireland (SFI) Programme, Frontiers for the Future: grant number 21/FFP-A/9898.

*Invited lecture: WG2*

# Synthetic applications and de novo design of haloperoxidases

Jan Deska,[1] Heather Jameson,[1] Juhana Aho,[1] Janne Naapuri[1,2]

jan.deska@helsinki.fi

[1] University of Helsinki, Department of Chemistry, A.I. Virtasen aukio 1, 00560 Helsinki, Finland
[2] Tampere University, Department of Materials Science & Environmental Engineering, Korkeakoulunkatu 7, 33720 Tampere, Finland

Haloperoxidases are oxidative enzymes that generate electrophilic hypohalite species from halide salts and hydrogen peroxide, enabling mild and selective halogenation reactions with broad synthetic utility. Their ability to replace hazardous molecular halogens with simple alkali halides makes them highly attractive tools in sustainable organic synthesis.[1]

Our group has systematically explored heme- and vanadium-dependent haloperoxidases in the halocyclization of allenic alcohols and carboxylates, where we developed biocatalytic protocols for aerobic 5-endo-trig [2,3] and 5-exo-trig bromocyclization [4] to brominated dihydrofurans with excellent yields and full stereochemical fidelity. Moreover, sequential one-pot coupling of the enzymatic halocyclizations with e.g. palladium-catalyzed cross-couplings delivered densely functionalized dihydrofurans in a single vessel. In addition, choline-based ionic liquids were introduced as dual-purpose additives,[5] simultaneously serving as substrate-solubilizing agents and hydrogen peroxide sources via choline oxidase. Building on this haloperoxidase expertise, we have now designed a de novo metallopeptide catalyst by engineering a histidine brace onto a carbohydrate binding module.[6] The resulting micro-enzymes (57-62 amino acids) that can be obtained through solid-phase peptide synthesis exhibit haloperoxidase-type reactivity in presence of vanadate, and while activities of the initial designs still remain low, they represent a novel motif to further explore in the future.

**Acknowledgements:** European Research Council (ABIONYS, 865885)

**References:**

1. G. T. Höfler, A. But, F. Hollmann, *Org. Biomol. Chem.* **2019**, *17*, 9267.
2. J. Naapuri, J. D. Rolfes, J. Keil, C. Manzuna Sapu, J. Deska, *Green Chem.* **2017**, *19*, 447.
3. J. Naapuri, G. A. Åberg, J. M. Palomo, J. Deska, *ChemCatChem* **2021**, *13*, 763.
4. J. Naapuri, P. K. Wagner, F. Hollmann, J. Deska, *ChemistryOpen* **2022**, *11*, e202100236.
5. M. Hallamaa, J. Naapuri, R. A. L. Silva, A. A. Rosatella, J. Deska, *ChemCatChem* **2025**, *17*, e202401216.
6. J. A. S. Aho, H. Jameson, L. Enders, J. Deska, *ChemRxiv* **2025**, 10.26434/chemrxiv-2025-spvxf

*Invited lecture: WG2*

# Use of conformational ensembles of enzymes to assign functional effects

Spela Mandl, Daniel J. Platero-Rochart, and Pedro A. Sánchez Murcia[1]

pedro.murcia@medunigraz.at

[1]Laboratory of Computer-Aided Molecular Design, Division of Medicinal Chemistry, Otto-Loewi Research Center, Medical University of Graz, Graz, Austria

The primary function of enzymes in biological environments is determined by their chemical configuration, which arises from a specific amino acid sequence as well as from post-translational modifications within the cell. Thus, by changing the enzyme sequence, its function can be altered. Over the years, significant progress has been made in the development of more stable enzyme scaffolds. More challenging, however, is estimating the impact of changes in an enzyme's amino acid sequence on its function, especially when these changes occur at positions remote from the active site. To address this question, our research group is developing metrics to describe, on a per-residue basis, the impact of such changes on enzyme dynamics, stability, and communication with the active site.[1,2] Together with experimental validation, we have analysed several defective human lysosomal hydrolases that cause diseases belonging to the group of lysosomal storage disorders with the final goal of predicting the impact of missense mutations on function.

**Acknowledgements:** Austrian FWF, City of Graz, Medical University of Graz.

**References**

1. B. Di Geronimo, Š. Mandl, S. Alonso-Gil, B. Žagrović, G. Reibnegger, C. Nusshold, P. A. Sánchez-Murcia, *J. Chem. Inf. Model.* **2025**, 65, 2650–2659 DOI: 10.1021/acs.jcim.4c02229.
2. Š. Mandl, B. Di Geronimo, S. Alonso-Gil, C. Grininger, G. George, U. Ferstl, S. A. Herzog, B. Žagrović, C. Nusshold, T. Pavkov-Keller, P. A. Sánchez-Murcia, *Protein Sci.* **2025**, 34, e70080 DOI: https://doi.org/10.1002/pro.70080.

*Invited lecture: WG3*

# Mechanism-inspired enzyme engineering of hydrophobic pockets for improved enzyme productivity – an experimentalist's perspective

Robert Kourist

kourist@tugraz.at

Graz University of Technology

The on-going digitalization of biotechnology changes the way how enzymes can be approved for biocatalytic application. Two examples illustrate the enormous impact of bioinformatics and machine learning on the engineering of powerful biocatalysts.

A first example deals with an artificial pathway for the synthesis of Tulipalin A, a very promising polymer precursor. While Tulipalin A can be isolated from the flowers of tulips and alstroemerias, its biosynthesis remains unknown. We propose a synthesis from isoprenyl acetate, which itself can be produced via the microbial hemiterpenoid metabolism. The crucial reaction step is catalyzed by a membrane-bound monooxygenase. The difficulty to obtain accurate structure information has been a serious obstacle for their optimization by enzyme engineering. Here we show how de novo structure prediction tools and molecular modeling by Rosetta docking can guide the generation of tailor-made enzyme variants with higher activity.

A second example presents the expansion of the substrate scope of bacterial arylmalonic acid decarboxylase (AMDase) inspired by the mechanism. The activity of bacterial arylmalonate decarboxylase is controlled by a hydrophobic pocket. Metadynamic simulations show that an increase of the hydrophobic surface in the active site by saturation mutagenesis increased the activity of the enzyme. Prompted by the finding that the enzyme has an unusually low stereoselectivity in the decarboxylation of 2-methyl-2-vinyl malonate, we investigated the mechanism by isotope labeling studies and QM/MM metadynamics calculations. The results indicated an alternative binding mode, which was exploited for the conversion of sterically hindered alpha-ethyl malonic acids that are not accepted by the wildtype enzyme.

**References:**

1. A. Nigl; V. Delsoglio; R. Kourist. *Angew. Chem. Int. Ed.* **2025**, 64, e202503464. DOI: 10.1002/anie.202503464s.
2. E. van der Pol; T. Schlatzer; G. Hoffka; B. Di Geronimo; R. Breinbauer; R. Kourist. *J. Am. Chem. Soc.* **2025**, 147, 39271–39283. DOI: 10.1021/jacs.5c10721.

*Invited lecture: WG3*

# Functional Characterization and Structure-Guided Engineering of a Thermostable (S)-Selective Amine Transaminase from *Streptomyces* sp. BV333

Stefania Patti[1], Ilaria Magrini Alunno[1,2], Simone A. De Rose[3], Michail N. Isupov[3], Sergio Riva[1], Daniela Monti[1], Jennifer A. Littlechild[3], Erica E. Ferrandi[1]

erica.ferrandi@scitec.cnr.it

[1]SCITEC-CNR, 20131 Milan, Italy
[2]Scuola Universitaria Superiore IUSS Pavia, Palazzo del Broletto, 27100 Pavia, Italy,
[3] Henry Wellcome Building for Biocatalysis, University of Exeter, Exeter EX4 4QD, United Kingdom

Amine transaminases (ATAs) are PLP-dependent enzymes that catalyze the stereoselective transfer of an amino group from an amino donor to prochiral ketone substrates, making them attractive biocatalytic tools for the synthesis of optically pure chiral amines.

Recently, our group identified an (*S*)-selective amine transaminase, Sbv333-ATA, in the genome of *Streptomyces* sp. BV333, a strain isolated from the rhizosphere of *Salvia pratensis* at the University of Belgrade. This enzyme exhibits remarkable thermostability and broad substrate specificity toward various amino acceptors [1,2]. In the present work, Sbv333-ATA was further functionally investigated and structurally characterized to assess its biocatalytic potential.

High-resolution crystal structures were solved for the native enzyme as well as for complexes with substrates and the inhibitor gabaculine. Structure-guided rational mutagenesis was subsequently employed to modulate substrate specificity. Among the generated variants, the W89A mutant showed significantly enhanced activity toward bulky substrates, highlighting its potential for further engineering. Overall, these results pave the way for the development of Sbv333-ATA as a versatile and robust biocatalyst for asymmetric synthesis [3].

**Acknowledgements:** This work was funded by the European Union, Next GenerationEU, Mission 4 Component 1, CUP D53C24003180006, PRIN Project “INTegration of directed Evolution, Rapid screening, and Intensification in flow for the Development of STereoselective biocatalytic synthesis of chiral Amines of pharmaceutical Relevance (INTERSTAR)”, project code 2022F22W44.

**References:**

1. J. Spasic, M. Mandic, J. Radivojevic, S. Jeremic, B. Vasiljevic, J. Nikodinovic-Runic, L. Djokic, *Biotechnol. Appl. Biochem.*, **2018,** 65, 822 -833
2. E. E. Ferrandi, J. Spasic, L. Djokic, Y. Vainshtein, R. Senthamaraikannan et al. *Catalysts,* **2021**, 11, 919.
3. S. Patti, S. A. De Rose, M. N. Isupov, I. Magrini Alunno et al. *Appl. Microbiol. Biotechnol.* **2025**, 109, 180.

*Invited lecture: WG3*

# Discovery and improvement of novel carbonic anhydrases towards accelerating post-combustion carbon capture

Io Antonopoulou, Ulrika Rova, Paul Christakopoulos

io.antonopoulou@ltu.se

Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental and Natural Resources Engineering, Luleå University of Technology, Porsön campus, 97187, Luleå, Sweden

Carbon Capture and Utilization and Storage (CCUS) is now openly discussed as a solution to decrease net $CO_2$ emissions, as well as a potential technology to move away from fossil carbon by using $CO_2$ 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 $CO_2$ capture is the use of the enzyme carbonic anhydrase (CA).CA is one of the fastest enzymes in nature, catalyzing the hydration of $CO_2$ to bicarbonate (CO2 + H2O <-->HCO3- + H+) which is the limiting step of $CO_2$ absorption. CA is a biocatalyst that can be employed alone in a biomimetic approach in aqueous media to capture $CO_2$, 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-sequestrating agents [2,3].

Aim of this presentation is to showcase enzyme mining towards discovery of ultra-stable CAs from harsh environments (i.e. hydrothermal vents) and the development of screening techniques to define their realistic range of operations [4]. A novel CA was discovered and 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.

**Acknowledgements:** The Swedish Energy Agency Project CO2-REDUCE is acknowledged for financial support. Part of the work presented was supported under a Short-Term Scientific Mission (STSM) grant by COZYME (CA21162) (Period: Feb-Apr 2024).

**References:**

1. A. Maciel, P. Christakopoulos, U. Rova, I. Antonopoulou, Chemosphere, 2022, 299, 134419.
2. M. Sjöblom, I. Antonopoulou, I.G. Gimenez, A. Maciel et al., ACS Sustainable Chemistry & Engineering, 2020, 8, 13672–13682.
3. A. Maciel, P. Christakopoulos, U. Rova, I. Antonopoulou, RSC Advances, 2024, 14, 6443-6461.
4. K. Rigkos, G. Saridis, I. Antonopoulou, A. Maciel, et al., Environmental Science & Technology, 2024, 58, 17732–17742.

*Invited lecture: WG3*

# Development of an enhanced enzymatic degradation system of polylactic acid

Adi Shalem[1], Fabian Glaser[2], Smadar Shulami[1], Omer Yehezkeli[1], Ayelet Fishman[1]

afishman@technion.ac.il

[1]Department of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa, Israel, 3200003

[2]Technion Center for Structural Biology, Technion-Israel Institute of Technology, Haifa, Israel, 3200003

Polylactic acid (PLA) is a bioplastic widely used in packaging, biomedicine, and 3D printing. However, its slow degradation in natural environments highlights the need for more effective biodegradation strategies. Enzymatic degradation offers a sustainable approach by breaking down PLA into reusable lactic acid monomers, yet current PLA-degrading enzymes exhibit limitations in efficiency and stability [1]. In this study, we employ directed evolution and rational protein engineering to enhance the activity and stability of a Cutinase-Like Enzyme (CLE) from Cryptococcus sp. S-2. PLA degradation rates are evaluated using various methods, including high-throughput fluorescence-based monitoring with dye embedded in PLA films and clear halo formation on emulsified PLA agarose gel. The enzyme was overexpressed in Escherichia coli, and expression was improved by approximately 100-fold by using E. coli SHuffle cells with periplasmic targeting. High-confidence models of CLE in complex with a PLA fragment were predicted using AlphaFold3, revealing a hydrophobic binding pocket that undergoes a conformational shift from an open to a closed state upon binding. Mutations of residues in the PLA binding site revealed that F52W and F52Y substitutions improved PLA degradation, whereas R28A and R28N exhibited reduced activities, highlighting the crucial role of R28. By optimizing enzymatic PLA degradation, we aim to achieve complete and efficient polymer degradation, enabling the reuse of lactic acid for biotechnological applications and contributing to a sustainable circular economy.

**Acknowledgements:** AF thanks the funding from the Israel Council for Higher Education – VATAT (#1024311) and the Israel Ministry of National Infrastructure, Energy and Water Resources (#2032809).

**References:**

1. A. Shalem, O. Yehezkeli, A. Fishman. *Appl. Microbiol. Biotechnol.*, **2024**, 108, 413.

*Invited lecture: WG3*

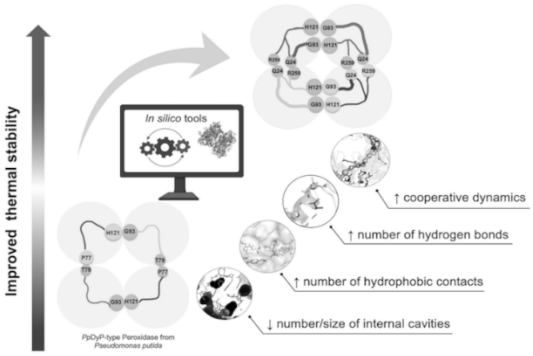
# Network Dynamics as Fingerprints of Thermostability in an In Silico-Engineered DyP-type Peroxidase

Lígia O. Martins[1], Carolina F. Rodrigues[1], Diogo Silva[1], Constança Lorena[1], Patrícia T. Borges[1], Laura Masgrau[2]

lmartins@itqb.unl.pt

[1]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av da República, 2780-157 Oeiras, Portugal;
[2]Dep.Chemistry, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

Stabilizing industrial enzymes is essential for advancing environmentally responsible bioprocesses, yet the structural basis of thermostability remains incompletely understood. Here, we engineered thermostable variants of a tetrameric dye-decolorizing peroxidase (DyP) using two independent open-source design algorithms, yielding two variant enzymes with significantly improved thermal performance and prolonged activity at elevated temperatures [1]. Subsequent recombination strategies minimize the mutational burden while maintaining or enhancing stability.




Structural and dynamic analyses of the thermostable variants revealed convergent features, including increased compactness, rigidity, and an enriched network of hydrogen bonds and hydrophobic interactions. Despite differing mutation profiles, stabilizing substitutions clustered in similar structural regions. Notably, integrating dynamic modeling with protein correlation network analysis uncovered a previously unrecognized stabilization mechanism: highly connected structural networks characterized by denser, more persistent intra- and inter-monomer interactions, greater internal cohesion, and enhanced cooperative dynamics. Tetramers exhibit long-range communication pathways and redundant routes, supporting coordinated motions that can hinder local unfolding and tetramer dissociation. These findings identify dynamic interaction networks as hypothetical critical determinants of protein stability and offer a previously unexplored framework for rational enzyme design.

Funded by the European Union

**References:**

1. Rodrigues, CF, Silva, D. Lorena, C, Borges, PT, Masgrau, Martins, LO. **2025**. **Network Dynamics as Fingerprints of Thermostability in an In Silico-Engineered DyP-type Peroxidase.** *ACS Catal* 15, 15395–15409.

*Invited lecture: STSM*

# Mapping the catalytic free-energy landscape of an engineered MHET hydrolase via QM/MM Simulations

Konstantinos Grigorakis[1], Christina Ferousi[1], Daojiong Wang[2], Natalia Kastana[1], Efstratios Nikolaivits[1], Marc van der Kamp[2], Evangelos Topakas[1*]

*vtopakas@chemeng.ntua.gr

[1]Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece

[2] School of Biochemistry, University of Bristol, Bristol, United Kingdom

Polyethylene terephthalate (PET) is the world's most extensively recycled polymer, the dominant material for beverage packaging, and valuable enough to drive sustained R&D into post-consumer recovery strategies.[1] Enzymatic depolymerization can fully convert PET into its monomers, terephthalic acid (TPA) and ethylene glycol (EG), enabling separation from mixed-plastic streams, upgrading to higher-value chemicals, and re-synthesis of virgin-quality PET, in contrast to the chain-degrading nature of conventional thermomechanical recycling.[2,3] Although various PET hydrolases (PETases) have been reported for this biorefinery route, their activity is inhibited by accumulation of the intermediate mono-(2-hydroxyethyl) terephthalate (MHET) and bis(2-hydroxyethyl) terephthalate (BHET).[4] Integrating an MHET hydrolase (MHETase), which converts MHET to TPA and EG, alleviates this bottleneck and restores full catalytic efficiency. Here, we report the engineering of a thermotolerant esterase from *Zhizhongheella caldifontis* (*Zc*EST) and its variants, including *Zc*MHETase (*Zc*EST_D355N) and *Zc*BHETase (*Zc*EST_D355S), which display up to 21-fold and 56-fold higher activity on MHET and BHET, respectively, compared to wild-type. Increased stability is also confirmed by thermal shift assays. To elucidate the molecular basis of this enhanced performance, we employed hybrid QM/MM molecular dynamics simulations using the GFN2-xTB method. These simulations mapped the catalytic free-energy landscapes, revealing that the D355N and D355S do not significantly lower the catalytic barriers for MHET and BHET hydrolysis, instead pointing to a binding-determined rate. Collectively, our findings establish ZcEST-derived hydrolases as potent candidates for biorecycling and demonstrate the power of combining experimental characterization with advanced computational modeling to guide rational enzyme design.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

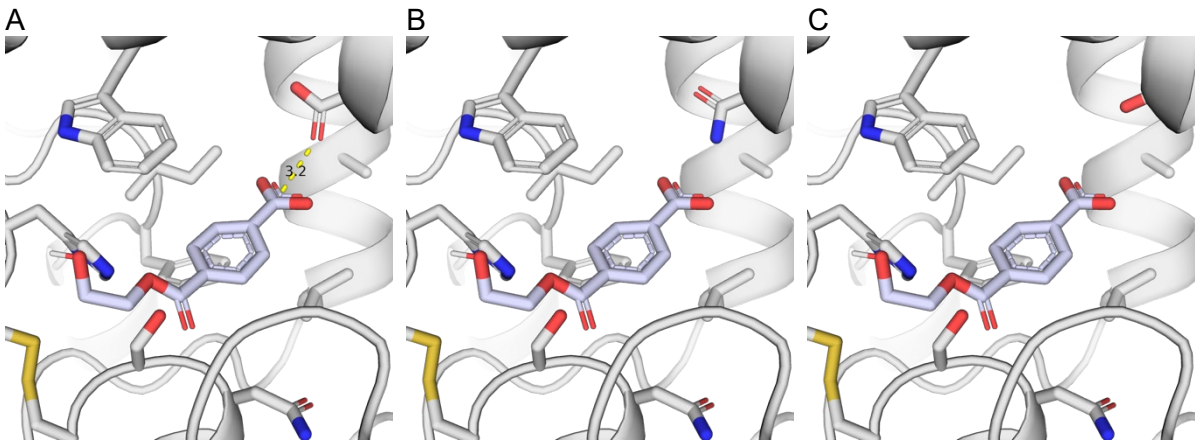



*Figure 1. Interactions in the active site of ZcEST (A). The negatively charged MHET species clashes with D355. Substituting D355 (ZcEST) for (B) D355N (ZcMHETase) and (C) D355S (ZcBHETase) allows for efficient and selective MHET and BHET depolymerization respectively*

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

**References:**

1. Sarda, P., Hanan, J. C., Lawrence, J. G. & Allahkarami, M. Sustainability performance of polyethylene terephthalate, clarifying challenges and opportunities. *Journal of Polymer Science* **60** (2022/01/01). https://doi.org/10.1002/pol.20210495
2. Kim, H. T. *et al.* Biological Valorization of Poly(ethylene terephthalate) Monomers for Upcycling Waste PET. *ACS Sustainable Chemistry & Engineering* **7** (November 12, 2019). https://doi.org/10.1021/acssuschemeng.9b03908
3. Kaabel, S. *et al.* Enzymatic depolymerization of highly crystalline polyethylene terephthalate enabled in moist-solid reaction mixtures. *Proceedings of the National Academy of Sciences of the United States of America* **118** (2021 Jul 13). https://doi.org/10.1073/pnas.2026452118
4. Barth, M. *et al.* Effect of hydrolysis products on the enzymatic degradation of polyethylene terephthalate nanoparticles by a polyester hydrolase from Thermobifida fusca. *Biochemical Engineering Journal* **93** (2015/01/15). https://doi.org/10.1016/j.bej.2014.10.012

*Invited lecture: STSM*

# Mechanistic Determinants of Substrate Specificity in Bacterial Glycoside 3-Oxidase

Tomás Frazão[1], Ferran Sancho[2], Patricia Borges[1], Lígia O Martins[1] and Pedro A. Sánchez-Murcia[3]

t.frazao@itqb.unl.pt, lmartins@itqb.unl.pt, pedro.murcia@medunigraz.at

[1]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av da República, 2780-157 Oeiras, Portugal
[2] Zymvol Biomodeling, C/ Pau Claris, 94, 3B, 08010 Barcelona, Spain
[3] Laboratory of Computer-Aided Molecular Design, Division of Medicinal Chemistry, Otto-Loewi Research Center, Medical University of Graz, Graz, Austria

Glycoside 3-oxidases (G3Ox) of bacterial origin are promising biocatalysts that oxidize diverse C-glycosides at the C3-position of their sugar moieties. These have been applied in natural sweeteners synthesis (1). However, the mechanism behind substrate oxidation remains unclear. Notably, rutin binds in the active site of PsG3Ox but is not oxidized, unlike the C-glycoside mangiferin (2). During this STSM, we investigated the molecular basis for this selectivity, implementing a computational workflow combining classical molecular dynamics (MD), QM/MM steered MD (sMD), and residue-level analysis.

Both systems displayed consistently short key catalytic distances. Surprisingly, rutin sampled shorter distance distributions. Analysis of non-catalytic contacts highlighted distinct interaction behaviour with previously identified mangiferin-binding residues, suggesting an alternative stabilization pattern. To probe reactivity, QM/MM-sMDs were performed, coupling hydride transfer and proton abstraction (collective-variable). Mangiferin trajectories yielded consistent work profiles with a well-defined maximum, whereas rutin showed higher variability. Though, both glycosides have identical maximum PMF values with exponential averaging, suggesting that the enzyme can oxidize rutin, in contrast to experimental results. SmarTSzyme (3) indicated altered stabilization contributions from both active site and distal residues to the enzyme–transition-state complex.

These results support a model in which rutin binds non-productively due to altered stabilization of the enzyme–substrate and/or enzyme–product, potentially trapping the glycoside and limiting turnover. To elucidate the oxidation mechanism and determine whether rutin or its product is retained in the active site, QM/MM-NEB calculations are in progress. This work identifies residues governing glycoside selectivity, highlighting targets for engineering efforts aimed at expanding the substrate scope toward alternative glycosides.

**References:**

1. Taborda A, et al., *Green Chem*, **2025**, 27, 1044-1053.
2. Taborda A, Frazão T, et. al, *Nat Commun*, **2023**, 14(1):7289.
3. Platero-Rochart D, Sánchez-Murcia PA., *ChemRxiv*, **2025**.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

*Invited lecture: STSM*

# Increasing the substrate specifies of N-carbamoyl-β-alanine amidohydrolase by computational modelling

Armen S. Sargsyan[1], Ani M. Paloyan[1], Sérgio M. Marques[2]

armenssargsyan@gmail.com

[1]Scientific and Production Centre “Armbiotechnology” National Academy of Sciences, Republic of Armenia
[2]Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Czech Republic

N-carbamoyl-β-alanine amidohydrolases (CβAAs) constitute an important class of industrial enzymes widely used in the production of optically pure non-proteinogenic amino acids and their derivatives. Despite their biotechnological relevance, biochemical and structural characterization of these enzymes remains limited. In particular, previously characterized CβAAs exhibit little to no activity toward aromatic and branched-chain substrates, restricting their industrial applicability [1].

The primary objective of this study was to design in silico variants of CβAA with altered substrate specificity toward aromatic N-carbamoylated amino acids, including N-carbamoyl-L-β-phenyl-β-alanine, N-carbamoyl-L-tyrosine, and N-carbamoyl-L-tryptophan. Initially, the three-dimensional structure of CβAA was analyzed to formulate hypotheses regarding its catalytic mechanism. Key residues involved in catalysis and substrate recognition were identified through literature analysis and computational prediction using HotSpot Wizard (Loschmidt Laboratories). Performance indicators and corresponding cutoff thresholds were defined to guide variant selection.

Active-site redesign was performed using the FuncLib approach (Fleishman Lab) to explore combinatorial mutations with improved structural stability and functional potential. In parallel, ancestral sequence reconstruction was conducted using FireProt-ASR to generate putative ancestral enzyme variants. Promising designs were further evaluated by molecular docking using AutoDock Vina to assess potential improvements in substrate binding affinity. As a result, multiple high-confidence mutant candidates were identified for each target substrate, including ten variants for N-carbamoyl-L-β-phenyl-β-alanine, eight for N-carbamoyl-L-tyrosine, and six for N-carbamoyl-L-tryptophan. These results provide a rational basis for experimental validation and future development of CβAA variants with expanded substrate specificity.

**Acknowledgements:** This work was supported by the COST Action: CA21162 and by the NAS RA within the framework of the ‘’Young Scientists’ Support Program’’: 24-YSIP-014. Computational resources were provided by the e-INFRA CZ project (ID:90254), supported by the Ministry of Education, Youth and Sports of the Czech Republic.

**References:**

1. Paloyan, A. Sargsyan, A. et al., 2023. Structural and biochemical characterisation of the N-carbamoyl-β-alanine amidohydrolase from *Rhizobium radiobacter* MDC 8606. FEBS J., Volume 290(23), pp. 5566-5580.

*Invited lecture: STSM*

# Rational Design and Computational Analysis of *Candida boidinii* Formate Dehydrogenase for Improved $CO_2$-Reducing Activity

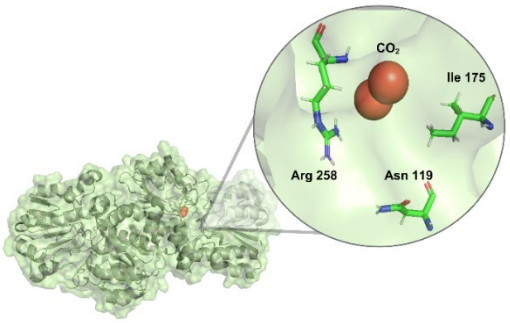
Sady Roberto Rodriguez[1], Eleftheria Sapountzaki[2], Oscar Romero[1], Marina Guillén[1] and Io Antonopoulou[2]

roberto.rodriguez@uab.cat

[1] Bioprocess Engineering and Applied Biocatalysis Group (ENG4BIO), Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

[2] Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental and Natural Resources Engineering, Luleå University of Technology, 97187 Luleå, Sweden.

Formate dehydrogenase (FDH) is a key enzyme in $CO_2$ fixation due to its ability to reversibly reduce $CO_2$ to formate under mild conditions and its suitability for integrated biocatalytic and electroenzymatic platforms for sustainable Carbon Capture and Utilization (CCU). The $NAD^+$-dependent FDH from *Candida boidinii* (CbFDH) is widely used due to its commercial availability and industrial relevance. However, it shows low affinity for $CO_2$, as it naturally favors formate oxidation over carboxylation (1). To address these limitations, computational approaches have become crucial in enzyme engineering to improve activity, selectivity, and stability. Leveraging these methods, rational protein design was employed to identify potentially mutable hotspots in the CbFDH structure that could enhance its $CO_2$-reducing catalytic activity through structure-based optimization of active site–substrate interactions, using the HotSpot Wizard 3.0 web server (2). Structural analysis identified non-essential residues suitable for mutation to facilitate hydride transfer from NADH to $CO_2$/bicarbonate, promoting formate formation (3). Molecular docking and dynamics simulations indicated that double and triple mutations in residues such as Gln197Thr, Val120Ser, and Ala229Met, significantly altered substrate proximity, suggesting improved catalytic efficiency. Notably, the Val120Ser + Ala229Met mutant displayed a more compact and stable conformation, with lower RMSD values and increased hydrophobic, ionic, and hydrogen-bond interactions in the active site. These *in silico* insights pave the way for experimental validation through the production and testing of selected enzyme variants, to evaluate the correlation between the modeled outcomes and actual catalytic behavior.




cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

*Figure 1. Three-dimensional structure of formate dehydrogenase from Candida boidinii (PDB ID: 5DN9) highlighting the active site residues involved in the capture of $CO_2$ and other inorganic carbonate species. The image was generated using PyMOL version 3.1.3.1.*

**Acknowledgements:** S.R.R., O.R and M.G. acknowledge Generalitat de Catalunya, and the 2021 SGR 00143 and project MEPLAB-$CO_2$ (TED2021-129732A-I00) funded by MCIN/AEI/10.13039/501100011033 and by the European Union "NextGenerationEU"/PRTR. This research is based upon work from COST Action COZYME CA21162, supported by COST (European Cooperation in Science and Technology) facilitating a collaboration between Universitat Autònoma de Barcelona and Luleå University of Technology.

**References:**

1. S. R. Rodriguez, O. Romero, M. Guillén. *ACS Sustain Chem Eng,* **2026**, 14, 86-98.
2. L. Sumbalova, J. Stourac, T. Martinek, D. Bednar, J. Damborsky. *Nucleic Acids Res* **2018**, 46, W1, W356–W362.
3. A. Maeir, L. M. Mguni, A.C.R. Ngo, D. Tischler. *ChemCatChem*, **2024**, 16, 21, e202401021

*Invited lecture: STSM*

# Deep-learning the committor function for the accurate characterization of the mechanism of sulphide oxidation by the bacterial flavin-dependent sulphide:quinone reductase

Rui P. P. Neves[1,2]*, Umberto Raucci[2], Michele Parrinello[2]

rui.neves@fc.up.pt

[1]LAQV,REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências Universidade do Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal
[2]Italian Institute of Technology, Genova, GE 16163, Italy

We report the optimization procedure of a feed-forward neural network outputing the committor function describing the enzymatic mechanism of suphide oxidation by the flavin-dependent sulphide:quinone reductase (SQR) of bacteria. SQR is a promising enzyme to target sulfur pollution as it uses the hydrogen sulphide pollutant gas as a reagent to produce sulphur as a feedstock.[1] Starting from umbrella sampling simulations at the PBE/MM level, we defined sets of geometrical descriptors discretizing enzyme:substrate states along sulphide oxidation. These were used to train the committor by employing the corresponding Kolmogorov functional as the loss function.[2] We then carried out on-the-fly probability enhanced sampling (OPES) simulations at the PBE/MM level biasing the trained committor function to obtain a thermodynamic and kinetic portrait of this enzyme, as well as an atomistic description of the catalyzed reaction mechanism. In particular, we used the OPES-$V_K$ scheme by Trizio et al., which combines the OPES and Kolmogorov bias to achieve efficient exploration of both the reaction space and the transition state region.[3, 4] The committor was optimized through an iterative active learning strategy, where the data from OPES simulations was included for subsequent training until the optimized committor model was obtained. Finally, we discuss the advantages of the OPES-$V_K$ scheme over umbrella sampling simulations, for the accurate representation of transition state ensembles.[4] The promising results drawn from the combination of OPES simulations with deep-learned committors should prove valuable for more successful rational enzyme engineering campaigns targeting enzyme active sites for improved activity and tuned specificity.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

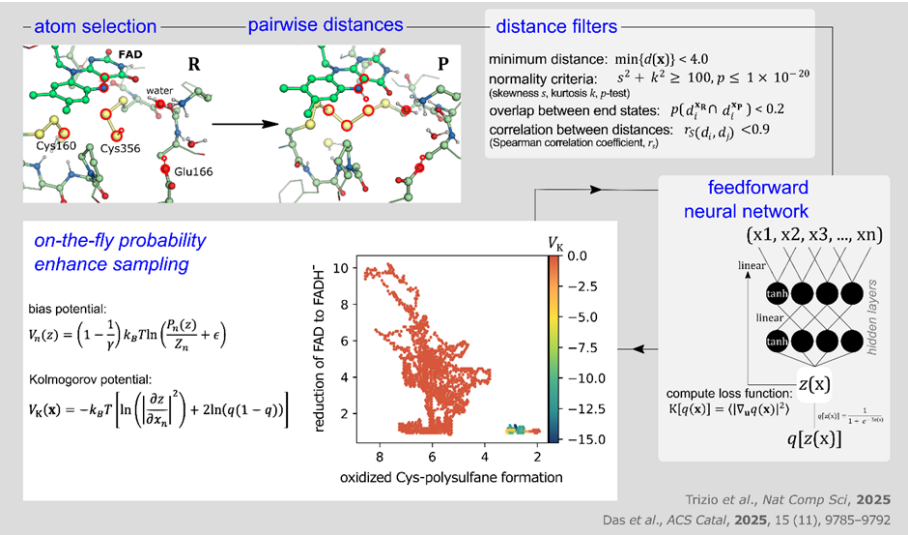



*Figure 1. General workflow for the committor training using the OPES+$V_K$ approach applied to the reaction mechanism of sulphide oxidation by bacterial sulphide:quinone reductase.*

**Acknowledgements:** This work received financial support from the PT national funds (FCT/MECI, Fundação para a Ciência e Tecnologia and Ministério da Educação, Ciência e Inovação) through the project UID/50006/2025 DOI 10.54499/UID/50006/2025 -Laboratório Associado para a Química Verde - Tecnologias e Processos Limpos, and COST Action COZYME, CA21162, supported by COST (European Cooperation in Science and Technology). R.P.P.N. further thanks the Atomistic Simulations group at the Center for Human Technologies (IIT, Genoa) and FCT for funding through the Individual Call to Scientific Employment Stimulus (Ref. 2021.00391.CEECIND/CP1662/CT0003 DOI: https://doi.org/10.54499/2021.00391.CEECIND/CP1662/CT0003).

**References:**

1. M. Marcia, et al., Proc. Natl. Acad. Sci. U. S. A., 2009, 106, 24, 9625-9630.
2. P. Kang, E. Trizio, and M. Parrinello, Nat. Comput. Sci., 2024, 4, 6, 451-460.
3. E. Trizio, P. Kang, and M. Parrinello, Nat. Comput. Sci., 2025, 5, 7, 582-591.
4. S. Das, et al., ACS Catal., 2025, 15, 11, 9785-9792.

*Invited lecture: STSM*

# High-throughput screening for enhanced HMFO expression

Garazi Ortiz-Orruño[1], Mirthe Hoekzema[2], Saskia Eder[2], Miloš Trajković[2], Oscar Romero[1], Marina Guillén[1]

Garazi.ortizdeorruno@uab.cat

[1]Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, Engineering School, 08193 Bellaterra (Cerdanyola del Vallès), Barcelona, Spain
[2]GECCO Biotech B.V., Groningen, The Netherlands

Bioprospecting has emerged as a powerful strategy to identify novel biocatalysts with specialized catalytic functions from diverse environmental metagenomes and genomic databases. However, enzymes identified through bioinformatic mining frequently face major challenges during experimental characterization, particularly low heterologous expression and solubility, which often limit their industrial applicability.

In this study, we address this critical bottleneck for three bioinformatically identified variants of Hydroxymethylfurfural oxidase (HMFO). These variants were selected for their ability to selectively catalyse individual steps of the oxidation cascade from 5-hydroxymethylfurfural (HMF) to 2,5-furandicarboxylic acid (FDCA), a key renewable monomer for polyethylene furanoate (PEF) production (1).

An initial high-throughput screening at microtiter plate scale was performed to evaluate expression conditions and solubility fusion tags, including SUMO, maltose-binding protein (MBP) and glutathione S-transferase (GST). The most promising constructs were subsequently scaled up to shake-flask cultures to validate expression trends and enable enzymatic activity assays. HMFO40 and HMFO21 were consistently obtained in soluble and active form, with MBP providing the most effective improvement in soluble expression for both enzymes. In contrast, HMFO26 proved to be the most challenging variant; although overall solubility remained limited, SUMO and GST fusions enabled partial recovery of soluble and active enzyme. Importantly, all successfully expressed variants retained catalytic activity consistent with their designed reaction specificities.

Overall, this work demonstrates the necessity of integrating bioinformatic bioprospecting with systematic experimental optimization. This synergy is vital to translate *in silico* discovery into functional biocatalyst production for sustainable FDCA synthesis (2).

**Acknowledgements:** All the authors acknowledge the project ROBUSTOO funded by the European Union (Grant agreement No 101135119), the COST Action CA21162 – COZYME, the Generalitat de Catalunya, and the 2021 SGR 00143.

**References:**

1. W. P. Dijkman, C. Binda, M. W. Fraaije and A. Mattevi, ACS Catal., **2015**, 5, 3, 1833-1839.
2. A. Carballido, A. Arias, J. Carro, Á. T. Martínez, F. J. Ruiz-Dueñas, G. Caminal, G. González, M. Guillen, O. Romero, K. Bonet, G. Ortiz-Orruño, D. Concha, G. Feijoo and M. T. Moreira, Ind. Crops Prod., **2026**, 240, 122625.

*Poster presentation (01): WG1*

# Some computational perspectives on residue roles in dynamic protein complexes

Vladimir Sladek[1], Shabeen Fatima[1], Antonija Tomić[2]

sladek.vladimir@savba.sk

[1] Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, 84538 Slovakia
[2] Division of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička cesta 54, Zagreb, Croatia

Specific interactions of large polymeric biomolecules, such as protein-protein interactions are indispensable for correct manifestation of their biological function. Both signalling and enzymes often rely on proper aggregation to supramolecular clusters. In recent years, computational techniques made a leap towards predicting biologically relevant structures of such complexes – AlphaFold3 [1] may be the prime example. On the other hand, the analysis of such structures relies heavily on wet-lab protocols that are complicated, time and resource consuming. Moreover, the interpretation of the role of individual residues with respect to the complex stability leaves a lot to be desired. We made a significant step in the direction of developing a computationally efficient method that addresses this problem [2]. The interpretation is well rationalised, based on first principle considerations [3] and simple mathematical analysis. To date, it was validated on static X-ray structures, whereas, in reality, such biomolecular complexes are a dynamic system. Here we present an extension of the method demonstrating that we can analyse also structural ensembles, e.g. from molecular dynamics, that allows for putting confidence intervals on the singular value centrality $C^{SV}$. Moreover, we show that the role of the residue is not a constant throughout the systems structural dynamics and that we can identify distinct clusters of structures in which residues behave differently. Such results can aid in rational interpretations of experimental observations in which the dynamic effects are usually averaged out. We discuss the examples of HIV-protease-1 and the DPP III – KELCH complex. Effects of water presence are shown.

**Acknowledgements:** We thank Antonija Tomić for delivering the structures and initiating the collaboration. Work supported by VEGA 2/0061/23, APVV-24-0594, APVV-24-0351.

**References:**

1. J. Abramson *et al*., *Nature* **2024**, 630, 493–500
2. V. Sladek *et al*., *J. Chem. Inf. Model*. **2024**, 64, 19, 7602–7615
3. V. Sladek*et al*., *J. Chem. Theory Comput*. **2018**, 4, 12, 6623–6631

*Poster presentation (02): WG1 and WG2*

# Dipeptide-Based Modeling of Protein Melting Temperature Using Linear and Nonlinear Machine Learning Approaches

Nurcan Vardar-Yel[1], Havva Esra Tütüncü[2], Yusuf Sürmeli[3]

nurcan.vardar@altinbas.edu.tr

[1]Department of Medical Laboratory Techniques, Altınbaş University, 34145 Istanbul, Turkey
[2]Department of Nutrition and Dietetics, Malatya Turgut Ozal University, 44210 Malatya, Turkey
[3]Department of Agricultural Biotechnology, Tekirdağ Namık Kemal University, 59030 Tekirdağ, Turkey

Protein melting temperature (Tm) is a key determinant of enzyme thermal stability and industrial applicability [1,2]. In this study, we investigated the relationship between sequence-derived features and Tm by integrating motif analysis with machine learning approaches. Conserved sequence motifs and short peptide patterns, including tripeptides, were initially identified to explore recurring signatures associated with thermal stability. These analyses revealed enrichment of specific residue combinations potentially contributing to enhanced stability. Subsequently, dipeptide composition was used as a quantitative descriptor to model Tm values. Both linear and nonlinear regression models were evaluated. Among linear methods, LASSO and Ridge regression were applied to assess feature importance and predictive performance. LASSO regression identified several dipeptides, particularly HC, WM, QQ, and IH, as strong positive contributors to Tm, suggesting their stabilizing role (Figure 1). Linear regression models demonstrated excellent predictive performance ($R^2 \approx 0.99$, RMSE ≈ 0.02), indicating that dipeptide contributions to Tm are largely linear and additive. To capture potential nonlinear effects, a Random Forest model was also employed. Although it identified overlapping important dipeptides and captured limited nonlinear patterns, its predictive performance was lower ($R^2 \approx 0.70$, RMSE ≈ 0.39) than that of linear models. This performance gap suggests that nonlinear interactions play a secondary role relative to dominant linear effects. Overall, our results demonstrate that protein melting temperature can be robustly predicted from dipeptide composition using linear machine learning models while maintaining biological interpretability. The integration of motif analysis with regression-based modeling provides complementary insights into sequence determinants of protein thermal stability.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

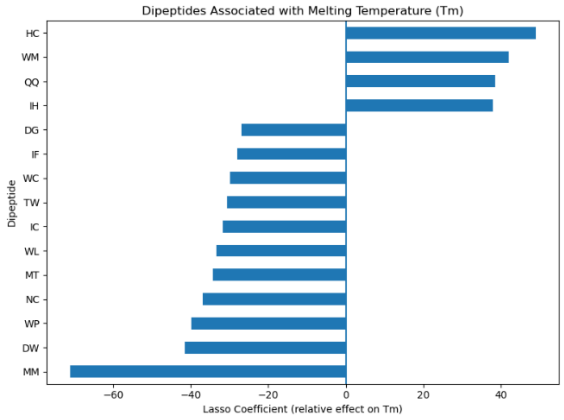



*Figure 1. Dipeptide contributions from Lasso Regression, highlighting effects on protein melting temperature*

**References:**

1. N. G. Nezhad, R. N. Z. R. A. Rahman, Y. M. Normi, S. N. Oslan, F. M. Shariff, T. C. Leow. Appl. Microbiol. Biotechnol., **2022**, 106, 13, 4845–4866.
2. C. Vieille, G. J. Zeikus. Microbiol. Mol. Biol. Rev., **2001**, 65, 1, 1–43.

*Poster presentation (03): WG2*

# pH-Driven Conformational and Protonation Changes in LipT6 Revealed by CpHMD Simulations

Marta S. P. Batista[1], Nitsan YehiShalom[2], Ayelet Fishman[3], Miguel Machuqueiro[1]

martaspbat@ciencias.ulisboa.pt

[1]BioISI - Biosystems & Integrative Sciences Institute, Lisbon, Portugal
[2]Inter-Departmental Program of Biotechnology, Technion-Israel Institute of Technology, Haifa, Israel
[3]Department of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa, Israel

Thermophilic lipases are valuable biocatalysts for industrial processes [1], such as converting waste oils into fatty acid methyl esters, more commonly known as biodiesel [2]. However, the performance of these thermophilic proteins often declines under acidic conditions, which are relevant for efficient biodiesel production. The lipase from Geobacillus stearothermophilus T6 (LipT6) is a promising candidate, with numerous published studies aimed at improving its stability and activity in high-methanol environments [3]. However, the molecular details of pH sensitivity remain insufficiently characterized. To address this gap, we performconstant-pH molecular dynamics (CpHMD) simulations [4] to investigate how pH-dependent protonation equilibria shape the structural and functional behavior of LipT6.

Using structural models of both the open (PDB:2W22) and closed (PDB:4X6U) conformations, we perform CpHMD simulations at pH 5, 7, and 8, enabling dynamic sampling of protonation states across all titratable residues. This approach allows us to explore how pH influences key functional regions, including the catalytic triad, metal-binding sites, and lid domain, and how these effects may differ between conformational states. The emerging trends provide insight into which residues are most responsive to pH changes, how protonation shifts may couple to conformational stability, and which regions could be targeted to enhance LipT6 performance under acidic conditions.

These preliminary computational findings will guide the rational design of mutations to improve LipT6 stability and activity at low pH. The resulting mutation candidates will form the basis for subsequent experimental validation, supporting the development of robust biocatalysts for sustainable biodiesel production.

**Acknowledgements:** AF thanks the funding from the Israel Council for Higher Education – VATAT (#1024311).

**References:**

1. A. Madhavan, K.B Arun, P. Binod, R. Sirohi, A. Tarafdar, R. Reshmyl. Bioresour. Technol., **2021**, 325, 124617.
2. S. Gihaz, D. Weiser, A. Dror, P. Sátorhelyi, M. Jerabek-Willemsen, L. Poppe. Chem. Sus. Chem., **2016**, 9, 3161–3170.
3. A. Dror, E. Shemesh, N. Dayan, A. Fishman. Appl. Environ. Microbiol., **2014**, 80, 1515–1527.
4. M. J. Yousef, N. F. B. Oliveira, J. N. M. Vitorino, P. B. P. S. Reis, P. Draczkowski, M. Majl. J. Chem. Theory Comput., **2025**, 21, 2655–2667.

*Poster presentation (04): WG2*

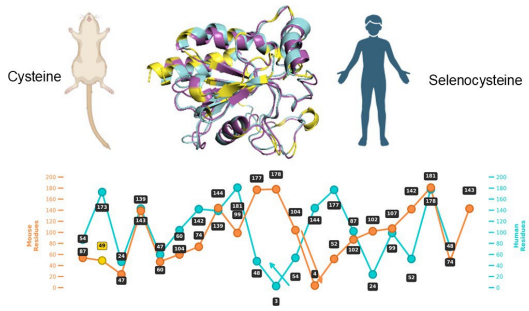
# Computational analysis of the free energy profile of the first step of Glutathione Peroxidase 6 (GPX6) catalysis: insights into the role of selenocysteine and species-specific variations

Nayanika Das[1], Vijay Baladhye[3], Jordi Villà-Freixa[1,2]

nayanikasekhar.das@uvic.cat

[1]Computational Biochemistry and Biophysics Lab (CBBL); Research Group on Bioinformatics and Bioimaging (BI2); Facultat de Ciències, Tecnologia i Enginyeries; Universitat de Vic − Universitat Central de Catalunya (UVic-UCC), Vic, Barcelona
[2]Institut de Recerca i Innovació en Ciències de la Vida i de la Salut a la Catalunya Central (IRIS-CC)
[3]Bioinformatics Centre, Savitribai Phule Pune University, Pune-411007 (MS), India

Glutathione peroxidase 6 (GPX6) displays remarkable evolutionary plasticity across mammals, including independent Cys-for-Sec substitutions in rodents despite ancestral Sec retention in primates. Here, we employ Empirical Valence Bond (EVB) simulations to map the free-energy landscape governing GPX6 catalysis and evolution, focusing on the proton-transfer step that converts selenol/thiol to selenolate/thiolate. Systematic EVB analysis of human and mouse homologs reveals that step-wise variants exhibit bidirectional hysteresis, following non-identical residue progressions driven by strong epistatic interactions among 20 proximal substitutions. Greedy-algorithm-guided mutational pathways connecting orthologs uncover pronounced asymmetric evolvability: progressive conversion of mouse GPX6 toward the human sequence monotonically stabilizes the transition state, whereas stepwise reversion of human GPX6 toward the mouse sequence induces early destabilization, effectively entrenching Sec dependence. Integrating these stepwise free energy transformations with previously studied phylogenetic reconstructions supports ancestral Sec optimality and reveals convergent rodent adaptation via compensatory mutational networks.




**References:**

1. Starr TN, Thornton JW. Epistasis in protein evolution. Protein Sci. 2016 Jul;25(7):1204-18. doi: 10.1002/pro.2897. Epub 2016 Feb 28. PMID: 26833806; PMCID: PMC4918427.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

2. Rees J, Sarangi G, Cheng Q, Floor M, Andrés AM, Oliva Miguel B, Villà-Freixa J, Arnér ESJ, Castellano S. Ancient Loss of Catalytic Selenocysteine Spurred Convergent Adaptation in a Mammalian Oxidoreductase. Genome Biol Evol. 2024 Mar 2;16(3):evae041. doi: 10.1093/gbe/evae041. PMID: 38447079; PMCID: PMC10958145.
3. Vardi-Kilshtain A, Roca M, Warshel A. The empirical valence bond as an effective strategy for computer-aided enzyme design. Biotechnol J. 2009 Apr;4(4):495-500.doi: 10.1002/biot.200800299. PMID: 19229886;PMCID: PMC2671572.
4. Oanca G, Åqvist J. Why Do Empirical Valence Bond Simulations Yield Accurate Arrhenius Plots?J Chem Theory Comput. 2024 Mar 26;20(6):25822591. doi: 10.1021/acs.jctc.4c00126. Epub 2024 Mar 7. PMID: 38452751; PMCID: PMC10976637

*Poster presentation (05): WG2*

# Bayesian optimization of ancestral arylmalonate decarboxylase for the asymmetric synthesis of sterically hindered profen derivatives

Lisa-Marie Krammer

lisa-marie.krammer@tugraz.at

TU Graz

Bacterial arylmalonate decarboxylase (AMDases) is a unique cofactor-independent enzyme capable of producing enantiopure α-chiral carboxylic acids, such as the non-steroidal anti-inflammatory drugs (NSAIDs) naproxen or ibuprofen. While the wild-type enzyme is strictly limited to substrates bearing α-substituents no larger than a methyl group, protein engineering efforts have expanded this scope. By introducing a "second" hydrophobic pocket, the conversion of α-ethyl substituted malonates has been unlocked[1]. In parallel, Ancestral Sequence Reconstruction (ASR) has successfully identified more stable variants, most notably the N131 ancestor, which exhibits an increased half-life time compared to the wild-type.

Despite these advancements, the catalytic efficiency towards sterically hindered substrates remains a challenge. Current engineered variants show significantly reduced activity when moving from methyl to ethyl or *n*-propyl α-substituents due to increased rotational flexibility and steric clashes within the active site. This highlights the need for a robust biocatalyst optimized specifically for bulky, pharmacologically relevant targets.

This project aims to address this by optimizing the AMDase towards α-hindered substrates, focusing on the synthesis of ethyl naproxen, a potent aldo-keto reductase 1C3 inhibitor. The N131 ancestor serves as the starting variant due to its superior thermal and operational stability.

Instead of traditional directed evolution, a machine learning-assisted approach using Bayesian optimization will be employed to map the mutant landscape of the hydrophobic pocket. This strategy aims to identify synergistic mutations that accomodate larger alkyl chains while preserving the high enantioselectivity of AMDases, potentially enabling industrial-scale production of bulky α-chiral building blocks.

**References:**

1. van der Pol, E. et al. Mechanistic Elucidation and Stereochemical Consequences of Alternative Binding of Alkenyl Substrates by Engineered Arylmalonate Decarboxylase. J. Am. Chem. Soc. 147, 39271–39283 (2025).

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

*Poster presentation (06): WG2*

# Structure-guided analysis of a pectin lyase from *Bacillus velezensis*

Marija Pavlović[1], Marija Stefanović[1], Marinela Šokarda Slavić[1], Tanja Lunić[2], Milan Kojić[3], Aleksandra Margetić[1], Nataša Božić[1]

marija.pavlovic@ihtm.bg.ac.rs

[1]University of Belgrade - Institute of Chemistry, Technology and Metallurgy – National Institute of the Republic of Serbia, Belgrade, Serbia
[2]University of Belgrade – Faculty of Biology, Belgrade, Serbia
[2]Institute of Virology, Vaccines and Serums “Torlak”, Belgrade, Serbia

Bacterial pectin lyases catalyze the depolymerization of pectic polysaccharides via a β-elimination mechanism and are widely applied in food, textile, and biomass-processing industries.

In our previous work, a highly active pectinase-producing strain, *Bacillus velezensis* 16B, was identified by whole-genome sequencing, and the *pelB* gene encoding a pectin lyase was annotated, cloned and heterologously expressed in *Escherichia coli*. The recombinant enzyme was purified by Ni-NTA affinity chromatography and experimentally characterized in terms of molecular mass, activity, and stability, establishing it as a robust and industrially relevant biocatalysts.

To support structure-guided enzyme engineering, a high-confidence 3D model of pectin lyase was generated using AlphaFold. Structural analysis revealed conservation of catalytically essential Asp and Arg residues and a well-defined active-site architecture characteristic of bacterial pectin lyases. This model served as a platform for computational investigation of enzyme–substrate interactions. Molecular docking of pectic oligomers was performed using AutoDock, allowing detailed mapping of binding modes and identification of residues contributing to substrate recognition and stabilization. Based on docking results, selected amino acids lining the catalytic cleft were subjected to *in silico* mutagenesis to evaluate their impact on substrate binding affinity and positioning. These computational analyses highlighted key interactions governing enzyme–substrate specificity and provided rational targets for future mutational studies.

**Acknowledgements:** This research has been financially supported by the Ministry of Science, Technological Development and Innovation of Republic of Serbia (Contract No. 451-03-136/2025-03/200026 and No. 451-03-136/2025-03/200168)

**References:**

1. M. Pavlović, M. Šokarda Slavić, M. Kojić, A. Margetić, M. Ristović, N. Drulović & Z. Vujčić, Food Chem., **2024**, 456, 140030.
2. S. S. Panda, J. Dey, S. R. Mahapatra, G. S. Kushwaha, N. Misra, M. Suar & M. Ghosh, Geomicrobiol. J., **2022**, 39, 294–305.

*Poster presentation (07): WG2*

# *In silico* study of the pH-dependent Au nanoparticle growth mechanism of a novel artificial metalloenzyme

Inês D. S. Pires[1], Carla Garcia-Sanz[2], Jose M. Palomo[2], Miguel Machuqueiro[1]

idpires@ciencias.ulisboa.pt

[1] BioISI–Instituto de Biossistemas e Ciências Integrativas, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal;
[2] Instituto de Catálisis y Petroleoquímica (ICP), CSIC, c/Marie Curie 2, 28049 Madrid, Spain

The design of artificial metalloenzymes promises increased activity and selectivity, enabling the catalysis of reactions for which no natural enzyme is presently known.[1] Gold nanoparticles (AuNPs) have been highlighted for their catalytic activity, which depends on AuNP size. Despite the finding that the increased size of the AuNP is correlated with reduced activity (due to factors like a reduced amount of exposed active sites and increased entropy),[2] there is still a minimum nanoparticle size required for catalysis, suggesting an optimal range of Au ions in the nanoparticle.

In previous works, Palomo and colleagues[3] have described the synthesis and characterization of a novel metalloenzyme with reductase-like activity. Taking advantage of the Au affinity of cysteine residues, *Geobacillus thermocatenulatus* (GTL) was modified, resulting in the formation of *in situ* AuNPs. They studied two variant systems (S114C and A193C), in which the AuNP grows at the surface or at an active site coordinated to the mutated cysteine. Their observations of nanoparticle size and shape varying with the mutated site and the experimental pH suggested significant differences in coordination between GTL and AuNP.[3]

To provide molecular insights into this system, we built a computational model to simulate AuNP growth at each mutation site across a range of pH values. Employing state-of-the-art methodologies such as Constant-pH Molecular Dynamics (CpHMD), we can study protein conformational and protonation dynamics.[4] To this end, we built three model systems: the apo protein and the GTL protein with the S114C and A193C mutations, where S114C is a surface mutation, and A193C is an internal mutation in the catalytic pocket. For the mutated variants, we studied multiple AuNP sizes (13, 35, 236, and 856 Au ions) at each mutation site. We will present preliminary results after running three 100 ns replicates of CpHMD simulations for each of these systems at pH 7, 8.5, and 10. These simulations allow us to investigate the impact of AuNP growth on the protein's secondary and overall structure. Conversely, we can study how the protein shapes and influences AuNP growth and analyze its sphericity preference and interfacial area across the various pH values and nanoparticle sizes examined for both mutations.

**Acknowledgements:** The authors would like to acknowledge the European Cooperation in Science and Technology (CA21162 COST Action), FCT, and BioISI for funding (2023.01155.BD and UID/04046/2025) and the Spanish Ministry of Science and Education for funding (PID2024-157256OB-I00).

**References:**


1. F. Nastri, M. Chino, O. Maglio, A. Bhagi-Damodaran, Y. Lu, A. Lombardi. *Chemical Society Reviews* **2016** 45, 18, 5020-5054.
2. A. Bano, A. Dawood, Rida, F. Saira, A. Malik, M. Alkholief, H. Ahmad, M. Asad Khan, Z. Ahmad, O. Bazighifan. *Scientific Reports* **2023** 13, 1, 12359.
3. C. Garcia-Sanz, B. De Las Rivas,J. M. Palomo. *Nanoscale* **2024** 16, 14, 6999-7010.
4. J. G. N. Sequeira,F. E. P. Rodrigues, T. G. D. Silva, P. B. P. S. Reis, M. Machuqueiro. *J Phys Chem B,* **2022,** 126, 40, 7870-7882.

*Poster presentation (08): WG2*

# Unraveling the Molecular Basis of Polyester-Polyurethane Hydrolysis: Structural Insights and Engineering of TfCut2

Agata Raczyńska[1,2], Oksana Kovalenko[1], Katarzyna Szleper[1], Tadeusz Bieg[3], Jeremy Esque[2], Rajendra Sharma[2], Katarzyna Papaj[1], Marzena Szawara[1], Kinga Plasa[1,3], Anna Kasprzycka[3], Isabelle André[2], Artur Góra[1]

agata.raczynska@polsl.pl

[1] Tunneling Group, Biotechnology Centre, Silesian University of Technology, Gliwice, Poland
[2] Toulouse Biotechnology Institute, TBI, Université de Toulouse, CNRS, INRAE, INSA, Toulouse, France
[3] Faculty of Chemistry, Silesian University of Technology, Gliwice, Poland

Polyurethanes (PURs) are chemically diverse synthetic polymers whose environmental persistence poses a significant ecological challenge [1]. Due to the structural heterogeneity of PUR waste, identifying the molecular determinants of enzymatic degradation remains a bottleneck for biocatalyst development. This study addresses these challenges through an integrated framework that combines automated computational modeling with experimental structural biology and enzyme engineering.

To explore PUR diversity, we developed PUR-GEN [2], a web server that automates the generation of PUR fragment libraries. These libraries, enriched with conformers and physicochemical property tables, offer a comprehensive representation of PUR structures. The application of PUR-GEN in molecular docking of over 400 generated oligomers demonstrates its utility as a robust pipeline for discovering and simulating PUR-degrading enzymes.

We also developed a computational framework to decipher the molecular basis of PUR binding and degradation using the model enzyme *Thermobifida fusca* cutinase (TfCut2) and the industrial polyurethane dispersion Impranil DLN. Crucially, we utilized high-resolution NMR spectroscopy to resolve the previously ambiguous chemical structure of Impranil DLN, providing a rigorous basis for our simulations. Computational analyses identified key interactions and guided the rational design of TfCut2 mutants with enhanced substrate affinity and turnover. These predictions were experimentally validated by assessing thermal stability and degradation rates, demonstrating that targeted engineering of substrate anchoring and product release significantly enhances catalytic efficiency. Collectively, this work establishes a direct link between *in silico* predictions and experimental outcomes, offering a new blueprint for designing enzymes capable of processing complex plastic waste.

**Acknowledgements:** We gratefully acknowledge Ministry of Science and Higher Education, Poland, "Diamond Grant" programme No. 0169/DIA/2020/49 (AR, KSz), Campus France (AR), EMBO under EMBO Solidarity grant no. SLG-5405 (OK), Polish high-performance computing infrastructure PLGrid (HPC Centres: ACK Cyfronet AGH) for providing computer facilities and support within computational grant no. PLG/2023/016344 and HPC resources of the Computing meso-center of Région Midi-Pyrénées (CALMIP, Toulouse, France).

**References:**

1. A. Raczyńska, A. Góra, I. André, Biotech. Adv., 2024, 77, 108439.
2. K. Szleper, M. Cebula, O. Kovalenko, A. Góra, A. Raczyńska, CSBJ, 2025, 27, 127.

*Poster presentation (09): WG2*

# Biochemical and Structural Characterization of a Novel Gluten-Hydrolyzing protease from *Bacillus velezensis* 16B

Marija Stefanović[1], Nenad Drulović[1], Mladen Đurđević[1], Marija Pavlović[1], Marinela Šokarda Slavić[1], Jelena Radosavljević[2], Aleksandra Margetić[1], Zoran Vujčić[2]

marija.stefanovic@ihtm.bg.ac.rs

[1]University of Belgrade - Institute of Chemistry, Technology and Metallurgy – National Institute of the Republic of Serbia, Belgrade, Serbia
[2]University of Belgrade – Faculty of Chemistry, Belgrade, Serbia

Gluten is a complex network of cereal proteins, primarily glutenins and gliadins, responsible for the viscoelastic properties of dough. It contains proline- and glutamine-rich sequences that are digestion-resistant and implicated in gluten-related disorders [1]. The development of targeted enzymatic strategies for gluten degradation offeres a sustainable alternative to conventional food-processing techniques. In this study, we report on comprehensive biochemical and structural characterization of a novel gluten-hydrolyzing protease from *Bacillus velezensis* 16B. Mass spectrometry identified the enzyme as a subtilisin BPN′ variant, a serine protease classified within the MEROPS S8A family. Furthermore, enzymatic activity was assessed against gluten and gliadin, and compared with other complex substrates, including zein, gelatin, and casein. The degree of gluten hydrolysis was quantified using the TNBS assay and further analyzed by SDS-PAGE and RP-HPLC. Kinetic parameters were determined using chromogenic p-nitroanilide substrates (AAVA and AAPF), revealing a preference for hydrophobic residues at the P1 position. The enzyme is synthesized as a zymogen (~39 kDa, 382 aa) and processed into a mature form (~26 kDa, 263 aa) following removal of a 30-aa signal peptide. The catalytic triad Asp32–His64–Ser221 was confirmed, and isoelectric focusing indicated an alkaline pI of ~9.0, consistent with optimal activity at pH 8–10 and stability up to ~60 °C. Structural insight were derived using homology-modeled and AlphaFold-predicted structures, alongside with molecular docking in AutoDock Vina targeting selected gluten-derived epitopes implicated in celiac disease. In parallel, ancestral sequence reconstruction based on homolog sequences sourced from UniProt and Open Enzyme Database data was conducted to investigate the evolutionary relationships of subtilisin-like proteases and identify structurally conserved regions related to substrate binding [2, 3]. These findings provide a basis for the rational engineering of subtilisin variants with improved activity toward immunogenic gluten epitopes.

**Acknowledgements:** This research has been financially supported by the Ministry of Science, Technological Development and Innovation of Republic of Serbia (Contract No. 451-03-136/2025-03/200026 and No. 451-03-136/2025-03/200168) and by the University of Belgrade – Institute of Chemistry, Technology and Metallurgy, under the Seed Research 2025 Grant Program for young scientists (“Novel Enzyme Solutions for Targeted Gluten Hydrolysis - GlutNZym”).

**References:**

1. P. R. Shewry & P. S. Belton, J. Cereal Sci., **2024**, 117.
2. M. A. Spence, J. Curr. Opin. Struct. Biol., **2021**, 69, 131–141.
3. L. Yuan, D. M. Bianchi & K. Arneson, Nucleic Acids Res., **2025**, 54, D1, D643–D651.

*Poster presentation (10): WG2*

# Regulating the Antioxidative Response by Designing Kelch Domain Mutants to Inhibit DPP III Activity in the Keap1–Nrf2 Signaling Pathway

Antonija Tomić[1], Antonia Matić[1], Filip Šupljika[2], Vladimir Sladek[3]

Antonija.Tomic@irb.hr

[1]Division of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička cesta 54, Zagreb, Croatia
[2]Faculty of Food Technology and Biotechnology, Department of Chemistry and Biochemistry, Pierottijeva 6, Zagreb, Croatia
[1]Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia

Dipeptidyl peptidase III (DPP III) has been identified as part of the Keap1 protein interaction network, suggesting its involvement in the cellular response to oxidative and electrophilic stress [1]. By binding to the Kelch domain of Keap1, DPP III disrupts the Keap1–Nrf2 pathway, preventing Keap1-mediated degradation of Nrf2 and thereby enhancing Nrf2's transcriptional activity in the nucleus, where it upregulates genes involved in reactive oxygen species detoxification, inflammation suppression, and cellular detoxification. While Nrf2 activation is protective in normal cells, its persistent overactivation has been linked to cancer progression. Increased DPP III expression has been associated with tumorigenesis, particularly in breast and colorectal cancers [2], where it enhances antioxidant responses and supports cancer cell survival through competitive binding to Keap1. Given the role of DPP III in oxidative stress regulation, our research focuses on identifying inhibitors that block DPP III binding to Keap1, thereby indirectly strengthening the Keap1–Nrf2 interaction. Our plan is to design a Kelch domain mutant with higher binding affinity for DPP III than endogenous Keap1. To achieve this, we employ a multidisciplinary approach that combines state-of-the-art molecular modeling (AlphaFold structure prediction, long MD simulations, and FMO-based residue-level binding analysis) with biochemical methods (protein expression and mutagenesis) and physicochemical techniques (ITC measurements), streamlining the identification of mutants that could act as molecular traps for overexpressed DPP III in cells.

**Acknowledgements:** This work was funded by the European Union under the NextGenerationEU Programme and by Croatian Science Foundation under the project IP-2025-02-7100.

**References:**

1. B.E. Hast et al., *Cancer Res.* **2013**, 73, 2199–2210; Hast et al., *Cancer Res.* **2014**, 74, 808–817.
2. T.-K. Choy et al., *Diagnostics* **2021**, 11, 1204; K. Lu et al., *Cancer Res.* **2017**, 77, 2881–2892; Y. Tong et al., *Cell Death Dis.* **2021**, 12, 529; S. Simaga et al., *Gynecol. Oncol.* **2003**, 91, 194–200.

*Poster presentation (11): WG2*

# Mechanistic Insights into Dioxygen Diffusion in the PHD2 Oxygenase from Long-Timescale Simulations

Brian Wiley[1], Simoni Furini[2], Carmen Domene[1*]

C.Domene@bath.ac.uk

[1]Department of Chemistry University of Bath, Bath BA2 7AX, United Kingdom,

[2]Department of Electrical, Electronic and Information Engineering 'Guglielmo Marconi', University of Bologna, via dell'Università 50, Cesena (FC) 47521, Italy

Understanding how dioxygen reaches buried catalytic centers in metalloenzymes is critical for elucidating enzymatic kinetics and informing therapeutic strategies. Here, we present over 20 microseconds of classical molecular dynamics simulations and Markov State Models of prolyl hydroxylase domain 2 (PHD2), a key enzyme regulating the hypoxia response via hydroxylation of HIF-1α. In addition to analyzing specific pathways of since PHD2 enzyme, we have also trained 3D Equivariant convolution models and message passing over graphs on both PHD2 and plant cysteine-dioxygenases (PCOs) to predict probabilities densities for $O_2$ in other cupin fold dioxygenases. From here we will show that we can use this information from long scale MD to speed up pathway insight on new dioxygenases. Our simulations reveal multiple diffusion pathways from solvent-exposed regions through the cupin fold into proximity (<6 Å) of the $Fe^{2+}$ active site. $O_2$ diffusion occurs on vastly different timescales, from rapid diffuse-in/diffuse-out events (~250 ps) to long residency within internal hydrophobic cavities spanning hundreds of nanoseconds and correlate to high densities predicted by our machine learning (ML). These findings suggest that these cavities act as kinetic modulators either delaying $O_2$ engagement with catalytic residues or retaining $O_2$ near the active site to enhance the probability of productive catalysis.

EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

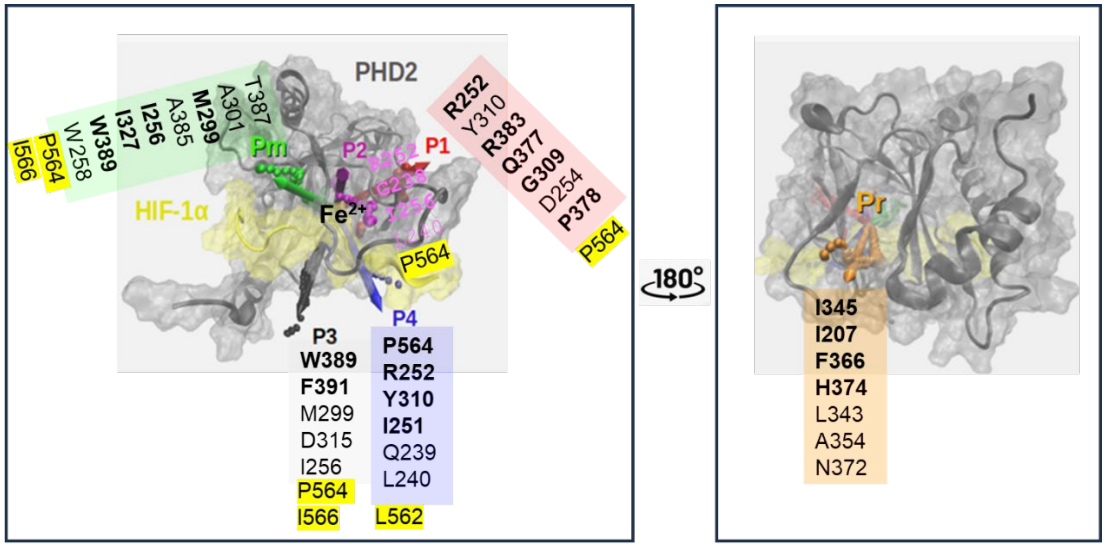



*Figure 1. Six distinct pathways for dioxygen access to the catalytic $Fe^{2+}$ centre in PHD2. Schematic representation of all six dioxygen diffusion pathways, annotated as P1 (red), P2 (pink), P3 (black), P4 (purple), Pm (green) and Pr (orange), with their most frequently contacted residues. Residues from HIF-1α are highlighted in yellow.*

**Acknowledgements:** C.D. acknowledges PRACE for awarding access to computational resources in CSCS, the Swiss National Supercomputing Service, in two of their Project Access Calls. This project also made use of computing time on U.K. Tier 2 JADE and Bede systems, and the Archer2 national facility, granted via the UK High-End Computing Consortium for Biomolecular Simulation, HECBioSim (http://hecbiosim.ac.uk), supported by EPSRC (grant no. EP/R029407/1 and EP/X035603/1).

**References:**

1. Domene, C.; Jorgensen, C.; Schofield, C. J. Mechanism of Molecular Oxygen Diffusion in a Hypoxia-Sensing Prolyl Hydroxylase Using Multiscale Simulation. *J Am Chem Soc* **2020**, *142* (5), 2253-2263.
2. Flashman, E.; Hoffart, L. M.; Hamed, R. B.; Bollinger Jr, J. M.; Krebs, C.; Schofield, C. J. Evidence for the slow reaction of hypoxia-inducible factor prolyl hydroxylase 2 with oxygen. *The FEBS Journal* **2010**, *277* (19), 4089-4099.

*Poster presentation (12): WG3*

# Structural Loop Dynamics Drive Enhanced Mn(II) Oxidation in *Pp*DyP

Patrícia Borges, Carolina Rodrigues[1], Constança Lorena[1], Vânia Brissos[1], Lígia O. Martins

pborges@itqb.unl.pt

[1]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

Microbial dye-decolorizing peroxidases (DyPs) are heme-containing enzymes active toward lignin-derived compounds, azo and anthraquinone dyes, and, to a lesser extent, metal ions such as Fe(II) and Mn(II)[1,2]. Given the central role of Mn(III) in lignin depolymerization, increasing attention has been directed toward bacterial DyPs, which display oxidative mechanisms reminiscent of classical fungal ligninolytic peroxidases. However, despite their biotechnological relevance, the molecular basis of Mn(II) oxidation in bacterial DyPs remains far less understood than in their fungal counterparts[3].

Here, we elucidate the structural and mechanistic determinants of Mn(II) oxidation in *Pseudomonas putida* DyP (*Pp*DyP) using a combination of X-ray crystallography, mutagenesis, and steady-state kinetics. We report the crystal structure of *Pp*DyP in complex with Mn(II), revealing a canonical metal-binding site formed by three conserved acidic residues, E135, E188, and E204, located on flexible loop regions limiting the heme cavity. Mutagenesis and steady-state kinetics demonstrate that these residues act cooperatively to coordinate Mn(II) and enable efficient electron transfer.

To investigate the role of local electrostatics and loop dynamics, we replaced the nearby residue E209 with alanine. The E209A substitution enhances catalytic efficiency and increases kcat by up to 4-fold relative to wild-type. Disruption of the inter-loop interactions effectively decouples the loops, increasing their conformational plasticity and loosening the heme-proximal region that defines the canonical Mn(II)-binding cavity. In the E209A variant, we identified an additional Mn(II) molecule near two carboxylate residues distal to the heme. However, the improved activity of the E209A variant was attributed to its greater flexibility near the canonical binding site. In contrast, the additional “non-canonical” Mn(II) binding site is mostly non-functional, as assessed by mutagenesis studies.

This work provides mechanistic insight into manganese oxidation by bacterial DyPs and establishes a rational framework for engineering improved peroxidases for lignin valorization and other oxidative biotransformations.

**Acknowledgements:** Fundação para a Ciência e Tecnologia, Portugal, grant 2022.02027.PTDC, MOSTMICRO-ITQB (UIDB/04612/2020 and UIDP/04612/2020), FCT CEEC contract 2022.00194.CCEIND (PTB), and PhD fellowship UI/BD/153388/2022 (CFR).

**References:**

1. D. Silva, C.F. Rodrigues, C. Lorena, P.T. Borges, L.O. Martins. *Biotechnol Adv*, **2023**, 65,108153.
2. A. Santos, S. Mendes, V. Brissos, L.O. Martins. *Appl Microbiol Biotechnol,* **2014**, 98:2053-65.
3. M.P. Singh, S.K. Vishwakarma, A.K. Srivastava. *Biomed Res Int,* **2013**, 180156.

*Poster presentation (13): WG3*

# Identification and application of oxidative enzymes from *Cerrena zonata* unraveled by a multi-omics and *in silico* approach

Valeriia V. Nikitenkova[1,2], Niklas Broel[1], Stefan Janssen[1], Binglin Li[1,3], Holger Zorn[1,4], Tatyana S. Zhuk[1,2], Martin Gand[1]

Martin.gand@lcb.chemie.uni-giessen.de

[1]Justus Liebig University, Heinrich-Buff-Ring 17, 35392, Giessen, Germany
[2]Igor Sikorsky Kyiv Polytechnic Institute, Beresteiskyi Ave, 37, 03056, Kyiv, Ukraine
[3]Northwest University, Tai Bai Bei Lu, 229, 710069, Xi'an, China
[4]Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Ohlebergsweg 12, 35392, Giessen, Germany

Wood-degrading fungi possess a complex suite of enzymes that target lignocellulosic biomass (LCB) as substrates. Their pivotal role in the natural carbon cycle underscores their potential as biorefinery catalysts converting plant polymers into value-added products. We investigated the white-rot fungus *Cerrena zonata* (CZO) using a comprehensive multi-omics strategy to catalogue its carbohydrate-active enzymes (CAZymes). Genomic sequencing identified 20,816 protein-coding loci, of which 487 encode putative CAZymes. Transcriptomic profiling during growth on LCB substrates revealed differential expression of CAZymes, consistent with a proteomic confirmation of a broad spectrum of degradative enzymes. Secretome analysis detected active laccases, manganese and versatile peroxidases, confirming the organism's oxidative capacity [1]. Transcriptomic data combined with *in silico* docking analyses indicated the presence of two P450 monooxygenases capable of oxidizing CH-bonds in hydrocarbon cage structures, explicitly diamondoid ketones, in the resting cells, illustrating the fungus' potential for biocatalytic transformations of recalcitrant substrates [2].

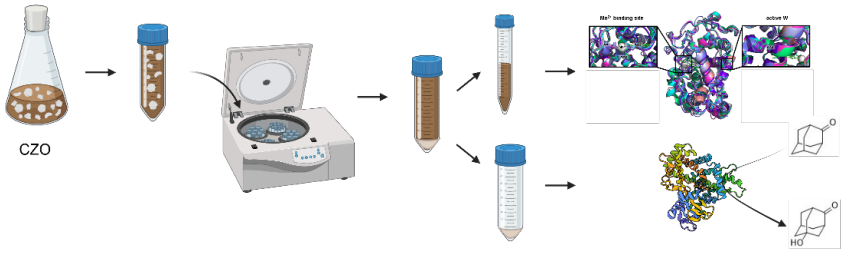



*Figure 1. Identification and application of oxidative enzymes by CZO [2].*

**Acknowledgements:** Financial support by the German Federal Environmental Foundation, Silesia-Clemens-Hanke Foundation and the Philipp Schwartz Initiative is gratefully acknowledged.

**References:**

1. N. Broel, F. Daumüller, A. Ali, J. Lemanschick, K. Maibach, C. Mewe, B. Bunk, C. Spröer, C. Baschien, H. Zorn, H. Schlüter, M. Rühl, S. Janssen, M. Gand, *Microbiol. Res.*, **2025**, 298, 128214
2. V. V. Nikitenkova, W. Albuquerque, M. Gand, S. Janssen, B. Li, H. Zorn, T. S. Zhuk, *Mol. Catal.*, **2026**, 592, 115741

*Poster presentation (14): WG3*

# Enzymes for synthesis and degradation of biobased polyesters: Automatic *in silico* screening and experimental validation

Anamaria Todea[1,2], Sara Fortuna[1], Demi Vattovaz[1], Danilo di Stefano[3], Emanuele Carosati[1], Monica Vlad[4], Lucia Gardossi[1]

[1] Dipartimento di Scienze Chimiche e Farmaceutiche, Università degli Studi di Trieste, Via L. Giorgieri 1, 34127, Trieste, Italy
[2] University Politehnica Timisoara, Faculty Chemical Engineering, Biotechnologies and Environmental Protection, Biocatalysis and Green Chemistry Group, V. Pârvan 6, 300223, Timisoara, Romania
[3] ESTECO SpA, Trieste, Italy
[4] MONAI Software, Cluj-Napoca, Romania

Considering the urgent need to develop and implement efficient biocatalytic processes in a timely and cost-effective manner, we devised a computational pipeline for the identification of optimal substrate–catalyst combinations. A series of bio-based monomers and enzymes was screened, and the computational results were correlated with experimental data obtained for the synthesis direction1 as well as with the data available for hydrolysis. The pipeline relies on well-established computational techniques including docking and molecular dynamics simulations to address three key challenges: (i) the identification of the optimal biocatalyst for the polymerisation and hydrolysis of a given substrate; (ii) the identification of optimal substrates for a given biocatalyst and, the (iii) the comprehensive characterisation of a given biocatalyst in terms of its predicted reactivity towards a pool of substrates. The process, based on subsequent calls to freely available well-known codes, was implemented in modeFRONTIER,2 a package equipped with graphical interface in which each call to external programs is conveniently represented by a node. Protein structures and protein-ligands interactions were investigated using molecular dynamics and docking methods. Specific software packages (e.g. Gromacs and AutoDock) were selected and integrated into the automatic workflow. The method was validated on a pool of six proteins and twenty substrates, and the predicted enzyme-substrate matches were subsequently compared with experimental data, thereby confirming the reliability of the predictions. Overall, this study demonstrates that automated computational workflows can be effectively employed for the rapid selection of enzymes capable of efficiently catalyzing the hydrolysis and/or synthesis of bio-based polyesters. The results represent a contribution to the rational eco-design of a new generation of tailored sustainable polymers and a step towards the in silico generation and screening of new biocatalysts for the polymer sector.

**References:**

1. A. Todea, S. Fortuna, C. Ebert, F. Asaro, S. Tomada, M. Cespugli, F. Hollan, L. Gardossi, “Rational Guidelines for the Two-Step Scalability of Enzymatic Polycondensation: Experimental and Computational Optimization of the Enzymatic Synthesis of Poly(glycerolazelate)” *ChemSusChem* **2022**, *15*, DOI 10.1002/cssc.202102657.
2. V. Ferrario, C. Ebert, A. Svendsen, W. Besenmatter, L. Gardossi, “An integrated platform for automatic design and screening of virtual mutants based on 3D-QSAR analysis” *J. Mol. Catal. B Enzym.* **2014**, *101*, 7–15.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

*Poster presentation (15): WG3*

# Data Processing and Kinetic Modelling of HPLC Reaction Time Courses with EnzymeML Tools

Max Häussler,[1,2] Lars-Hendrick Köppl,[2] Jan Range,[1] Jennifer Andexer,[2] Jürgen Pleiss[1]

max.haeussler@ibtb.uni-stuttgart.de

[1] Institute of Biochemistry, Stuttgart, Germany
[2] Institute for Pharmaceutical Sciences, Freiburg, Germany

The digitalisation of biocatalysis is essential for data-driven kinetic modelling and reaction optimisation. Yet experimental reaction time course data are rarely available in a format suitable for computational analysis. The results of a manual evaluation of chromatographic measurements are not machine-readable, the experimental metadata are incomplete, and the data analysis is not reproducible. These limitations severely restrict the scalable integration of time-course data into modelling pipelines.[1,2,3]

To address this, EnzymeML provides a standardised, machine-readable framework for biocatalytic experiments, ensuring complete representation of reaction conditions and metadata.[4] The newly developed Python tool chromhandler builds on this standard to enable automated processing of reaction time course data. In this workflow, chromatograms are ingested and processed by chromhandler, which performs peak fitting, aligns and annotates peaks across sampling points, and constructs calibration models to convert peak areas into concentration profiles for a complete time-course series. The resulting concentration time courses, enriched with reaction conditions and metadata, are stored in the EnzymeML format. The resulting document forms a consistent and reproducible basis for kinetic analysis.

The workflow was applied to study the kinetics of an S-adenosyl-L-homocysteine hydrolase (SAHH) system.[5] Reaction progress data were obtained from HPLC and analysed using Bayesian inference to estimate kinetic parameters and to examine model plausibility. This methodology permits discrimination among alternative kinetic models, including contributions from side reactions and inhibitory effects, while propagating data uncertainty throughout the analysis. All results were stored in the FAIR-compliant EnzymeML format, assuring that modelling results remain reproducible and reusable.

**Acknowledgements:** DFG FOR5596, DFG CRC1333

**References:**

1. Pleiss, J. ACS Catalysis 14 (2024): 2709-2718.
2. Range, J. FEBS Journal, 19 (2022): 5864-5874
3. Behr, A.S., et al. Processes 12 (2024): 597.
4. Lauterbach, S., Dienhart, H., Range, J., et al. Nat Methods 20 (2023): 400–402
5. LH. Koeppl, D. Popadić, R. Saleem-Batcha, et al., Commun. Biol 7 (2024): 380

*Poster presentation (16): WG3*

# Assessment of Pullulanase Variant Stability under Operational Conditions

Višnja Kosić[1], Marina Ristović[1], Sanja Stojanović[1], Sérgio M. Marques[2,3], David Bednar[2,3], Biljana Dojnov[1], Nataša Božić[1]

visnja.kosic@ihtm.bg.ac.rs

[1]University of Belgrade, Institute of Chemistry, Technology and Metallurgy, Njegoševa 12, Belgrade, Serbia
[2]Masaryk University, Loschmidt Laboratories, Department of Experimental Biology, Faculty of Science, Kamenice 5/C13, 625 00 Brno, Czech Republic
[3]International Clinical Research Centre, St. Anne's University Hospital Brno, Pekarska 53, Brno 656 91, Czech Republic

The industrial use of pullulanase (EC 3.2.1.41) is frequently restricted by insufficient thermal stability and solubility, particularly for mesophilic enzymes operating outside their native temperature range. Identifying variants with improved robustness remains a key challenge in the development of pullulanase-based biocatalytic processes.

In this study, a set of engineered variants of the *Bacillus paralicheniformis* 9945a pullulanase obtained using an automated structure- and sequence-based design pipeline, was experimentally screened to identify candidates with enhanced robustness at elevated temperatures. Thermal stability was assessed by real-time PCR–based thermal shift analysis. Melting temperature measurements revealed that the most pronounced Tm increases were observed for variants in which stabilizing disulfide bonds were introduced, as well as for mutants designed to improve solubility by reducing aggregation-prone regions. The selected mutants were subsequently subjected to operational thermal stability assays, focusing on residual catalytic activity following prolonged incubation at application-relevant temperatures.

This two-step evaluation strategy enabled discrimination between variants exhibiting increased structural stability and those retaining catalytic function under sustained thermal stress, highlighting the importance of integrating melting temperature analysis with functional stability assessments. Based on these findings, a subset of variants was selected for subsequent biocatalytic studies and further mutational exploration.

**Acknowledgements:** This research was supported by the Science Fund of the Republic of Serbia, #7347, Unique enzyme solutions for obtaining added value products from corn – UniCorn and COST Action COZYME. This project was also supported by the European Union's Horizon 2020 Research and Innovation Programme under grant agreements No. 101136607.

*Poster presentation (17): WG3*

# A Bacterial Cell Platform for Functional Evaluation of Cytochrome P450 Oxidoreductase Variants

Michel Kranendonk[1]

michel.kranendonk@nms.unl.pt

[1]NOVA Medical School, Universidade NOVA de Lisboa

Understanding the molecular determinants that govern enzyme activity, electron transfer, and regulation is a central objective of the COZYME COST Action, which focuses on heme- and flavin-containing model enzymes. In this context, we have developed *Escherichia coli*–based whole-cell models employing a bi-plasmid expression system for the coordinated co-expression of human cytochrome P450 oxidoreductase (CPR) and its redox partner cytochrome P450 (CYP). This system enables independent and fine-tuned control of CPR and CYP expression levels, allowing systematic modulation of their relative stoichiometry and functional coupling (1, 2).

Compared with single-plasmid systems or in vitro reconstitution approaches, the bi-plasmid configuration more closely mimics the physiological electron transfer process, providing a robust framework to investigate CYP–CPR interaction efficiency across different enzyme combinations. The models are cost-effective, and scalable.

We have demonstrated the applicability of these bacterial systems for dissecting structure–function relationships of CYPs and CPR, including the functional impact of genetic polymorphisms, disease-associated mutations, and engineered variants, with particular emphasis on CPR (4–7) and CYP-specific case studies such as CYP1A2 (3). Importantly, the platform supports both targeted analysis of individual variants (8, 9) and medium- to high-throughput screening of random CPR mutant libraries (10) using whole-cell activity assays (11).

Overall, these *E. coli*–based models represent a versatile and complementary toolset to advance mechanistic understanding, enzyme benchmarking, and rapid functional assessment of redox enzymes relevant to pharmacogenetics, toxicology, and enzyme engineering.

**Acknowledgements:** *Funding:* UID/04923 - Comprehensive Health Research Centre, from the Portuguese Fundação para a Ciência e a Tecnologia—FCT; *Collaborators:* Francisco Esteves and Diana Campelo.

**References:**

1. Kranendonk, M., Carreira, F., Theisen, P., Laires, A., Fisher, C. W., Rueff, J., Estabrook, R. W., and Vermeulen, N. P. (1999) Escherichia coli MTC, a human NADPH P450 reductase competent mutagenicity tester strain for the expression of

human cytochrome P450 isoforms 1A1, 1A2, 2A6, 3A4, or 3A5: catalytic activities and mutagenicity studies. Mutat. Res. 441, 73–83

2. Duarte, M. P., Palma, B. B., Laires, A., Oliveira, J. S., Rueff, J., and Kranendonk, M. (2005) Escherichia coli BTC, a human cytochrome P450 competent tester strain with a high sensitivity towards alkylating agents: involvement of alkyltransferases in the repair of DNA damage induced by aromatic amines. Mutagenesis. 20, 199–208
3. Palma, B. B., Silva E Sousa, M., Vosmeer, C. R., Lastdrager, J., Rueff, J., Vermeulen, N. P. E., and Kranendonk, M. (2010) Functional characterization of eight human cytochrome P450 1A2 gene variants by recombinant protein expression. Pharmacogenomics J. 10, 478–488
4. Moutinho, D., Marohnic, C. C., Panda, S. P., Rueff, J., Masters, B. S., and Kranendonk, M. (2012) Altered human CYP3A4 activity caused by Antley-Bixler syndrome-related variants of NADPH-cytochrome P450 oxidoreductase measured in a robust in vitro system. Drug Metab. Dispos. 40, 754–760
5. McCammon, K. M., Panda, S. P., Xia, C., Kim, J.-J. P., Moutinho, D., Kranendonk, M., Auchus, R. J., Lafer, E. M., Ghosh, D., Martasek, P., Kar, R., Masters, B. S., and Roman, L. J. (2016) Instability of the Human Cytochrome P450 Reductase A287P Variant Is the Major Contributor to Its Antley-Bixler Syndrome-like Phenotype. J. Biol. Chem. 291, 20487–20502
6. Kranendonk, M., Marohnic, C. C., Panda, S. P., Duarte, M. P., Oliveira, J. S., Masters, B. S. S., and Rueff, J. (2008) Impairment of human CYP1A2-mediated xenobiotic metabolism by Antley-Bixler syndrome variants of cytochrome P450 oxidoreductase. Arch. Biochem. Biophys. 475, 93–99
7. Campelo, D., Lautier, T., Urban, P., Esteves, F., Bozonnet, S., Truan, G., and Kranendonk, M. (2017) The Hinge Segment of Human NADPH-Cytochrome P450 Reductase in Conformational Switching: The Critical Role of Ionic Strength. Front Pharmacol. 8, 755
8. Esteves, F., Urban, P., Rueff, J., Truan, G., and Kranendonk, M. (2020) Interaction Modes of Microsomal Cytochrome P450s with Its Reductase and the Role of Substrate Binding. IJMS. 21, 6669
9. Esteves, F., Almeida, C. M. M., Silva, S., Saldanha, I., Urban, P., Rueff, J., Pompon, D., Truan, G., and Kranendonk, M. (2023) Single Mutations in Cytochrome P450 Oxidoreductase Can Alter the Specificity of Human Cytochrome P450 1A2-Mediated Caffeine Metabolism. Biomolecules. 13, 1083
10. Esteves, F., Campelo, D., Gomes, B. C., Urban, P., Bozonnet, S., Lautier, T., Rueff, J., Truan, G., and Kranendonk, M. (2020) The Role of the FMN-Domain of Human Cytochrome P450 Oxidoreductase in Its Promiscuous Interactions With Structurally Diverse Redox Partners. Front Pharmacol. 11, 299
11. Esteves, F., Campelo, D., Urban, P., Bozonnet, S., Lautier, T., Rueff, J., Truan, G., and Kranendonk, M. (2018) Human cytochrome P450 expression in bacteria: Whole-cell high-throughput activity assay for CYP1A2, 2A6 and 3A4. Biochem. Pharmacol. 158, 134–140

*Poster presentation (18): WG3*

# Stabilizing the thermophilic *Dm*PETase to improve PET degradation by computational protein engineering

Konstantinos Makryniotis[1], Konstantinos Grigorakis[1], Sergio M. Marques[2,3], Efstratios Nikolaivits[1], David Bednar[2], Evangelos Topakas[1]

* vtopakas@central.ntua.gr

[1] Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece
[2] Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic
[3] International Clinical Research Center, St. Anne's University Hospital Brno, Pekarska 53, 656 91 Brno, Czech Republic

Despite recent international negotiations and global diplomatic efforts, plastic pollution continues to rise, posing a serious global threat. Polyethylene terephthalate (PET), the most widely produced polyester, used in textiles and beverage packaging, is limited to biodegradation due to its semi-crystalline and semi-aromatic structure.[1] PET hydrolases (PETases) are enzymes able to depolymerize PET into water soluble intermediates, with degradation enhanced near the glass transition temperature ($T_g \approx 70$ °C), where increased chain mobility facilitates enzyme accessibility. Consequently, PETase stability in such high temperatures has been considered a key requirement for efficient degradation. However, prolonged exposure of PET to temperatures near $T_g$ promotes crystallinity increases that hinder enzymatic depolymerization, making reaction time a critical parameter. Efficient PET degradation therefore requires not only thermostable enzymes but also high catalytic turnover.[2] The recently characterized thermophilic *Dm*PETase, which is less affected by PET crystallinity increase than the benchmark LCC$^{ICCG}$ while maintaining comparable catalytic efficiency, represents a promising template for engineering improved PETases.[2] Hereby, we aimed to enhance the stability of *Dm*PETase by designing disulfide bridges and single point mutations with automated pipelines. Two 200 ns molecular dynamics simulations of a *Dm*PETase homology model, generated using SWISS-MODEL,[3] were performed with Amber to assess residue flexibility.[4] Representative snapshots from these simulations were analyzed with Disulfide by Design 2.0[5] to identify potential disulfide bridges. In parallel, stabilizing point mutations were predicted using PROSS[6] and FireProt 2.0,[7] based on structural models generated by SWISS-MODEL and AlphaFold 2.0.[8] To identify the most promising mutations, candidates were benchmarked against reported engineered variants of *Is*PETase and LCC and further evaluated using machine learning-based MūtCompute predictions.[9] Selected point mutations were validated by in silico mutagenesis and visual inspection in PyMOL, as well as ΔΔG calculations using Rosetta.[10] Three variants containing an additional disulfide bond and two single-point variants were generated by site-directed mutagenesis. Thermal shift assays revealed one variant (*Dm*PETase_$T_L$) with enhanced melting temperature, while its increased thermostability was further confirmed through enzymatic assays in high temperatures. In parallel, PET degradation experiments showed that *Dm*PETase_$T_L$ exhibited a 2.0-fold increase in released hydrolysis products. Overall, engineering *Dm*PETase thermostability can positively impact PET degradation activity providing a framework for developing more efficient PET-depolymerizing biocatalysts.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

**Acknowledgements:**

This work has been financially supported by the European Union's Horizon HORIZON-WIDERA-2023-ACCESS-02 programme under grant agreement No. 101159570-Twinn4MicroUp project, while the work of Konstantinos Makryniotis was supported by the Hellenic Foundation for Research and Innovation (HFRI) under the 5th Call for HFRI PhD Fellowships (Fellowship Number: 19298). Presented project's results are based upon work from COST Action CA21162 COZYME, supported by COST (European Cooperation in Science and Technology).

**References:**

1. Makryniotis, K.; Papi, M.; Nikolaivits, E.; Topakas, E.; Papi, M.; Nikolaivits, E. Structure-Guided Engineering of a Fungal Feruloyl Esterase Enhances Both MHET Conversion and Lignocellulose Breakdown. Int. J. Biol. Macromol. 2025, 148892. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2025.148892.
2. Makryniotis, K.; Nikolaivits, E.; Gkountela, C.; Vouyiouka, S.; Topakas, E. Discovery of a Polyesterase from Deinococcus Maricopensis and Comparison to the Benchmark LCCICCG Suggests High Potential for Semi-Crystalline Post-Consumer PET Degradation. J. Hazard. Mater. 2023, 455 (May), 131574. https://doi.org/10.1016/j.jhazmat.2023.131574.
3. Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., De Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T. SWISS-MODEL: Homology Modelling of Protein Structures and Complexes. Nucleic Acids Res 2018, 46 (W1), W296–W303. https://doi.org/10.1093/NAR/GKY427.
4. Case, D. A., Aktulga, H. M., Belfon, K., Ben-Shalom, I. Y., Berryman, J. T., Brozell, S. R., Cerutti, D. S., Cheatham, T. E. , I., Cisneros, G. A., Cruzeiro, V. W. D., Darden, T. A., Forouzesh, N., Giambaşu, G., Giese, T., Gilson, M. K., Gohlke, H., Goetz, A. W., Harris, J., Izadi, S., Izmailov, S. A., Kasavajhala, K., Kaymak, M. C., King, E., Kovalenko, A., Kurtzman, T., Lee, T. S., Li, P., Lin, C., Liu, J., Luchko, T., Luo, R., Machado, M., Man, V., Manathunga, M. K. M., Miao, Y., Mikhailovskii, O., Monard, G., Nguyen, H., K.A. O'Hearn, Onufriev A., Pan F., Pantano S., Qi R., Rahnamoun A., Roe D.R., Roitberg A., Sagui C., Schott-Verdugo S., Shajan A., Shen J., Simmerling C.L., Skrynnikov N.R., Smith J., Swails J., Walker R.C., Wang J., Wang J., Wei H., Wu X., Wu Y., Xiong Y., Xue Y., York D.M., Zhao S., Zhu Q., Kollman P.A. Amber 2023 Reference Manual; University of California: San Francisco, 2023.
5. Craig, D. B., Dombkowski, A. A. Disulfide by Design 2.0: A Web-Based Tool for Disulfide Engineering in Proteins. BMC Bioinformatics 2013, 14 (1), 346. https://doi.org/10.1186/1471-2105-14-346.
6. Goldenzweig, A., Goldsmith, M., Hill, S. E., Gertman, O., Laurino, P., Ashani, Y., Dym, O., Unger, T., Albeck, S., Prilusky, J., Lieberman, R. L., Aharoni, A., Silman, I., Sussman, J. L., Tawfik, D. S., Fleishman, S. J. Automated Structure- and Sequence-Based Design of Proteins for High Bacterial Expression and Stability. Mol Cell 2016, 63 (2), 337–346. https://doi.org/10.1016/j.molcel.2016.06.012.
7. Musil, M., Stourac, J., Bendl, J., Brezovsky, J., Prokop, Z., Zendulka, J., Martinek, Bednar, D., Damborsky, J. FireProt: Web Server for Automated Design of Thermostable Proteins. Nucleic Acids Res 2017, 45 (W1), W393–W399. https://doi.org/10.1093/nar/gkx285.
8. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A. W.,

Kavukcuoglu, K., Kohli, P., Hassabis, D. Highly Accurate Protein Structure Prediction with AlphaFold. Nature 2021 596:7873 2021, 596 (7873), 583–589. https://doi.org/10.1038/s41586-021-03819-2.
9. Lu, H., Diaz, D. J., Czarnecki, N. J., Zhu, C., Kim, W., Shroff, R., Acosta, D. J., Alexander, B. R., Cole, H. O., Zhang, Y., Lynd, N. A., Ellington, A. D., Alper, H. S. Machine Learning-Aided Engineering of Hydrolases for PET Depolymerization. Nature 2022, 604 (7907), 662–667. https://doi.org/10.1038/s41586-022-04599-z.
10. Kellogg, E. H., Leaver-Fay, A., Baker, D. Role of Conformational Sampling in Computing Mutation-Induced Changes in Protein Structure and Stability. Proteins: Structure, Function, and Bioinformatics 2011, 79 (3), 830–838. https://doi.org/10.1002/prot.22921.

*Poster presentation (19): WG3*

# Evaluation of reducing sugar levels in wine using enzymatic methods

Gianina Marian (Țoca) [1], Gabriela Râpeanu[1], Iuliana Aprodu[1], Nicoleta Stănciuc[1]

tocagianina@gmail.com

[1]Dunărea de Jos University of Galati, Faculty of Food Science and Engineering, Galati, Romania

The determination of sugars in wines is of significant economic interest due to their role as a quality parameter of wine. The hexoses glucose and fructose are the major sugars in grapes, and their content mainly depends on factors such as variety, ripening stage, harvest time, climate, and agricultural practices. Their levels in must range between 170 and 260 g/L, while in wines, both sugars are present in lower concentrations due to the fermentation process, during which they are converted into ethanol by yeast. The residual monosaccharides in wine reflect the degree of alcoholic fermentation and can indicate potential fermentation blockages, non-compliant additions, or technological deviations, which is why their accurate and reproducible determination is fundamental in quality control. In international oenological practice, various analytical methods have been used to quantify sugars, among which classical chemical methods, such as the Schoorl method, have played an important role in the traditional laboratory due to their simplicity and long history of use.

The main objective of this study was to develop an enzymatic method for residual monosaccharides in wines able to identify possible frauds in the collected wine samples. The enzymatic method is based on the fact that glucose and fructose are phosphorylated by adenosine triphosphate (ATP) through an enzymatic reaction catalyzed by hexokinase (HK), resulting in glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P). Glucose-6-phosphate is first oxidized by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH) to gluconate-6-phosphate and reduced NADPH. The amount of reduced nicotinamide adenine dinucleotide phosphate obtained corresponds to the amount of glucose-6-phosphate and glucose. The reduced nicotinamide adenine dinucleotide phosphate is determined by measuring absorbance at 340 nm. After the fructose-6-phosphate is converted into glucose-6-phosphate through the action of phosphoglucoisomerase (PGI). Glucose-6-phosphate reacts again with nicotinamide adenine dinucleotide phosphate to produce gluconate-6-phosphate and reduced nicotinamide adenine dinucleotide phosphate, proportional to the amount of fructose in the wine, which is determined by measuring the absorbance at 340 nm. The sugar content represented by glucose + fructose is expressed in g/L glucose + fructose.

The results revelead after analysis of 127 wine samples (white, red and rose) that the difference between the sugar values found by both methods (school and enzymatic) was less than 1%.

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

*Poster presentation (20): WG3*

# Tailoring Oxidative Enzymes and Catalytic Cascades for Efficient Biocatalysis

Yasmina Mekmouche, Alexandre Ciaccafava, A. Jalila Simaan, Thierry Tron, Ioannis Pavlidis, Katia Duquesne, Véronique Alphand

y.mekmouche@univ-amu.fr

In the search for more sustainable catalytic processes, enzymes have emerged as powerful catalysts due to their high chemo-, regio-, and stereoselectivity under mild conditions. Oxidative enzymes play a central role in this context, enabling the use of molecular oxygen as an environmentally benign oxidant. Among oxidative biocatalysts used in industrial biocatalysis, laccases and Baeyer-Villiger monooxygenases (BVMOs) appears as valuable oxidation catalyst reactions using molecular oxygen as the terminal oxidant.[1] Laccases are multicopper oxidases that catalyze single-electron oxidation reactions coupled to the four-electron reduction of oxygen to water, whereas BVMOs are flavin-dependent monooxygenases that catalyze the insertion of one oxygen atom into carbonyl compounds, leading to the formation of esters or lactones. Limitations such as restricted substrate scope, enzyme stability, and inefficient electron transfer still constrain their broader application. To address these challenges, complementary strategies combining enzyme engineering, catalytic optimization, and cascade design are currently being explored to improve biocatalysis.

A first axis investigates laccase-based catalytic systems. Structural analysis and surface mapping are used to identify accessible residues suitable for the site-selective grafting of photocatalysts or redox mediators.[2] This approach aims to enable controlled electron transfer to the active sites of the enzyme while preserving enzymatic activity. In parallel, oriented immobilization strategies on conductive electrodes or porous materials are being explored to improve enzyme robustness and facilitate integration into heterogeneous catalytic or electroenzymatic systems.[3]

A second axis focuses on the emerging investigation of type II Baeyer-Villiger monooxygenases, such as 2,5-diketocamphane monooxygenase (DKCMO).[4] These enzymes display attractive stability but suffer from significant drawbacks that reduce catalytic efficiency (decoupling reaction, narrow substrate scope). Ongoing work focuses on understanding the factors influencing catalytic efficiency and aims to develop variants with improved performance.

A third axis focuses on the development of multi-enzyme catalytic cascades involving BVMO's and dehydrogenases (alcohol dehydrogenase, ADH) to develop a redox neutral catalytic system.[5] Fusion protein strategies are being investigated to spatially organize enzymes and facilitate intermediate transfer between catalytic steps.

Together, these complementary approaches aim to contribute to the development of robust oxidative biocatalytic systems and to provide new tools for sustainable chemical synthesis.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

**References**


1. (a) Cardullo N., et al. RSC Chem. Biol., 2022, 3, 614; (b) Fürst M. J. L. J., et al. ACS Catal., 2019, 9, 11207; (c) Paul C. E., et al. Biotechnology Advances 2021, 51, 107712.
2. (a) Yang F., et al. ChemPlusChem 2023, 88, e202300156; (b) Robert V., et al. ChemPlusChem 2017, 82, 607; (c) Modenez I. de A., et al. Chem. Eur. J. 2025, e202404633.
3. (a) Contaldo U., et al. J. Mater. Chem. A 2023, 11, 10850 ; (b) Lalaoui N., et al., ACS Catal. 2016, 6, 1894 ; (c) Gentil S., et al., Chem. Eur. J. 2020, 26, 4798.
4. Rollig R., et al. ChemBioChem, 2022, 23, e202200293.
5. Ménil S., et al. Biotechnol. Bioeng., 2019, 116, 2852.

*Poster presentation (21): WG3*

# Tuning the polyesterase promiscuity of Antarctic MoPE: Biochemical and docking insights into substrate-specificity shifts

Konstantinos Makryniotis[1], Panagiota Karampa[2], Maria Dimarogona[2], Efstratios Nikolaivits[1], Evangelos Topakas[1]

snikolai@chemeng.ntua.gr

[1] Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece
[2] Laboratory of Structural Biology and Biotechnology, Department of Chemical Engineering, University of Patras, Patra, Greece

The global surge of synthetic polyesters has intensified the search for enzymes that depolymerise these materials under mild conditions. Production of biodegradable polyesters such as poly(butylene adipate-co-terephthalate) (PBAT), polycaprolactone (PCL) and poly(3-hydroxybutyrate) (PHB) is rising steeply, yet the catalytic landscape on these emerging plastics remains largely unmapped. Polyesterases are inherently promiscuous, but protein engineering campaigns have focused almost exclusively on PET, leaving activity and specificity on alternative polyesters understudied and untuned.

Here we selected the mesophilic, versatile polyesterase MoPE from *Moraxella* sp. as a scaffold and introduced mutations previously shown to enhance benchmark PETases (LCC and IsPETase) to test whether single substitutions could re-route specificity toward PBAT, PCL and PHB without sacrificing stability. Five single and two double variants were constructed and successfully expressed in *Escherichia coli*, purified and screened on PBAT, PCL and PHB at 30 °C.

Variant V96T boosted PCL degradation 10-fold relative to wild-type MoPE. On PBAT, Y188W/F220I released 1.2-fold more terephthalate-derived products but 3-fold fewer aliphatic products than WT, whereas D127G and Y188W showed the inverse preference, keeping total monomer release comparable. Docking of representative oligomeric models traced these shifts to altered π-stacking and H-bond networks around PBAT's aromatic moiety. On PHB, the double variant doubled hydrolysis, while V96T matched WT activity. These variants remained equally stable at 30 °C, but Y188W/F220I displayed 2-fold higher thermostability after 24 h at 40 °C.

Our data demonstrate that minimal mutation loads can rebalance polyester specificity in cold-active enzymes, providing tuneable biocatalysts for mixed-polyester waste streams.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

*Poster presentation (22): WG3*

# Yeast surface display of the human kappa opioid receptor to enhance solubility

Mária Tomková[1], Mária Repovská[1], Erik Sedlák[1,2]

erik.sedlak@upjs.sk

[1] Center for Interdisciplinary Biosciences, Technology and Innovation Park, University of Pavol Jozef Šafárik, Jesenná 5, 041 01 Košice, Slovakia
[2] Department of Biochemistry, University of Pavol Jozef Šafárik, Moyzesova 11, 041 54 Košice, Slovakia

G protein-coupled receptors (GPCRs) are cell-surface proteins that mediate cellular responses to diverse endogenous signaling molecules. Despite their importance, structural determination of GPCRs remains challenging, with only a limited number of crystal structures resolved to date due to difficulties associated with their expression and purification (1). The human kappa-opioid receptor (hKOR), a member of the GPCR superfamily, is widely expressed in the central nervous system and peripheral tissues, where it binds endogenous opioid peptides (dynorphins) to regulate pain, mood, reward, and cognitive functions (2). In this study, we aim to develop a soluble hKOR variant using directed evolution, ribosome display, and yeast surface display **(Fig. 1)** to facilitate its structural analysis and biophysical characterization.

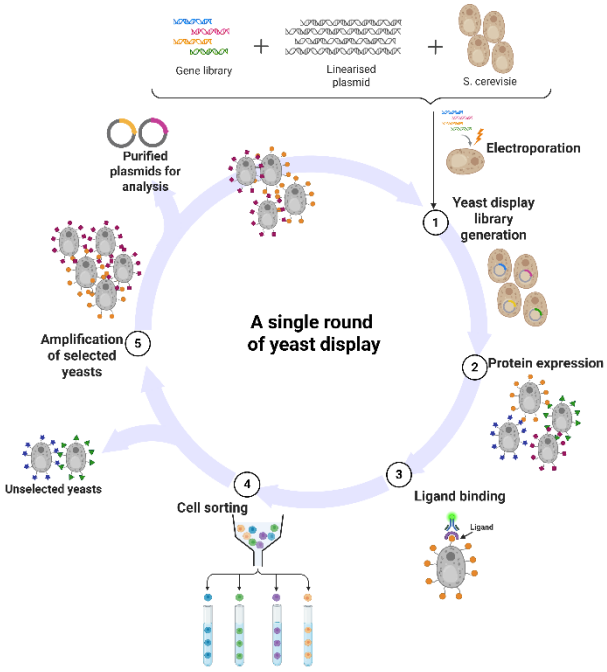



*Figure 1. Schematic representation of one round of yeast display.*

A diverse hKOR DNA library was generated by randomizing 40 surface-exposed hydrophobic residues to hydrophilic ones. Four rounds of ribosome display were performed to reduce library complexity. The enriched library and a linearized yeast display vector (pYD) were subsequently co-transformed into *Saccharomyces cerevisiae* EBY100 via electroporation and

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

homologous recombination. Surface expression was induced and confirmed by confocal microscopy **(Fig. 2)** and flow cytometry (FACS) using anti-c-myc labelling (Alexa Fluor 488) and dynorphin binding (Alexa Fluor 647). Three iterative rounds of FACS sorting enriched for full-length, potentially functional hKOR variants.

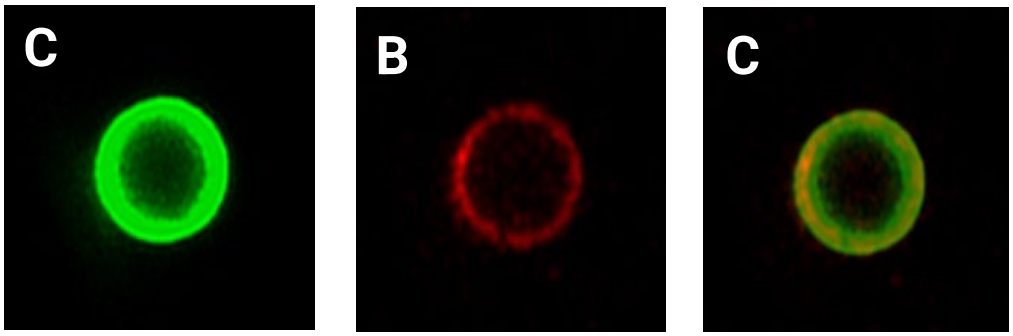



*Figure 2. Confocal microscopy images of Saccharomyces cerevisiae EBY100 displaying hKOR. Cells were labelled with anti-c-myc antibody (Alexa Fluor 488, green signal) to detect surface expression and fluorescently labelled dynorphin (Alexa Fluor 647, red signal) to assess ligand binding.*

In conclusion, successful yeast transformation was confirmed by selective growth on tryptophan-deficient media. Flow cytometry revealed progressive enrichment of the population labelled with anti-c-myc and dynorphin binding, indicating improvements in solubility.

**Acknowledgements:** The authors would like to thank Dr. Veronika Huntošová for her help with microscopy analysis and preparation of Figure 2. Funded by the EU NextGenerationEU through the Recovery and Resilience Plan for Slovakia under projects No. 09I01-03-V04-00041 and 09-I02-03-V01-00021.

**References:**

1. A.D. Rawlings, Biochem. *Soc. Trans.,* **2016**, 44, 3, 790–795.
2. M. Dalefield, *et al*., *Front. Pharmacol.,* **2022**, 13, 837671.

*Poster presentation (23): WG3*

# Directed Evolution by Ribosome Display Optimizes Haloalkane Dehalogenases for Bioremediation

Ivana Timková[1], Michal Nemergut[1], Rastislav Varhač[2], Erik Sedlák[1]

ivana.timkova@upjs.sk, michal.nemergut@upjs.sk, rastislav.varhac@upjs.sk, erik.sedlak@upjs.sk

[1]Center for Interdisciplinary Biosciences, Technology and Innovation Park, P.J. Šafárik University in Košice, Jesenná 5, 041 54 Košice, Slovak Republic
[2]Institute of Chemistry, Faculty of Science, P.J. Šafárik University in Košice, Šrobárova 1014/2, 040 01 Košice

Protein modification strategies for biocatalyst optimization include rational design, relying on structural analyses, and directed evolution via experimental selection from variant libraries. Here, we applied ribosome display (RD) — a display technique for directed evolution of haloalkane dehalogenases (HLDs), hydrolytic enzymes from the α/β-hydrolase superfamily that detoxify halogenated aliphatic pollutants. HLDs feature a variable cap and conserved α/β-hydrolase domain, with a catalytic pentad in an interfacial cavity accessed via two tunnels (Figure 1). Despite their promise, native HLDs often underperform industrially, necessitating optimization via protein engineering [1] [2].

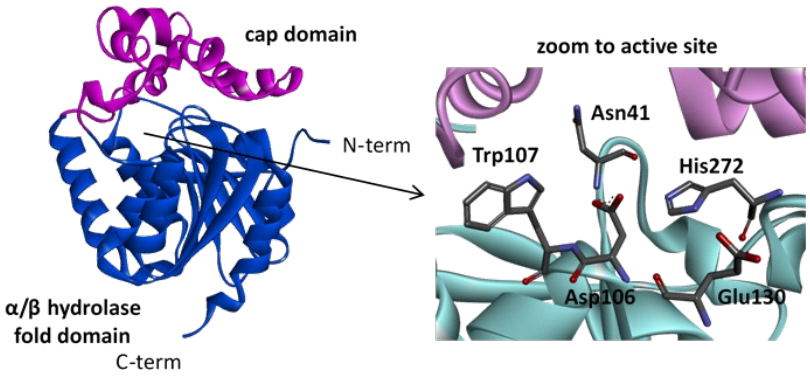



*Figure 1. Tertiary structure of HLD DhaA and topology of catalytic pentad inside the active site of DhaA [PDB: 4HZG].*

Using DhaA_115 H272N variant as a model, we generated a library via error-prone PCR and performed four rounds of RD selection against immobilized biotinylated HaloTag ligand. The post-selection variants RD4-32 and RD4-37, upon expression and characterization, both displayed diminished thermostability (as evaluated by circular dichroism and differential scanning calorimetry); however, enhanced enzymatic activity toward 1-chlorobutane, 1-iodobutane, and 1-bromobutane was observed exclusively in RD4-37. Preliminary structural insights link these gains to mutations in access tunnels.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

This work validates RD as a robust, structure-independent tool for directed evolution, enabling rapid development of industrially viable HLD biocatalysts for environmental detoxification.

**Acknowledgements:** This work was supported by the EU NextGenerationEU through the Recovery and Resilience Plan for Slovakia under the project No. 09I03-03-V04-00112 and by the grant agency of the Ministry of Education, Science, Research, and Sport of the Slovak Republic (grant no. VEGA 1/0074/22).

**References:**

1. D. B. Janssen, *Curr. Opin. Chem. Biol.*, **2004**, 8, 150-159.
2. J. Damborský, J. Koca, *Protein Eng*., **1999**, 12, 11, 989-998.

*Poster presentation (24): WG3*

# Defluorination by Design: ML guided Enzyme Engineering

Aron Wanz [1], Daniel Kracher[1], Tommaso Ancilli[2], Robert Peharz[2]

Aron.wanz@tugraz.at

[1] Graz University of Technology, Institute of Molecular Biotechnology, Graz, Austria
[2] Graz University of Technology, Institute of Machine Learning, Graz, Austria

The environmental persistence of fluorinated hydrocarbons stems from the high thermodynamic stability of the C-F bond. With the highest dissociation energies of single bonds in organic chemistry, these anthropogenic pollutants cannot be degraded by naturally evolved metabolic pathways.

While nature lacks a specific defluorinase, Dehaloperoxidase (DHP) from the marine annelid *Amphitrite ornata* represents a rare, multifunctional hemoglobin. It is capable of switching from oxygen transport to peroxidase activity, demonstrating a latent ability to facilitate the oxidative dehalogenation of various halogenated phenolic compounds.

To expand the substrate specificity towards efficient defluorination of fluorinated phenols, we apply a machine learning based enzyme evolution strategy via the introduction of an adapted workflow, which joins kinetic enzyme characterisation of combinatorial variant libraries, Bayesian Optimisation guided machine learning and predictive computational methods.

We are trying to not only establish an engineering platform towards carbon fluoride bond cleavage, but also elucidate the evolutionary origins and trajectory of multifunctional hemoglobins in annelids, as these enzymes may serve as valuable foundation for further engineering approaches, while expanding the sequence space of DHPs.

**Acknowledgements:** LEAD project: DigiBioTech, TU Graz, Austria; BioTechMed-Graz, Austria.

*Poster presentation (25): WG3*

# Biochemical and computational tools for the study and valorization of white-rot fungal xyloglucanase *Abi*XegA

Grigorakis, K. [1], Zhara, A.[2], Pentari, C.[1], Daskalakis, V.[3], Topakas, E.[1], and Zerva, A.[2]*

anazer@aua.gr

[1] Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece
[2] Laboratory of Enzyme Technology, Department of Biotechnology, School of Applied Biology and Biotechnology, Agricultural University of Athens, 75 Iera Odos Street, 11855 Athens, Greece
[3] Department of Chemical Engineering, University of Patras, Caratheodori 1, University Campus, GR 265 04 Patras, Greece

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 substrates[1]. In order to shed light on the enzymatic degradation of xyloglucan, a novel xyloglucanase from the basidiomycete *Abortiporus biennis* (*Abi*XegA), with unknown structure was studied. Initially, the structure of xyloglucanase was predicted by AlphaFold 2.0[2] starting from the aminoacid sequence. In order to identify binding preferences, the modelled structure with 17 xyloglucan 10-mer ligand variants were subjected to a total of 17 μs molecular dynamics (MD) simulations in GROMACS, 250ns each in 4 replicates, containing all combinations of xylose and galactose decorations. Effective binding poses were sampled and were evaluated using ligand binding free energies, as calculated by the MMGBSA method. The enzyme was heterologously produced in *P. pastoris* as the host and purified to determine its biochemical and catalytic properties. *Abi*XegA was found to be a glycoprotein with relatively strict substrate specificity, since it is only active in xyloglucan and glucomannan, 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.

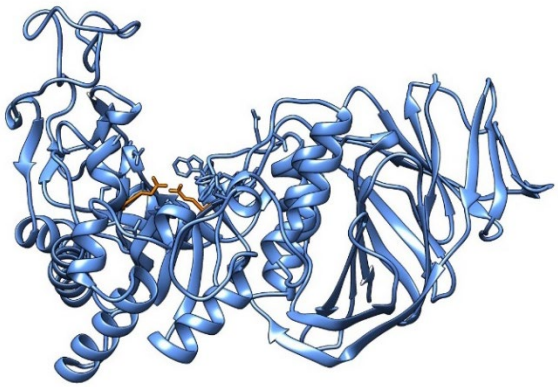


*Figure 1. Alphafold model structure of AbiXegA. The proposed catalytic residues are highlighted in orange.*

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

**References:**

1. P. Sun, X. Li, A. Dilokpimol, B. Henrissat, R.P. de Vries, M.A. Kabel, M.R. Mäkelä, *iScience*, **2022,** 25, 103666.
2. J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S.A.A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, *Nature,* **2021,** 596, 583–589.

*Poster presentation (26): WG3*

# Uncovering the Evolutionary Origin of Enantioselectivity in Borneol-type Dehydrogenases

Jasmin Zuson[1], Carl P.O. Helmer[2], Bruno Di Geronimo[3], Andrea M. Chánique[1], Katarína Kavčiaková[1], Rosa Teijeiro-Juiz[2], Sebastian Brickel[4], Ivana Drienovská[5], Daniel Kracher[1], Shina Caroline Lynn Kamerlin[3,6], Bernhard Loll[2], Robert Kourist[1]

zuson@tugraz.at

[1] Graz University of Technology, Institute of Molecular Biotechnology, Graz, Austria
[2] Free University of Berlin, Institute of Chemistry and Biochemistry, Berlin, Germany
[3] Georgia Institute of Technology, School of Chemistry and Biochemistry, Atlanta, USA
[4] Uppsala University, Department of Chemistry – BMC, Uppsala, Sweden
[5] Vrije Universiteit Amsterdam, Department of Chemistry and Pharmaceutical Sciences, Amsterdam, The Netherlands
[6] Lund University, Department of Chemistry, Lund, Sweden

The exceptional stereoselectivity of enzymes is crucial for industrial applications, enabling the development of biocatalyst-based synthesis routes for enantiopure compounds. Yet rationalizing how enzymes discriminate between highly hydrophobic substances within hydrophobic active sites remains challenging. This is particularly difficult in the family of plant borneol-type dehydrogenases (BDHs), where closely related sequences exhibit striking differences in enantioselectivity [1,2].

To identify the mutations that led to the high specificity of *Salvia rosmarinus* BDH1 (*Sr*BDH1)[1], we applied ancestral sequence reconstruction (ASR) to trace an evolutionary trajectory from a non-selective ancestor to the highly enantioselective *Sr*BDH1[1]. By combining experimental data with crystal and *de-novo* protein structures of ancestral nodes, we identified key mutations driving stepwise diversification (Figure 1). Targeted substitutions and substrate-enzyme interaction modelling revealed further insight into the determinants behind the selectivity towards the highly hydrophobic substrate borneol.

This work highlights the importance of context-dependent evolutionary changes in enzymes, where intricate substrate-enzyme interactions determine stereoselective catalysis. It demonstrates how computationally guided enzyme engineering tools can decode natural evolutionary principles in underrepresented enzyme families.

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

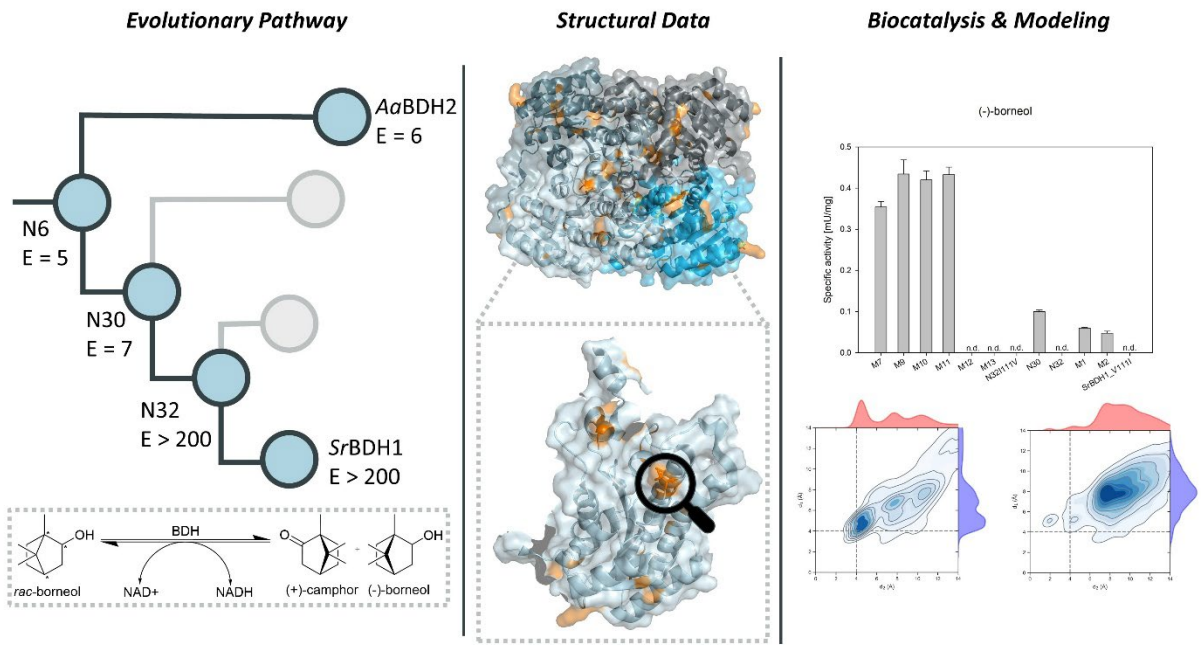



*Figure 1. Ancestral sequence reconstruction of BDH enzymes as a guide to identifying peripheral and active site mutations in the structure leading to changes in enantioselectivity and -specificity.*

**Acknowledgements:** FWF Austrian Science Fund, https://ror.org/013tf3c58, 10.55776/COE17, 10.55776/P34280; European Commission, 101072731; The Vasser Wooley Foundation; Georgia Tech; Georgia Research Alliance.

**References:**

1. A.M. Chánique et al., *ChemCatChem*, **2021**, Vol. 13, Issue 9, 2262-2277.
2. R. Croteau, *Arch. Biochem. Biophys.*, **1978**, Vol. 188, Issue 1, 182-192.