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Enhancing Enzyme Stability for Biotechnological Applications

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Abstract: Enzymes are emerging as a central element of green chemistry due to their high selectivity, biodegradability, and biocompatibility. However, their application in biotechnology is often limited by their poor stability under non-native conditions. Such an instability eventually compromises their catalytic efficiency and economic viability. To date, no single solution exists to universally solve the challenge of enzyme stability. Herein, we summarized the main strategies that have been developed to address this challenge, including enzyme discovery, protein engineering, enzyme immobilization, and computational tools. Beyond stability, this account also highlights recent technologies to improve biocatalytic efficiency. All these approaches are illustrated by examples of our most recent research work. Ultimately, enhancing enzyme stability and activity will have a broad impact for biocatalytic processes in biomedicine, food processing, and chemical manufacturing, among other biotechnology areas.

Keywords: Biocatalysis · Biotechnology · Enzyme stability · Protein structure · Sustainability

Ana I. Benítez-Mateos started her independent career as SNSF Ambizione Group Leader at ETH Zürich in October 2023. She obtained her PhD in 2019 with Prof. F. López-Gallego at CICbiomaGUNE (Spain). Then, she joined the group of Prof. F. Paradisi, first at the University of Nottingham (UK) and later at the University of Bern (Switzerland), under a Seal of Excellence Postdoctoral Fellowship and an Innosuisse Innovation Project. During her PhD and Postdoc, she investigated the co-immobilization of enzymes and cofactors and their integration into continuous flow reactors for chemical manufacturing. Her current research aims to design and develop more robust and sustainable biocatalytic systems by harnessing the unique properties of proteins from extremotolerant animals.

1. Introduction: Enzyme Stability

Enzymes are the molecular machines that drive and regulate most of the chemical reactions essential to life. Over millions of years, enzymes have evolved and adapted to their biological environment and the corresponding physico-chemical conditions (*i.e.* temperature, pH).[1] This has led to a diverse range of enzymatic reactions, each with distinct properties and highly specific functions. Enzymes show an exquisite stereo-, chemo-, and enantioselectivity towards their substrates. From a biotechnological point of view, this makes enzymes an attractive toolbox of biocatalysts for diverse applications such as chemical synthesis, biodegradation, and medical biosensing, among others.[2,3] Moreover, enzymes are biodegradable and biocompatible, making them excellent catalysts for sustainable chemistry.[4,5]

Nevertheless, enzymes often suffer from low stability when exposed to abiotic stress conditions (*i.e.* high temperature, extreme pH) to which they are not adapted, thus compromising their catalytic efficiency. Typically, two types of stability are described:[6]

- *Storage (or shelf) stability*: ability of enzymes to maintain their structural integrity and activity when stored as a solution, lyophilized powder, or in the immobilized form.
- *Operational stability*: retention of enzymatic activity while in use.

Enzymes that exhibit high activity while maintaining their stability for long times are considered ideal biocatalysts for biotechnological applications. However, although a certain degree of flexibility is essential for enzymatic activity, excessive structural mobility can compromise stability as enzymes are more susceptible to denaturation. This balance between activity and stability (though not yet fully understood) is paramount for biocatalysis.[7,8]

Therefore, the question is: *how can we enhance enzyme stability without compromising their activity?* To address this challenge, various approaches have emerged, though no single solution is universally effective for all enzymes and all types of abiotic stress.[9] Four main strategies can be highlighted: enzyme discovery from extremophiles; enzyme immobilization; addition of stabilizing agents; and protein engineering and bioinformatics (Fig. 1). In the following paragraphs, I will provide a broader context of each approach, while also highlighting our recent contributions to the field and current research focus.

1.1 *Enzyme Discovery from Extremophiles*

Organisms that require extreme environmental conditions for optimal growth and survival are known as extremophiles.[10] Enzymes from those organisms (extremozymes) are highly attractive for biotechnological applications requiring very harsh conditions.[11] One of the most well-known examples is the polymerase chain reaction (PCR) technique, which is used in diverse applications, from clinical diagnostics to biosensing in agriculture. The polymerase catalysing this reaction (with optimal performance at 72 °C) was obtained from *Thermus aquaticus*, the first extremophile discovered in Yellowstone Park (USA).[12]

Traditionally, enzyme discovery has been carried out by culturing microorganisms under the desired conditions and screen-

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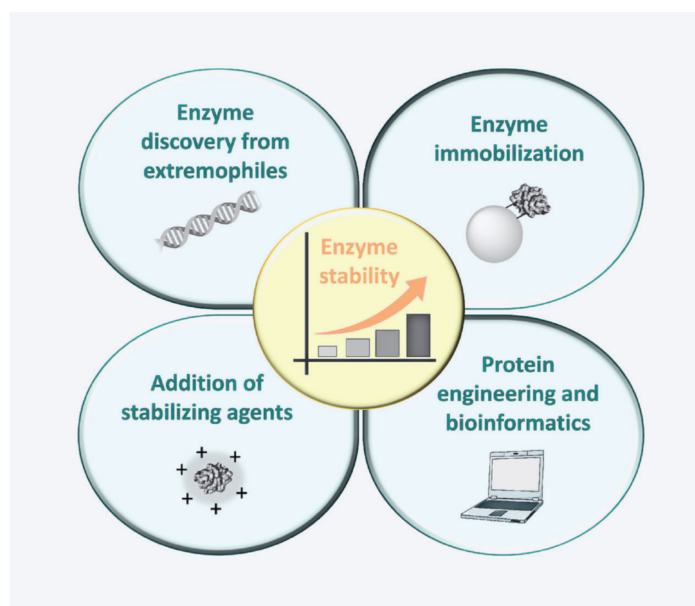


Fig. 1. Strategies for enzyme stability described in this account.

ing them for enzyme production. Over the past few years, more sophisticated methodologies, such as metagenomics and genome mining, have enabled us to expand the portfolio of extremozymes in biotechnology without direct contact with the organism of origin.[10]

However, extremophiles represent a very specialized type of organism that cannot grow or perform well under moderate (mesophilic) conditions. Hence, their enzymes often have limited applicability due to a lower catalytic activity under 'normal' conditions. On the contrary, the so-called extremotolerant organisms can survive extreme environments but prefer moderate conditions. As a result, they exhibit both flexibility and robustness, offering a balance of the advantages found in extremophiles and mesophiles.

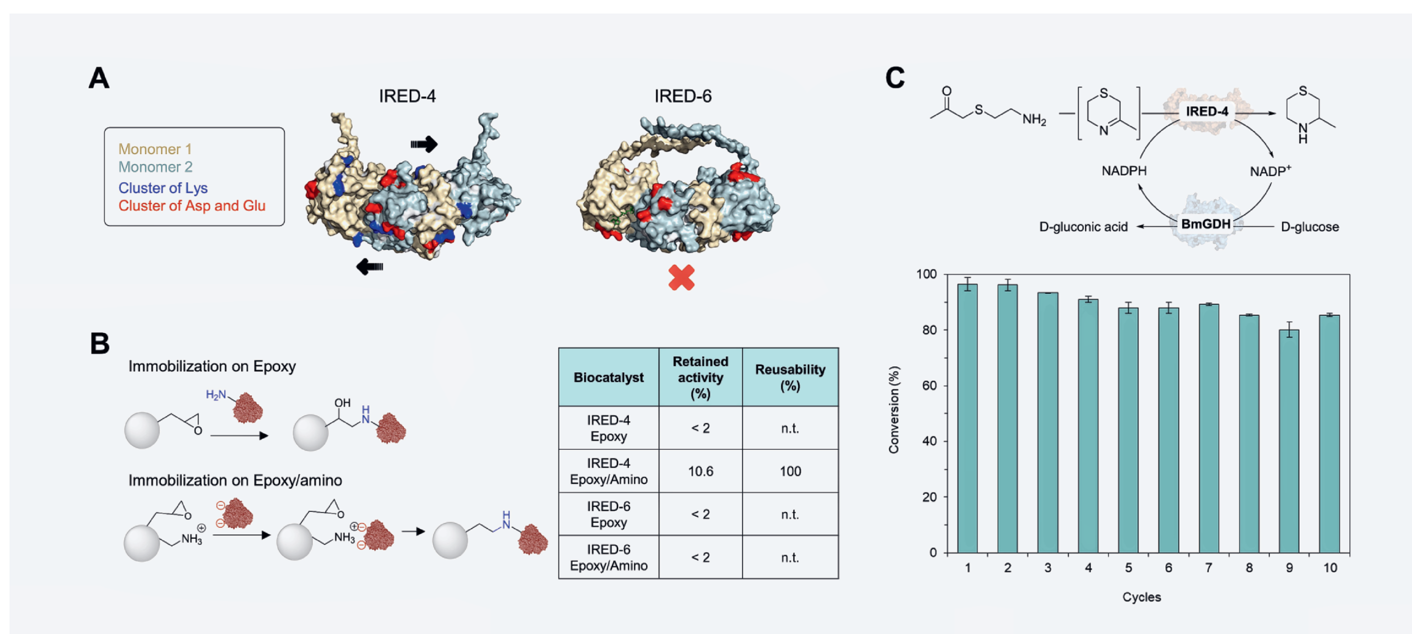
A halotolerant (tolerant to high salt concentrations) organism that has served as a source for enzymes for biotechnology is *Halomonas elongata*.[13] We have described the application of two novel nucleoside phosphorylases for the synthesis of antiviral and anticancer drug molecules. Both enzymes have shown remarkable co-solvent stability, maintaining up to 60% of activity in the presence of 50% organic co-solvent (DMSO).[14,15] Now, we are focusing our research on extremotolerant organisms that are less common as a source of enzymes, such as extremotolerant animals that may offer tolerance to a wider scope of reaction conditions.

1.2 *Enzyme Immobilization*

One of the most applied techniques to stabilize enzymes is the attachment of the enzyme to a solid support, which can stabilize their 3D structure while offering other benefits such as reusability.[16,17] Once the enzymes are immobilized, the larger biocatalytic particles can be easily filtered from the reaction bulk and reused. This is a key aspect for the integration of enzymes into flow reactors that can intensify the biocatalytic reaction.[18–20]

There are a wide variety of protocols for enzyme immobilization using different solid supports (*i.e.* crosslinked agarose, methacrylate, silicates), various binding chemistries (*i.e.* covalent multipoint attachment, hydrophobic adsorption, ionic interactions), and even post-immobilization treatments (*i.e.* polymer coating).[21] The selection of the protocol for a given enzyme has traditionally relied on an empirical approach. During the last several years, more rational and predictive strategies have emerged, driven by the availability of enzyme structure databases, advanced predictive models, and the rapid development of computational tools.[22,23] These advancements can accelerate the screening process of immobilization protocols while saving resources.

In our recent work with industrial partners, bioinformatics were applied to analyse the enzyme structures and guide the immobilization strategies.[24] Our focus was on imine reductases



Biocatalyst	Retained activity (%)	Reusability (%)
IRED-4 Epoxy	< 2	n.t.
IRED-4 Epoxy/Amino	10.6	100
IRED-6 Epoxy	< 2	n.t.
IRED-6 Epoxy/Amino	< 2	n.t.

Fig. 2. IRED immobilization driven by bioinformatics and immobilization screening. A) Structure analysis of IRED. Black arrows indicate the forces during covalent immobilization by lysines (Lys) that may disassemble the dimeric structure. Red cross indicates that immobilization of IRED-6 by covalent binding is not possible due to the lack of anchoring residues (Lys). B) Schemes of IRED immobilization and results after immobilization of IRED-4 and IRED-6 (n.t. = not tested). Retained activity (%) indicates the IRED activity after immobilization. Reusability (%) refers to retained activity on the third cycle of consecutive operation. C) Reaction scheme when a glucose dehydrogenase (BmGDH) was added to recycle the cofactor, and stability of biocatalysts when the biotransformations were carried out in batch. Images were adapted from Benítez-Mateos *et al.* with permission from the American Chemical Society, ref. [24]

(IREDs), a class of enzyme notoriously difficult to immobilize irreversibly due to significant loss of activity. Structural bioinformatic analysis revealed that covalent immobilization often leads to rigidification of the dimeric interface and catalytic site. Additionally, it revealed the lack of anchoring residues (Lys) in some IREDs that prevent their covalent immobilization (Fig. 2A). To overcome this, we applied a hybrid immobilization protocol combining covalent (epoxy) and non-covalent (amine) binding that allowed them to maintain their activity (8–39%) (Fig. 2B). Moreover, these protocols resulted in a high reusability (up to 100% after 3 cycles). This stability enabled the reuse of the biocatalysts for several reaction cycles and the successful integration of IREDs (together with a cofactor-recycling enzyme) into flow reactors for the biosynthesis of chiral heterocyclic amines (space-time yield (STY): 20.7 g $L^{-1}h^{-1}$), which are valuable building blocks in pharmaceutical manufacturing (Fig. 2C).

Integrating multiple enzymes into flow reactors is indeed a challenge due to the distinct optimal conditions required for each enzyme. Recently, we have contributed to research work in which an enzymatic cascade was assembled to transform 1,ω-diols into 1,ω-amino acids, which are relevant monomers for bioplastic production.[25] Firstly, six enzymes were co-immobilized onto agarose microbeads to perform the conversion of 1,ω-amino acids into 1,ω-hydroxy acids. We then developed a second heterogeneous biocatalyst by co-immobilizing three additional enzymes on methacrylic microbeads. Both biocatalysts were packed into two reactors connected in a telescoped configuration to enable an in-line cascade (Fig. 3). The high stability provided by the immobilization allowed continuous operation over >24 h. This work set a record for the number of immobilized enzymes assembled in a cell-free biosynthetic cascade operating in flow, although the productivity could be further improved.

1.3 *Addition of Stabilizing Agents*

Enzymes in solution can also be stabilized by the addition of stabilizing agents that minimize unfolding, aggregation, and loss of activity caused by thermal, chemical, or mechanical stress.[9] Salts, sugars, polyols, surfactants, reducing agents, amino acids, and proteins are the most commonly added molecules. For instance, the addition of dithiothreitol (DTT) as a reducing agent or ethylenediaminetetraacetic acid (EDTA) as a metal chelator avoids the oxidation of enzymes (and proteins in general) that may cause activity loss. For preservation or transportation purposes, glycerol and ethylene glycol are cryo-protectants that prevent the formation of crystals when enzymes are stored at -20 ºC.[26] Other small stabilizing molecules are amino acids, including arginine, glycine, and proline, which can help maintain enzyme structure by stabilizing hydrophobic regions of the enzyme structure. Moreover, antimicrobial agents such as sodium azide inhibit contamination of enzyme solutions due to microbial growth that can also affect the activity and stability of enzymes.

Proteins (*i.e. bovine serum albumin* (BSA)) are larger biomolecules that are often superior stabilizing agents for enzyme stability compared to small-molecule stabilizers.[9] They offer multiple and synergistic modes of protection, preventing enzyme aggregation and degradation. Proteins act as crowding agents, closely mimicking the natural environment of enzymes in which there are millions of proteins. Furthermore, proteins are biocompatible and less likely to interfere with enzyme function compared to some synthetic stabilizers or salts that may affect ionic strength or pH. Therefore, proteins are not only used as stabilizing agents for the purification and storage of enzymes, but also during their biotechnological applications, *i.e.* therapeutic formulations, industrial chemical manufacturing. One of the current research goals of our group is the discovery and application of new proteins that can enhance the shelf-life and the performance of enzymes while being a sustainable and cost-effective alternative to current stabilizers.

1.4 *Protein Engineering and Bioinformatics*

The engineering of proteins to improve their stability has been an active research area over the last 30 years, since the emergence

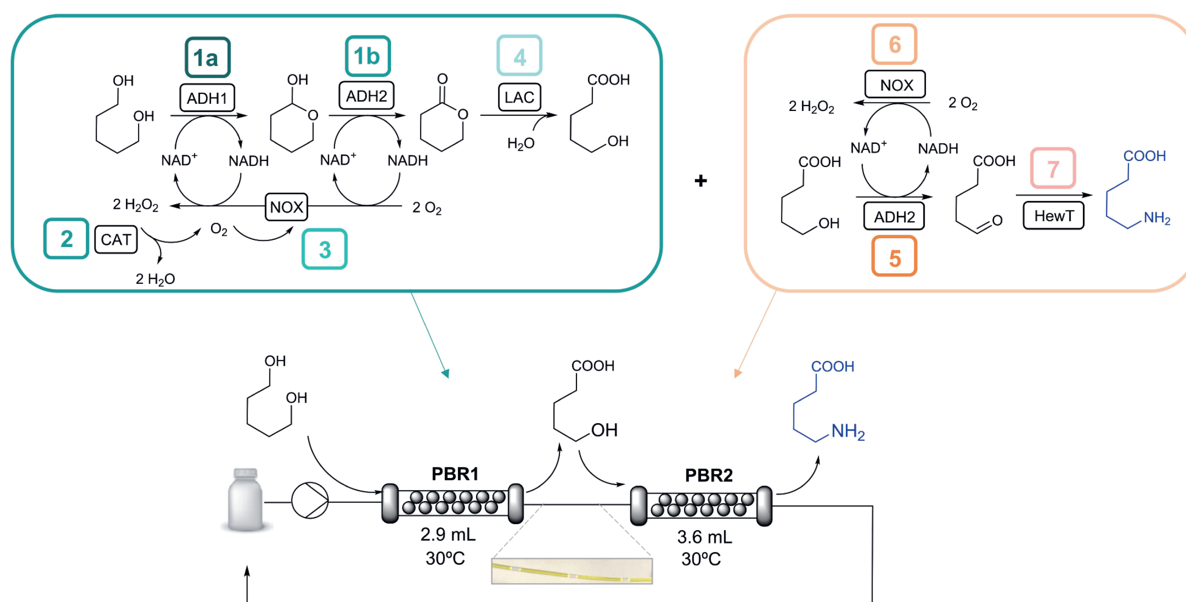


Fig. 3. Telescoped multi-enzyme cascade for the continuous-flow synthesis of ω-amino acids. Enzymes in the green panel (alcohol dehydrogenase 1 (ADH1); alcohol dehydrogenase 2 (ADH2); lactonase (LAC); catalase (CAT); and NADH oxidase (NOX)) were co-immobilized on agarose microbeads. Enzymes in the orange panel (NOX, ADH2, and ω-transaminase (HeωT)) were co-immobilized on methacrylic microbeads. The two packed-bed reactors (PBR) were connected in line. Recirculation of the unreacted reagents allowed an increase in yield. Moreover, a bi-phasic system was naturally formed between the two PBRs, which allowed to provision of more O_2 to PBR2. Images were adapted from Santiago-Arcos *et al.* ref. [25], CC BY 4.0 https://creativecommons.org/licenses/by/4.0.

of techniques such as epPCR, DNA shuffling, or directed mutagenesis.[27,28] To date, two main strategies have had a strong influence in the field of enzyme engineering to improve both stability and activity. These are directed evolution (recognized with the Nobel Prize in Chemistry in 2018)[29] and rational enzyme design. While directed evolution mimics natural selection on a lab scale, rational design targets specific modifications based on detailed knowledge of the enzyme structures. Once again, the trade-off between stability and activity becomes a challenge, as modifications aimed at higher enzyme stability can impact enzyme activity unfavourably. Additionally, screening and identifying those beneficial modifications among all the possible options is a tedious task.

Bioinformatic tools have emerged in the last decade, intending to alleviate those issues by *in silico* screening protein modifications.[30] Databases (*i.e*. BRENDA, FireProtDB) offer the foundational data needed to train computational models and guide design.[31,32] In the very last years, new computational approaches such as machine learning (ML) and artificial intelligence (AI) methods have already revolutionized how we modify or even *de novo* design new enzymes.[33,34] Undoubtedly, the predictive platforms AlphaFold and RoseTTa Fold (recognized with the Nobel Prize in Chemistry in 2024) have been a key source of predicted protein models that are opening new doors for the understanding, analysis, and improvement of enzyme stability.[35,36]

Although these strategies are not part of our mainstream research, we often use protein engineering (*i.e.* addition of peptide tags for enzyme immobilization, design of more stable fusion proteins)[14] and bioinformatics (*i.e.* structural analysis to rationalize enzyme immobilization)[24] to support our enzyme stability studies.

2. Enhancing Enzyme Reactions Beyond Stability

Making enzymatic biotransformations more efficient and sustainable is the ultimate goal that our research pursues.[37] As mentioned several times throughout this manuscript, both stability and activity are crucial factors to transfer biocatalytic reactions into biotechnological applications. Thus, we also investigate other aspects of enzymatic catalysis, apart from enzyme stability, to develop more effective biocatalytic systems.

Very recently, we have contributed to research studies in which enzymatic activity has been enhanced by creating liquid-liquid condensates with distinctive properties from the surrounding reaction bulk.[38,39] The formation of those membraneless compartments is driven by specific intrinsically disordered regions (IDRs) domains that are fused to the enzyme (Fig. 4A). The formation of certain biomolecular condensates allows for the recruitment of substrates and cofactors, thus enhancing the enzymatic rate within the condensates. In this work,[38] we achieved > a 2-fold activity increase within the condensates compared to the enzyme in solution (Fig. 4B). Further work in this field demonstrated that biomolecular condensates can also act as local pH buffers, maintaining a different pH from the surrounding reaction bulk.[39] In the assembly of a bi-enzymatic cascade, a lipase with an optimal pH ≥7 was successfully coupled to an oxidase requiring more acidic conditions (pH 6). The bi-enzymatic reaction performed in two phases showed a higher product formation compared to the homogenous biocatalytic system.

3. Conclusions

In most natural proteins and enzymes, evolution has optimized function over stability. Therefore, many natural enzymes lack enough resilience to stress conditions that are often present in industrial processes. This significantly impacts the efficiency and economic viability as well as the sustainability of a process. For this reason, enzyme stability remains a critical determinant in the successful application of biocatalysts for biotechnology, making enzyme stabilization a research area of increasing interest.

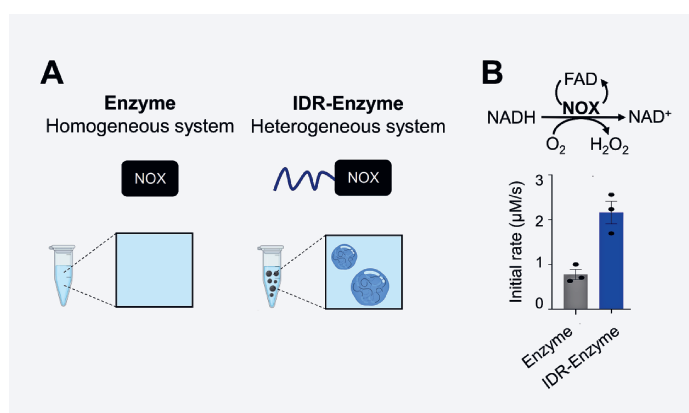


Fig. 4. Enzymatic reactions in biomolecular condensates. A) Scheme of the enzyme (NADH oxidase; NOX) forming liquid-liquid phase separation only when fused to the intrinsically disordered region (IDR). B) Reaction scheme and enzymatic activity of NOX in homogeneous and heterogeneous systems. Images were adapted from Gil-Garcia *et al*. ref. [38], CC BY 4.0 https://creativecommons.org/licenses/by/4.0.

Over the past decade, substantial progress has been made in understanding the molecular determinants of enzyme stability through advances in structural protein biology and computational modelling. Despite these advances, challenges in transferring lab-scale biocatalytic reactions to industrial setups persist, especially for multi-step biotransformations. Multidisciplinary approaches that combine biophysics, synthetic biology, and process engineering are likely to reach more effective solutions. To sum up, continued innovation is paramount to expand the applicability of biocatalysis across different areas of biotechnology, from biomedicine to food processing, or environmental remediation.

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