COST ACTION CA21162

COZYME

Computational Redesign of Enzymes

1st Working Group Meeting

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MEETING BOOKLET

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COST ACTION CA21162

Computational Redesign of Enzymes

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Computational Redesign of Enzymes

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About CA 21162 COZYME

Enzymes are essential for life, enabling the required biological chemistry to occur. Owing to their unparalleled chemical and eco-friendly properties, enzymes are also industrially relevant. For example, enzymes are applied in food and pharma, while they are also included in laundry detergents. Despite their staggering chemical potential, the industrial use of enzymes is lagging behind. This is mainly because enzymes do not tolerate the conditions of their potential applications. To exploit their industrial use, enzymes have to be improved to withstand these process conditions often with additional tuning of their activity. This is typically accomplished by directed-evolution, which is laborious because it requires the experimental screening of massive mutant libraries to find the desired variants. This has been addressed by the development of computational enzyme engineering tools that show great promise by harnessing the power of a computer to create and screen large virtual libraries or to predict beneficial mutations. This dramatically speeds up and improves the efficiency of a protein redesign campaign. The COZYME (COmputationally assisted design of enZYMEs) Action comprises a Pan-European collaborative network aimed at developing and implementing state-of-the-art computational tools for rapid enzyme improvement. This will solve a key bottleneck in biotechnology: the exploitation of industrially relevant enzymes. Specifically, the Action focuses on four issues:

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

Committees

COZYME Core Group

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Dr. Anamaria Todea	ITC Conference Manager, University of Trieste
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Prof. Lucia Gardossi	WG2 Vice Leader, University of Trieste
Prof. Jürgen Pleiss	WG3 Vice Leader, University of Stuttgart

Local organizer

Prof. José M. Palomo	Chemical Biology and Biocatalysis Group. Institute of Catalysis and Petrochemistry (ICP), The Spanish National Research Council (CSIC)

Agenda 1st WG meeting

March 20th, Fuengirola, Spain

Time	Program	Speaker
09:00 – 09:30	Registration and Welcome	
09:30 – 09:50	Introduction	Action chair: **Prof. Marco Fraaije**
WG1 Session	Chair: **Dr. Sérgio Marques** (WG1 leader)	
09:50 – 10:00	WG1 introduction	**Dr. Sérgio Marques**
10:00 – 10.30	WG1 keynote lecture	**Dr. Sérgio Marques**
10:30 – 11:00	**Coffee break**	
11:00 – 11:20	WG1 presentation 1	**Dr. Mehdi D. Davari**
11:20 – 11:40	WG1 presentation 2	**Dr. Maximilian Fürst**
11:40 – 12:00	WG1 presentation 3	**Dr. Artur Góra**
WG2 Session	Chair: **Dr. Aleksandra Maršavelski** (WG2 leader)	
12:00 – 12:10	WG2 introduction	**Dr. Aleksandra Maršavelski**
12:10 – 12.40	WG2 keynote lecture	**Prof. Lucia Gardossi**
12:40 – 14:30	**Group photo and Lunch**	

14:30 – 14:50	WG2 presentation 1	**Dr. Sara Fortuna**
14:50 – 15:10	WG2 presentation 2	**Prof. Maciej Szaleniec**
15:10 – 15:30	WG2 presentation 3	**Dr. Pedro Sánchez-Murcia**
15:30 – 16:00	**Coffee break**	
WG3 Session	Chair: **Prof. Lígia O. Martins** (WG3 leader)	
16:00 – 16:10	WG3 introduction	**Prof. Lígia Martins**
16:10 – 16.40	WG3 keynote lecture	**Prof. Jürgen Pleiss**
16:40 – 17:00	WG3 presentation 1	**Prof. Dirk Tischler**
17:00 – 17:20	WG3 presentation 2	**Prof. Marina Lotti**
17:20 – 17:40	WG3 presentation 3	**Dr. Ani Paloyan**
17:40 – 18:30	MC meeting for MC members / Drinks on the rooftop	
20:00 – 23:00	**Dinner**	

Speaker	Title
WG1 Session	
Dr. Sérgio Marques	*Computational tools for design of enzyme stability*
Dr. Mehdi D. Davari	*Transforming protein engineering through the power of in silico methods*
Dr. Maximilian Fürst	*High-throughput predictions and screening of protein variants*
Dr. Artur Góra	*Water molecules as a key for enzymes interior understanding and reshaping*
WG2 Session	
Prof. Lucia Gardossi	*Optimization of catalytic properties: integrating tools for computational platform development*
Dr. Sara Fortuna	*Automatic screening of enzymes for synthesis and biodegradation of renewable polyesters*
Prof. Maciej Szaleniec	*Engineering tungsten aldehyde oxidoreductase from Aromatoleum aromaticum in Aromatoleum evansii expression system*
Dr. Pedro Sánchez-Murcia	*First steps for the prediction of enzymatic reaction energy barriers using machine learning*
WG3 Session	
Prof. Jürgen Pleiss	*FAIR and scalable biocatalytic experimentation and integration with computational modelling*
Prof. Dirk Tischler	*Functional annotation: from genes to processes*
Prof. Marina Lotti	*Features of cold active enzymes*
Dr. Ani Paloyan	*Hydantoinase process enzymes and more*

Abstracts Working group 1

Computational optimization of global enzyme properties

Integrating artificial intelligence and multiscale modeling: Empowering the discoveries in protein design and engineering in the data-driven era

Mehdi D. Davari, Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle, Germany (Email: mehdi.davari@ipb-halle.de)

The advent and emergence of “big-data” in biotechnology combined with advances in artificial intelligence (AI) have created unique opportunities for **“data-driven” modelling methods**. Our current focus is on the development of AI-based methodologies to (i) enzyme discovery and mining (ii) enzyme design, and (iii) creation of tailor-made proteins by exploring the biodiversity or combinatorial mutagenesis landscapes. We are developing-through hybrid biophysics-AI deployments, predictive methods to accelerate *in silico* high-throughput screening of enzymes, provide unique insight into protein sequence landscapes, and assist large-scale engineering campaigns for biocatalysis.

Our future research will focus on the **integration of multiscale simulation techniques and AI-based methods** to elucidate fundamental molecular mechanisms and evolution of enzymes with emphasis on biocatalysis. In this regard, we aim to contribute to the emerging field of integration of highly interdisciplinary methods, including state-of-the-art computational biophysics methods (e.g., quantum mechanical, molecular dynamics, and QM/MM simulations), with statistical and AI-approaches with the overarching goal to advance our knowledge of enzyme catalysis and evolution. The key for realizing this goal is to **bridge between theory and experiment**. The guiding principle throughout method development should be always to compare and validate the simulations results with experimental data, in close collaboration with experimental groups. These synergistic approaches are of key importance to addressing major global challenges (e.g., environmental and health issues such as microplastics) and to ultimately achieving a more sustainable future based on “bio-inspired” and “bio-based” technologies.

Our holistic vision is to **transform computational modelling as an efficient approach so that great challenges in protein design can be addressed through combined “multiscale” simulation and “data-driven” AI modeling approaches.** We believe that advancing the “integrated *in silico* and hierarchical” approaches, which blend with protein biophysics and protein engineering experiments, will trigger a quantum leap in the discoveries in design of proteins. It is expected that through the convergence of quantum computing and AI a tremendous computational power will be available to simulate complex systems for disruptive application such as holistic *in silico* design of enzymes, enzymatic processes and cascades in close future.

High-throughput predictions and screening of protein variants

Maximilian Fürst, University of Groningen, The Netherlands

The Fürstlab is the computational protein (re)design group at the University of Groningen, established in June 2022. Our main research interest lies in the combination of computational protein design and high-throughput screens for protein engineering towards biotechnological and synthetic biology applications. One core question in our research is how a large number of computationally predicted protein variants can be efficiently generated and tested experimentally. In addressing this need by developing and applying high-throughput protein function assays (such as *in vivo* assays, protein display, or droplet screens) and combining those with deep sequencing, we aim to generate large sequence-function datasets used as input for improved or new computational design approaches. To that end, our lab applies an interdisciplinary approach involving computational biology / bioinformatics, molecular / synthetic biology, and biochemistry. As protein engineers, we aim to install novel or improved functionalities in natural enzymes, and we are particularly interested in enzymes and proteins that interact with nucleic acids and nucleotide-derived cofactors. While our initial projects are aimed at improving inherent protein parameters such as stability, we also aim to engineer enzymatic reactivities in the future.

Water molecules as a key for enzymes interior understanding and reshaping

Artur Góra, [1]Tunneling Group, Biotechnology Centre, Silesian University of Technology, ul. Krzywoustego 8, 44-100 Gliwice, Poland

Water molecules maintain enzymes' structures, functions, stabilities and dynamics. Therefore, they are ideal probes for investigation of enzymes' properties which allows us to understand the mechanisms responsible for macromolecules interactions, signal transduction and catalysis. The analysis of water molecules' distribution and trajectories allows to study proteins' interactions with small molecules, composition, tunnel and cavities dynamics and functionality, transportation network and location of key residues. Such studies of the dynamics of tunnels, cavities and other types of intramolecular voids buried inside macromolecule's core remain a challenging task due to the ephemeral character of these elements. Moreover, the experimental methods hardly provide insight into accessibility of such structures, transport of small ligand(s) or solvent exchange between protein interior and the environment.

We would like to present a comprehensive overview of several studies performed by us in the last few years using water tracking software which provided insight and understanding into proteins' function and evolution which then facilitated enzyme redesign and drug discovery. By combining molecular dynamic simulations with our software, we got access to information about flow direction, and regions in enzymes in which small molecules are stuck or trapped. By analysing the local distribution analysis, we were able to determine the approximate energy profile of a particular passage and finally understand changes in enzyme performance due to particular mutations. The extensive comparison of interior dynamics within the enzyme family helps us to understand tunnels network evolution. The analysis of the protein interior from the perspective of water molecules can be used as a versatile and exhaustive approach that enables enzymes understanding and re-engineering.

Abstracts Working group 2

Computational optimization of catalytic properties

Automatic screening of enzymes for synthesis and biodegradation of renewable polyesters

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Plastic pollution, specifically plastics derived from fossil fuels, is one of the most challenging environmental issues of the past years. The solution offered by bioeconomy is represented by renewable bio-based polymers [1]. Even though several bio-based plastics have been commercialized in the last years, e.g. polylactic acid, polyhydroxy-alkanoates, and more recently polybutylene succinate, the range of monomers derived from renewable resources expands [2]. Biocatalysis can boost such innovation, leveraging on enzymes that overcome the limitations of conventional chemical strategies by catalyzing, under highly selective and mild conditions, the targeted modification, synthesis or degradation of polymers and, most importantly, biobased polymers [3]. Hydrolases, such as lipases and cutinases, were successfully used for *in vitro* polycondensation of bio-based diacids and polyols, leading to biodegradable polyesters with controlled structures [4]. In parallel the capacity of several cutinases and lipases to degrade polyesters was evaluated by several groups [5]. The possibility to correlate structural features of a polymer with the catalytic properties of an enzyme would allow the rational design of environmental safe new tailor-made biodegradable polymers. However, the experimental screening of new enzymes is very expensive and time-consuming [6]. *In silico* screening, using appropriate bioinformatics tools, can be an effective aid in accelerating the selection of optimal biocatalysts [7]. Previously, we reported the development of an automatic workflow for the generation and selection of virtual mutants endowed with amidase activity [8]. In the present study we apply the concept for 5 diols, 5 diacids, and combinations (dimers) all biobased and 6 enzymes: 3 lipases and 3 cutinases. Automatic workflow matches the chemical structure with the most effective enzyme structure using as a scoring function (docking energy and the geometric compatibility for the near attack conformation (productive poses) of dimers (hydrolysis and elongation) and diacids (synthesis). In this framework we developed an automated pipeline that embraces well-established computational techniques based on docking and molecular dynamics simulations aiming at: (i) the identification of the optimum biocatalyst for the oligoesters synthesis and hydrolysis of a given substrate, (ii) the identification of the optimum substrates for a given biocatalyst. The same process can be further employed for (iii) the full characterization of a given biocatalyst in terms of reactivity towards several substrates. Thanks to the integration of docking and molecular dynamics simulations, together with the geometrical assessment of productive poses. The pipeline, here implemented in modeFRONTIER software allows to select, from a pool of enzymatic structures, the optimum biocatalyst for catalyzing the synthesis and/or the hydrolysis of polyesters.
The automatic pipeline implemented in the present study for *in-silico* substrates and enzymes screening is described in Figure 1.

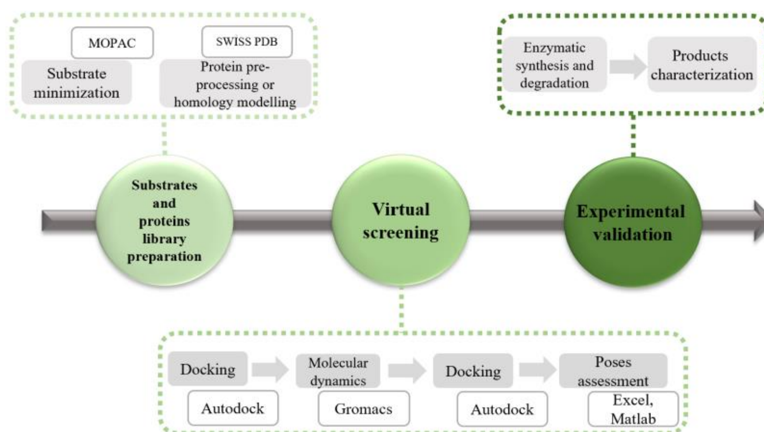

Figure 1. Process flow diagram implemented for *in-silico* substrates and enzymes screening.

The modeFRONTIER software (Figure 2), connected different codes through a graphic user interface.

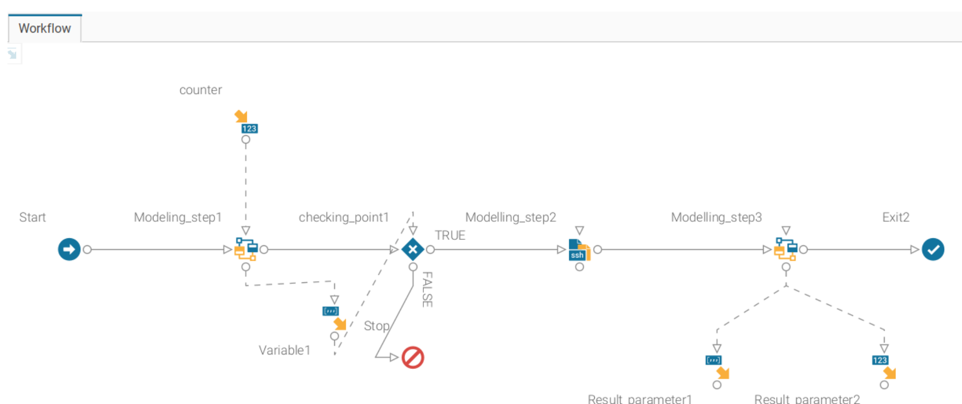

Figure 2. Automatic computational procedure for *in-silico* enzyme screening. From left to right: Substrates and enzymes preparation, docking, molecular dynamics, docking and data analysis.

At the end of the automatized computational flow, the scoring procedure was accomplished by computing the percentage of productive poses determined based on NAC (Figure 3b). These were identified by taking as a reference C atom from carboxylic (for chain elongation) and ester groups (for hydrolysis). The docking free energies (red squares) correspond to the sum of intermolecular energies (including desolvation energies), internal energies, and torsional free energies. Large, negative, values are associated with favorable conformations.

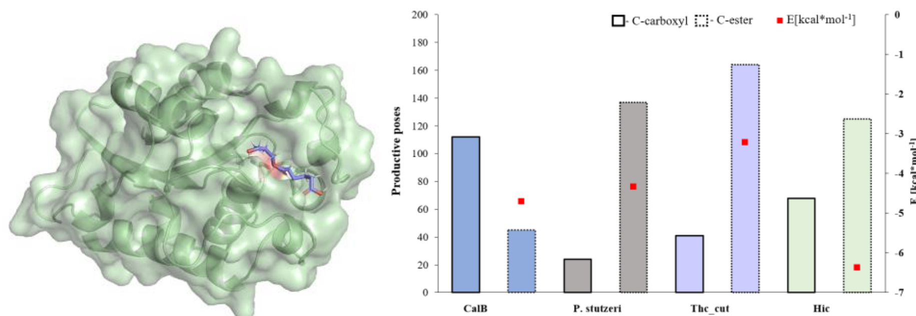

Figure 3. Conformation of the dimer containing 1,4-butandiol and adipic acid in the active site of the Hic cutinse with respect to the catalytic Ser 105 (left). Comparison of NAC productive poses obtained by analyzing the attack of the two different acyl groups (carboxylic acid = black line; ester group = black dots) resulted from the automatic flow for a specific adipic acid based dimer. Red squares correspond to the average of the docking free energies of the corresponding productive poses (right).

This study demonstrates that automatic computational work-flows can be used for the fast selection of enzymes efficient in catalyzing the hydrolysis and/or synthesis of bio-based polyesters. The results represent a contribution for the rational eco-design of tailored new sustainable polymers and a first step towards the *in silico* generation and screening of new biocatalysts [9] for the polymer sector.

Acknowledgements

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Engineering tungsten aldehyde oxidoreductase from *Aromatoleum aromaticum* in *Aromatoleum evansii* expression system

Agnieszka Winiarska, Maciej Szaleniec,
Jerzy Haber Institute of Catalysis and Surface Chemistry Polish Academy of Sciences

Keywords: Tungsten aldehyde oxidoreductases, carboxylic acid reduction, hydrogenase, enzyme kinetics

The W-dependent aldehyde oxidoreductase from the facultative anaerobic, denitrifying bacterium *Aromatoleum aromaticum* (AOR_{Aa}) catalyses the oxidation of aldehydes to carboxylic acids and the respective reverse reaction. The AOR_{Aa} consists of three subunits compared to the single one of previously known archaeal AORs (α_2) and, therefore, must exhibit a more complex structure. However, the quaternary structure of the enzyme remained unknown. Recently we have shown that the enzyme exhibits a fascinating case of an enzymatic nanowire system. Cryo-EM single particle analysis resulted in a 3D reconstruction of a filamentous complex with an overall resolution of 3.4 Å. The resolved structure showed a composition of multiple ab-protomers forming a filament on top of a single FAD-binding γ-subunit. Although much different than expected, this novel structure was confirmed by mass photometry analysis. The tungsten cofactor of AOR_{Aa} is localised in the large W-cofactor-carrying b-subunits, which branch out of the connecting nanowire structure formed by the oligomer-forming α-subunits. Electric conductivity is provided throughout the nanowire and into the branched-out β-subunits by a chain of connected Fe_4S_4 clusters, which also links up to the FAD cofactor in the γ-subunit, facilitating the use of NAD^+, benzyl viologen (BV) and ferredoxin as redox mediators [1]. The geometry of the W cofactor in AOR_{Aa} was found to be highly similar to that of the respective cofactor in *Pyrrococus furiosus* AOR [3], which we previously had reinterpreted by theoretical modelling (QM-only, QM:MM) [4]. We have also proven that AOR_{Aa} accepts hydrogen as an electron donor for reducing either carboxylic acids or NAD^+, which can be used to produce valuable aldehydes or recycle NADH in biochemical cascade reactions [2]. Finally, we have demonstrated that AOR can be efficiently immobilised on a GC electrode and used in biocatalytic/electrochemical systems for the synthesis of organic compounds or as a platform for biosensors [5].

Based on these findings, we will develop the enzyme further, using a rational-based design based on predictions from QMMM models of the reaction. We aim to enhance the (side) activity of the enzyme with hydrogen and to increase its affinity for carboxylic acids (i.e. the natural product of the native reaction). Furthermore, we will develop a platform technology for efficient directed evolution in *A. evansii*.

Furthermore, we would like to develop chimeric enzymes using different AOR-like catalytic subunits found in various homologous strains. The nanowire nature of the enzyme provides an unprecedented opportunity to construct an enzymatic system with enhanced substrate range due to the assembly of different types of catalytic units on the same nanowire core.

Finally, we would like to develop whole-cell cascade systems which utilise AOR's double activity (H_2-dependent reduction of carboxylic acid and NADH recycling) and other enzymes (such as aldolases or alcohol dehydrogenases) to transform reactive aldehydes into other more valuable and stable compounds.

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Prediction of enzyme catalysis by computing reaction energy barriers and using Machine Learning

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Introduction of mutations in enzyme scaffolds allows the modification of properties such as catalytic efficiency or substrate specificity. Nowadays, directed evolution method[1] is the golden experimental approach for getting a desired scaffold with particular properties.[2] However, such method is both time- and resource-consuming. On the other hand, computational methods allow the generation and evaluation of virtual enzyme variants in a rational way. Hence, they stand as an appealing solution for reducing the size of the mutational landscape to be experimentally explored. Indeed, different efficient tools are already available for the scientific community.[3,4] Nevertheless, the use of *in-silico* methods for the prediction of catalytic activities in in protein engineering campaigns is still in a development phase.

In this communication I will present our first results in the use of kernel regression methods to predict catalytic energy barriers calculated via steered QM/MM MD simulations.[5] As showcase, we selected the second catalytic step of the hydrolysis of mono-(2-hydroxyethyl)terephthalic (MHET) acid by the enzyme MHETase from *Ideonella sakaiensis*.

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Abstracts Working group 3

Experimental evaluation and characterization

Functional annotation: from genes to processes

Dirk Tischler, Ruhr University Bochum, Microbial Biotechnology

The Microbial Biotechnology group (MBT) is working in the field of functional prediction of biocatalysts; so to say from gene to process. We focus mainly on oxidoreductases and among those we have several topics which are of interest for the COZYME action. Current projects involve: The structural elucidation of FAD-dependent styrene/indole monooxygenases to explain and improve the enantioselectivity of these biocatalysts for selective epoxidation and sulfoxidation. As we lack structural data comprising substrate and cofactor binding, we currently employ homology modelling and molecular dynamics to computationally guide enzyme engineering. A related flavoprotein monooxygenase study involves BVMO-like N-hydroxylases from siderophore biosynthetic pathways. Those are NADPH/FAD dependent and interesting targets for biocatalysis and potential drug targets. Here, we engineer the enzymes for either altered substrate preferences or higher process stability to employ it in enzymatic cascades towards novel N-activated drugs. The product of those enzymes is either N-hydroxy amine or amino acid which can be used by another enzyme to form N-N-containing cyclic compounds, KtzT. This model enzyme is structurally unexplored. By means of a computational approach we now can guide its evolution to become more promiscuous. In another project we work on a heme containing membrane protein which does use the heme-cofactor as interface between two protamers and as an active site element to polarize the substrate. Sophisticated protein production and purification strategies allowed to solve the mechanism and the structure of this interesting biocatalyst. Additionally, we work on other enzymes such as azo reductases, glutathione transferases, eugenol oxidases, and various dehydrogenases.

Features of cold active enzymes

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One major topic of our laboratory is related to enzymes produced by cold-adapted microorganisms, so called "cold active enzymes", in particular glycosidases and esterases. These enzymes are endowed with the ability to maintain high activity at low temperatures. Very often they are heat sensitive and can undergo inactivation/denaturation even at mild temperatures. Nevertheless, we observed a few notable exceptions. For example, a cold active beta-glycosidase from *Marinomonas* sp., in addition to be active at 4°C, displays highest activity at 55°C and is thermostable. Our results suggest that each enzyme family appears to have followed different pathways of adaptation. The availability of biocatalysts active at low temperatures and thermolabile can be of interest for industrial application.

We are also studying the robustness of enzymes, in particular lipases, exposed to harsh experimental conditions, for example to various organic solvents.

Recent papers

Marchetti A., Orlando M., Mangiagalli M. *, Lotti M. (2022) A cold-active esterase improves its mesophilic properties through Mn2+ binding. FEBS J https://doi.org/10.1111/febs.16661

Mangiagalli M., Ami D., de Divitiis M., Brocca S., Catelani T., Natalello A.*, Lotti M. * (2022) Short-chain alcohols inactivate an immobilized industrial lipase through two different mechanisms, Biotechnology J 2022;2100712. https://doi.org/10.1002/biot.202100712

Orlando M., Buchholz P.C.F., Lotti M., Pleiss J. (2021) The GH19 Engineering Database: Sequence diversity, substrate scope, and evolution in glycoside hydrolase family 19 ***PLoS ONE***, 2021, 16(10 October), e025681

Mangiagalli M, Barbiroli A, Santambrogio C, Ferrari C, Nardini M, Lotti M, Brocca S. (2021) The activity and stability of a cold-active acylaminoacyl peptidase rely on its dimerization by domain swapping. International Journal of Biological Macromolecules 181 (2021) 263–274

Mangiagalli M. and Lotti M. (2021) Cold-active β-galactosidases: insight into cold adaption mechanisms and biotechnological exploitation. Mar. Drugs 2021, 19, 43. https://doi.org/10.3390/md19010043

Mangiagalli M, Lapi M, Maione S, Orlando M, Brocca S, Pesce A, Barbiroli A, Camilloni C, Pucciarelli S, Lotti M*, Nardini M* (2021) The co-existence of cold activity and thermal stability in an Antarctic GH42 β-galactosidase relies on its hexametric quaternary arrangement. FEBS J. 288: 546–565

Hydantoinase process enzymes and more

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We are a newly established group in the laboratory of Protein technologies of SPC Armbiotechnology NAS RA (SCIENTIFIC AND PRODUCTION CENTER “ARMBIOTECHNOLOGY” NAS RA). Optically pure D- and L-amino acids are valuable intermediates for the preparation of semisynthetic antibiotics, pesticides, and other products of interest for the pharmaceutical, food, and agrochemical industries. This goal can be achieved using the so-called "hydantoinase process" by opening the ring of the corresponding racemic hydantoins followed by decarbamoylization using hydantoinases and carbamoylases with the desired enantiomeric activity. Currently, our efforts are focused on adapting the substrate specificity of the "hydantoinase process" enzymes to desired substrates through site-directed mutagenesis. We are going to use in silico strategies in enzyme construction processes along with traditional strategies.

Along with the "hydantoinase process" enzymes, we are also intensively working on L- and D-acylases with the same goal to separate the corresponding racemic mixtures of amino acids into enantiomeric constituents. There is also experience in the use of transaminases for the synthesis of optically active amino acids from their optically inactive precursors, oxo acids. In particular, we used Erwinia carotovora aromatic and aspartic transaminases in the biosynthesis of L-phenylalanine from phenylpyruvic acid. Enzymes of great biotechnological interest, such as proteases, β-galactosidases, carbohydrate-active enzymes (amylases, cellulases, hemicellulases, etc.), are also in the field of our deep scientific interests.

We are going to improve the processing characteristics (substrate specificity, thermal and/or pH stability, oxidative stability, etc.) of these enzymes through site-directed mutagenesis, again using in silico strategies in parallel with traditional strategies.

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