Increasing the Sustainability of Biocatalytic Processes

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Abstract: The number of industrial processes that include one or more enzyme-mediated steps is growing rapidly, together with our understanding of how biocatalysts can be adapted to efficiently perform chemical reactions, often only remotely related to their natural reactivity. While enzymes as naturally biodegradable molecules are the most sustainable approach to catalysis, a more global vision of the overall sustainability of enzymatic process must be considered. Here an insight of how sustainability can be further improved when enzymes are immobilized on solid supports is discussed.

Keywords: Biocatalysis · Flow chemistry · Sustainability

Prof. Francesca Paradisi is the Chair of Sustainable Pharmaceutical Chemistry at the University of Bern since 2019. She holds an MSc and a PhD in Organic Chemistry from the University of Bologna. She then joined the group of Prof. Engel at University College Dublin, Ireland. In 2006 she started her independent career in UCD, and then moved to the University of Nottingham, UK, in 2016. Biocatalysis as a sustainable approach to the synthesis of valuable products is the focus of her research. Her group developed several enzyme-based processes in continuous flow, with immobilized biocatalysts, reducing the gap between academic discovery and industrial application.

1. Introduction

The fast evolution of enzyme engineering in the last decade has impacted dramatically the number of industrial processes that include one or more biocatalytic steps.[1] One of the most impressive examples is the Merck synthesis of Islatravir which has been achieved exclusively enzymatically in 2019, reducing the number of synthetic steps from 12 to 5.[2] What is particularly clear is that obtaining the catalyst is no longer a problem: a total of nine enzymes, most of which had to be carefully engineered, is required for the accomplishment of such a synthesis and in fact four of those enzymes serve exclusively as auxiliary systems to shift equilibria and recycle cofactors. The concern that biocatalysts are 'expensive', a niche technology, and incompatible with the majority of industrial reactors has been dismantled. However, it is no longer enough to consider only the absence of metal-based catalysts, or organic solvents, or any other energy-demanding set ups, as the end goals in terms of sustainability, co-factor recycling, recovery and reuse of enzyme catalysts, and minimal waste are all essential parameters to evaluate. To put it in perspective, in 2015, de María and Hollmann published a review[3] in which they highlighted a number of misconceptions about the 'greenness' of biocatalysis, and how they can be addressed. For example, the often major dilution of reagents

which are not particularly soluble in water leads to a very poor ratio of auxiliary reagents to product, with concentration of products in the low mM range and a high E-factor (mass waste/mass product). The addition of co-solvents or the use of better suited media such as deep eutectic solvents can offer a solution.[3] More recently, Woodley reviewed further the sustainability aspect of biocatalysis, specially in terms of scalability of enzyme-mediated reactions. In terms of affordability of a process, the target working concentration which should be aimed at is over 100 g L^{-1} for low-priced products ($<5 \text{ USD kg}^{-1}$) while for high-priced products ($>100 \text{ USD kg}^{-1}$) it is sufficient to stay between 10 and 50 g L^{-1},[4] and this requires new approaches and the development of very robust biocatalysts which will likely have to cope with increasing amounts of co-solvents.

In our research group, we have steadily evolved towards including sustainability in all aspects of the process design, with particular attention to the stabilization and recovery of the biocatalyst to the overall process safety (Fig. 1).

Fig. 1. Concept for sustainability in biocatalysis.

For immobilization of enzymes, we have reported on several methods which can significantly improve the lifespan of a biocatalyst, and these are not limited to single enzyme loading, but can exploit different chemistries leading to a rational immobilization approach for multi-enzyme systems.[5–7]

A core application of immobilized enzymes is the possibility of housing them in a packed-bed reactor for continuous biotransformations, achieving in many cases high substrate loading.[6,8–11]

Here, the most recent advances in terms of sustainability, which have been developed in our laboratory to target protein immobilization, will be discussed.

2. Sustainable Immobilization Technologies

The concept of enzyme immobilization is quite straightforward: one or more biocatalysts are anchored onto a support generating a

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heterogenous system that can be separated from the reaction mixture, enabling its recovery. When the bonding between the catalyst and the support is covalent, the catalyst will not leach into the reaction bulk, and it can be reused several times. Despite normally inducing some loss in catalytic efficiency, enzyme immobilization is accepted as an excellent approach to enzyme stabilization.

Several aspects, however, must be considered. First, there is not a single universal approach to enzyme immobilization, which means that every biocatalyst needs to be tested on several supports and with different chemistries to identify the optimal system. Then, the support itself is a chemical entity, normally manmade as is the case for all commercial resins and, in many cases, it represents the least 'green' element in the system (polystyrene and acrylic resins are among the most commonly used). Finally, even though the immobilized enzyme has a longer lifespan, eventually it will inactivate, and everything is disposed of as waste.

2.1 Rational Approach to Enzyme Immobilization

A way to minimize the waste generated by the simple and unavoidable problem of screening resins and chemistries would be to have points of reference on what chemistry may be more suitable for a specific enzyme and possibly which support gives the highest chance of favorable immobilization.

In 2021, Dr. Roura Padrosa in our team developed CapiPy (Computer assistance for protein immobilization – Python), the first bioinformatic tool centered on protein immobilization.[12] CapiPy provides a user-friendly interface to important open-source and newly developed bioinformatic tools (generation of a protein model, identification of the active site, and identification of clusters of reactive amino acids on the surface) that help to guide the user towards making an informed decision on the choice of immobilization techniques (Fig. 2). The first version of CapiPy also retrieves papers from the literature relevant to the input protein sequence.
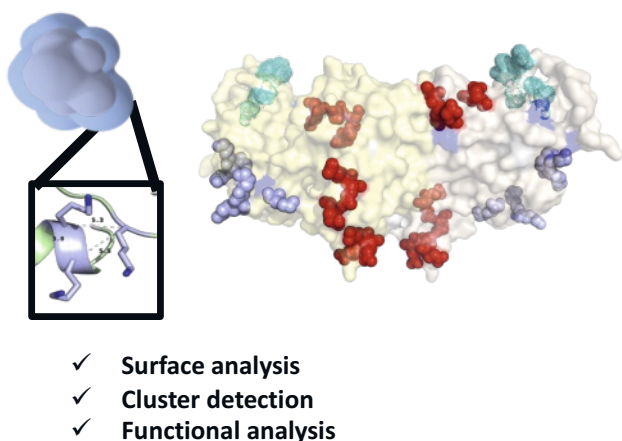
✓ **Surface analysis**
✓ **Cluster detection**
✓ **Functional analysis**

Fig. 2. Example of visual results obtained with CapiPy.
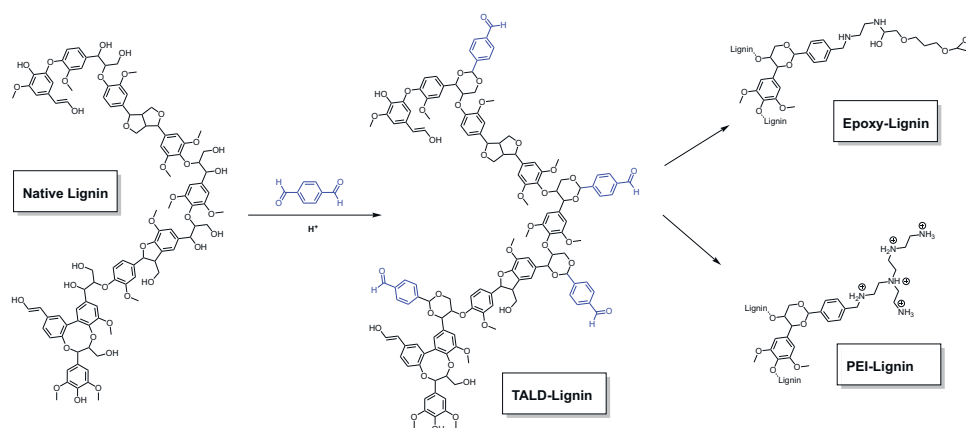
Since its development, we have used CapiPy in different projects, significantly streamlining the immobilization step.[13,14]

Our efforts in this field are continuing with the aim of creating a large searchable database on protein immobilization where standardized metrics can be collected and used to increase the prediction capacity of the software.

2.2 Sustainable Supports

Acrylic supports are commonly used as matrices for immobilization of enzymes. They are readily available through different suppliers and can harbor a range of functionalities for covalent attachments through nucleophilic residues on the surface of the enzyme, generally lysines. In our group we have relied on this type of support on many occasions[10,13,15,16] because it offers a significant advantage in continuous flow, given its non-swelling properties. However, it is clearly not the most sustainable of supports as it is not easily recyclable. Agarose resins have a significantly lower environmental impact, often provide an excellent microenvironment for the enzyme (highly hydrophilic), but they may not be sufficiently robust in harsher reaction conditions.[17]

Recently, in collaboration with Prof. Luterbacher in EPFL, we explored the possibility of using lignin as a support for immobilization of biocatalysts.[18] The extraction process optimized by the Luterbacher group involves a terephthalic aldehyde-stabilization step (TALD-Lignin),[19] and the aldehyde can then be used to develop a series of functionalized lignins with a range of reactive groups (epoxy, amine, aldehyde, metal chelates), which are ideal to test a range of chemistries for immobilization (Fig. 3). As a proof of concept, we immobilized four different enzymes on differently functionalized lignin, achieving immobilization yields between 64–100%. While the recovered activity (a key parameter in immobilization) was low (up to 17%) the immobilization *via* ionic interactions (PEI-Lignin) was better than through covalent bonds (Epoxy-Lignin), as it is normally observed with standard supports. In the absence of covalent bonding, as mentioned, leaching of the enzyme is normally observed. However, when we tested the stability of one of our most exploited catalysts (*Halomonas elongata*, ω-Transaminase - HeWT), even the PEI-lignin offered excellent reusability over eight reaction-cycles in batch (Fig. 4).

Clearly, the hydrophobic structure of lignin increases the non-specific interaction with the protein, avoiding leaching into the reaction bulk even after repeated uses, with complete retention of activity. The lignin support also induced higher stability of the enzyme at extreme pH (11–12) which was less remarkable on previously tested supports.

HeWT is pyridoxal phosphate (PLP) dependent, and it is added exogenously to reaction mixtures to ensure its availability during the reaction cycle. It has been shown, however, that PLP
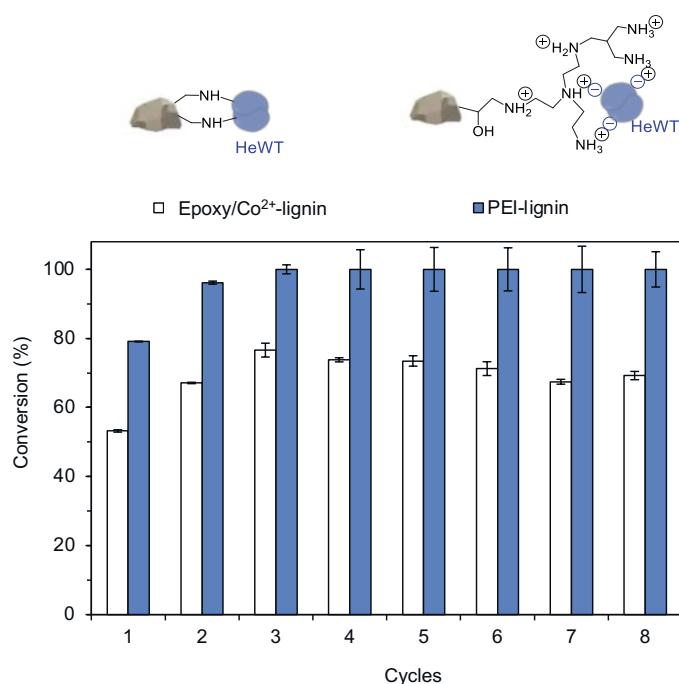
Fig. 3. Modification of lignin leads to typical functionalization for biocatalyst immobilization.

Native Lignin

TALD-Lignin

Epoxy-Lignin

PEI-Lignin

Fig. 4. Reusability of the immobilized HeWT immobilized *via* covalent (white bars) and non-covalent (blue bars) bonding on lignin.

Fig. 5. Stability testing under flow conditions of different non-covalent immobilization systems.

can be co-immobilized *via* ionic interactions on a support, which then acts as a local reservoir.[20,21]

PEI-Lignin was incubated with both HeWT and PLP, yielding a support which was self-sufficient and just as efficient over time as when PLP was added in the medium.

PEI-Lignin as a support also outperformed previously tested reversible preparations on acrylic resins when packed in a reactor for continuous bio-transformations. It is, in fact, commonly observed that non-covalent immobilization chemistries, under flow conditions, are not sufficiently resistant to the set up and eventually the catalyst is lost downstream causing a steady decline in conversion. This was not the case with this renewable support (Fig. 5).

2.3 *Covalent yet Reversible Chemistry*

When covalent attachment is a must (high ionic strength of the reaction medium, high concentration of polar substrates, *etc.*) in terms of sustainability it is important to consider the life cycle of the overall system. While the enzyme is biodegradable, the support may not be. In terms of circular economy, if we can recover and reuse the (non-biodegradable) support multiple times we would reduce the environmental impact of our heterogenous preparation. We therefore invested in the development of reversible covalent chemistry which could enable the reuse of the support.

By genetically inserting in the enzyme a poly-cysteine tag ((6x)Cys-tag), we introduced a chemical handle in the structure that can be used to form disulfide bonds with resins modified with thiol functional groups. The (6x)Cys-tag is also located in a specific protein region (normally at the C- on the N terminus), therefore the immobilization would be no longer through multi-point attachments of the exposed lysines (which are randomly distributed, and vary in location in every protein), but selectively through the tag, leading to reduced structural distortion (if any), and clearer orientation of the biocatalyst on the resin.

Unlike weaker reversible immobilization strategies, the disulfide bonds keep the enzyme covalently bound to the support avoiding lixiviation of the enzyme during the reaction, but it can be selectively reversed, under precise chemical conditions.

The protocol is very straightforward: starting from a resin with epoxy functionalities, thiol groups are generated by reaction
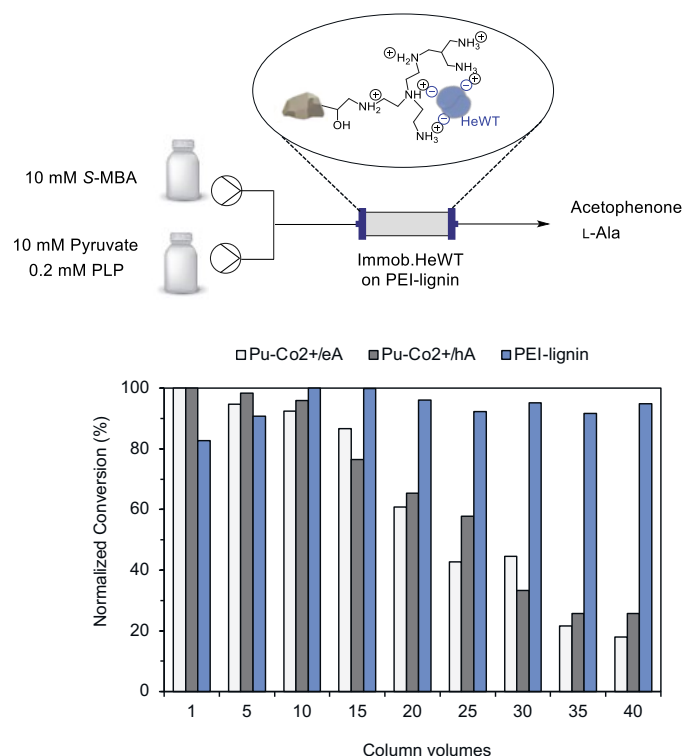
with Na_2S, and this is followed by a second step with DTNB (2,2'-dinitro-5,5'-thiobenzoic acid). The resin is incubated with the Cys-tag enzyme to yield the immobilized biocatalyst (Fig. 6).
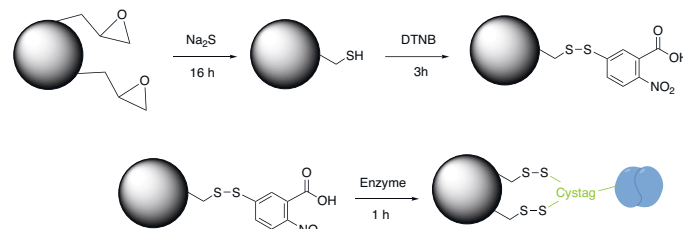

Fig. 6. Scheme of the resin preparation and enzyme immobilization *via* a disulfide bond.

We carried out this chemistry using epoxy-agarose beads to immobilize a novel purine nucleoside phosphorylase from *Halomonas elongata* (HePNP).[14] The structure analysis of HePNP performed with CapiPy[12] shows that no additional cysteines are exposed on the enzyme surface, and therefore only the Cys-tag can be involved in the disulfide bond formation (Fig. 7). When compared with other covalent immobilization strategies (either *via* aldehyde chemistry or epoxy groups), the Cys-tag approach is equally excellent in terms of immobilization yield (94%) and gives a recovered activity of 37%, equivalent to the epoxy-immobilization, and significantly better than aldehyde chemistry (29%).

This preparation was perfectly stable when incubated with up to 5 mM DTT for 24 h (Fig. 8), and this is particularly relevant in those cases where a reducing agent is required in specific reactions.

However, when a higher concentration of DTT was applied (50 mM), the enzyme could be efficiently cleaved from the support enabling its recycling for the immobilization of fresh enzyme. The efficiency of the re-immobilization reaches 90% and the recovered activity is identical to the first round.
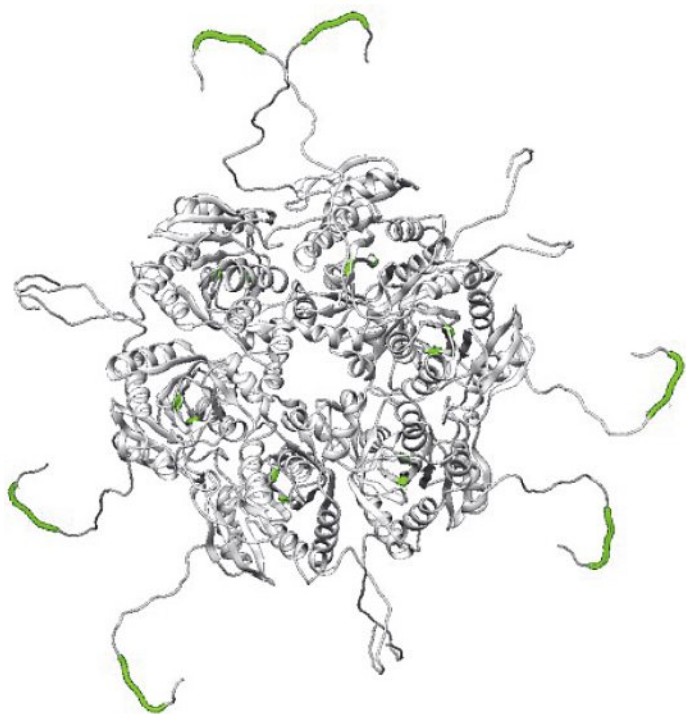
Fig. 7. Analysis of structure of HePNP containing the (6x)Cys-tag at the N-terminal. Cysteines are represented in green.
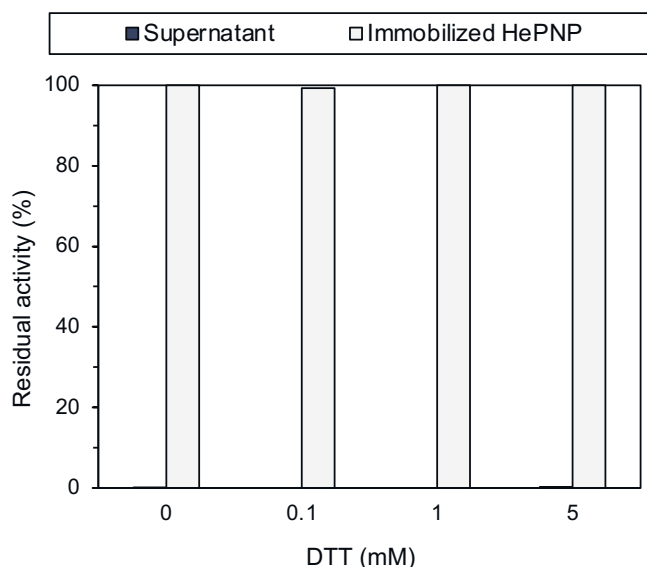

Fig. 8. Stability of the (6x)Cys-tag HePNP and the support following incubation with DTT for 24 h.

When the catalyst was tested in biotranformations in batch and in flow, it showed again very good performance.

3. Conclusions

Biocatalysis as a discipline offers very valid complementary approaches to traditional organic synthesis, and it is steadily growing as a go-to solution in the preparation of many industrial products. The technology to support the implementation of biocatalysis is also pacing up, providing solutions to the major drawbacks of using sizable biological molecules in large-scale processes. We should not forget that even enzyme-mediated reactions, once the chemistry is established, could be further improved from a sustainability point of view. In this paper I wanted to highlight some of the projects we have worked on that focused on further minimizing the environmental impact of enzyme chemistry. By adopting a more targeted approach to enzyme immobilization, for example, we can save time while minimizing waste, moving away from a trial-and-error approach. Further sustainable measures can be found in the choice of supports, favoring biodegradable ones, and in equally performing immobilization chemistries which enable the reuse of the support.

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