Towards Greener and More Cost-efficient Biosynthesis of Pharmaceuticals and Fragrance Molecules

Ana I. Benítez-Mateos$*

$SCS-Metrohm Award for best oral presentation in Catalysis Sciences and Engineering

Abstract: Enzymes are natural catalysts which are gaining momentum in chemical synthesis due to their exquisite selectivity and their biodegradability. However, the cost-efficiency and the sustainability of the overall biocatalytic process must be enhanced to unlock completely the potential of enzymes for industrial applications. To reach this goal, enzyme immobilization and the integration into continuous flow reactors have been the cornerstone of our research. We showed key examples of the advantages of those tools for the biosynthesis of antivirals, anticancer drugs, and valuable fragrance molecules. By combining new strategies to immobilize biocatalysts, innovative bioengineering approaches, and process development, the performance of the reactions could be boosted up to 100-fold.

Keywords: Biocatalysis · Enzyme immobilization · Flow chemistry · Sustainability

1. Introduction

Biocatalysis, where enzymes are utilized to carry out chemical reactions, has advanced a long way in the last few years, and is now considered another tool in organic chemistry.\(^1\) The recent developments in bioinformatic tools, together with protein engineering and directed evolution are expanding the possibilities for biocatalytic reactions.\(^3,4\) In fact, biocatalysis has moved from the typical use of bulk enzymes in laundry detergents, to tailor-made enzyme cascades for the synthesis of fine chemicals such as pharmaceuticals (i.e. Islatravir). Yet, some of the main points on the to-do-list for enzymatic reactions to allow them to be incorporated into industrial processes is the improvement of their stability, cost-efficiency and sustainability.\(^3,4\) Although enzymes are considered ‘green catalysts’ due to their biological origin and the use of water as the main solvent, there are critical parameters such as cofactor-recycling, waste reduction, and biocatalyst reusability, that must be improved to meet the sustainability and cost-efficiency requirements of industry.\(^5\)

Pharmaceuticals and fragrances are two of the main products for which new biocatalytic approaches have emerged during the last years.\(^2\) Therefore, the design and development of greener enzymatic processes for their production is of high interest.

2. Four Strategies to Enhance the Sustainability and Efficiency of Biocatalysts

We have looked into the main aspects that must be improved when preparing a biocatalyst and we have developed four new strategies to overcome sustainability and efficiency issues.

2.1 Enzyme Immobilization: Recycling the Support

Enzyme or cell immobilization is a strategy that generally improves the stability of biocatalysts by their entrapment, crosslinking, or attachment to a support.\(^6\) Moreover, immobilized biocatalysts can easily be separated from the reaction bulk enabling the biocatalyst to be reused for a new reaction cycle.

For industrial applications, the attachment of a biocatalyst to a support (i.e. microbeads of methacrylate, agarose, silica) is usually preferred because the final biocatalyst preparation is robust and easy to handle.\(^7\) To this end, a variety of immobilization chemistries are available to link the enzyme to the support. Reversible immobilization chemistries provide in general a better biocatalyst activity and also enable cleaving of the enzyme once inactive, allowing the reuse of the support to immobilize a fresh enzyme solution. In fact, recycling of the support is of utmost importance for biocatalytic applications since the cost of the support can be very high (1000–2000 CHF/kg).\(^8\) On the other hand, reversible chemistries such as hydrophobic and ionic bonds may be weak interactions, posing the risk of enzyme lixiviation to the reaction bulk. Therefore, covalent irreversible binding is often chosen for enzyme immobilization.

In order to exploit the benefits of reversible enzyme immobilization while eliminating the potential risk of enzyme lixiviation, we developed a covalent but irreversible binding chemistry. To this end, the protein was genetically labelled with a (6x)Cys-tag that allowed the covalent binding through disulfide bonds to a support
functionalized with thiol groups (thiobenzoic acid) (Fig. 1). This immobilization strategy allowed the biocatalyst reusability for multiple reaction cycles during the synthesis of pharmaceuticals, even under continuous flow conditions, while maintaining the biocatalytic activity. Once the biocatalytic activity dropped below 50%, the inactive enzyme was removed from the support by incubation with a reducing agent (DTT). Finally, the support was reused for the immobilization of a fresh enzyme solution (Fig. 1).

**2.2 Enzyme and Cofactor Co-immobilization**

Once (cofactor-dependent) enzymes have been immobilized, the need for addition of costly cofactors remains one of the biggest challenges for the cost-efficiency of the biocatalytic process. From an economic point of view, the reusability of cofactors is also of interest as some cofactors can reach the price of 10,000 CHF/mmol. To overcome this issue, we developed a novel strategy based on the reversible co-immobilization of cofactors together with the immobilized enzymes. This strategy benefits from the phosphate group that is present in most of the natural cofactors for the ionic adsorption onto supports functionalized with positively-charged amino groups (Fig. 2). To prove this, we firstly co-immobilized two enzymes (alcohol dehydrogenase and formate dehydrogenase) on epoxy-activated microbeads. Secondly, the immobilized enzymes were coated with a cationic polymer, polyethylenimine (PEI). This enabled the co-immobilization of the cofactor (NAD⁺) by ionic interactions, so that the cofactor could move from the matrix of the support to the active site of the enzymes and back without leaving this micro-environment. The resulting self-sufficient biocatalyst showed a 10-fold higher accumulated TTN (mol product/mol NAD⁺) over 10 reaction cycles when compared with the same biocatalyst but adding the soluble cofactor for each reaction cycle.

Having this simple but efficient strategy in hand, we exploited its potential for the co-immobilization of different enzymes with the corresponding cofactors: transaminases with PLP, ketoreductases with NADPH, and an oxidase with FAD and NADH. This versatile technique allowed the reusability of the cofactors for more than 50 times maintaining the biocatalytic conversion >90%. More recently, the group of Prof. López-Gallego has advanced the co-immobilization of cofactors by harnessing the ribose moiety of adenylated cofactors for the reversible binding to microbeads activated with boronic acid.

**2.3 Reuse of Water and Additives in Flow Reactors**

The integration of immobilized enzymes into continuous flow reactors is an attractive strategy for the intensification of biocatalytic processes. Yet, enzymes still work under very diluted conditions and may need the continuous addition of excipients (i.e., donors, surfactants, co-solvents) to carry out the enzymatic reactions. In 2018, Contente and Paradisi developed a closed-loop system which allowed the in-line recovery and recirculation of by-products and waste waters for their reusability into flow biocatalytic processes. Based on this concept, we have developed several flow set-ups: 1) to recover the cofactor during the synthesis of a pharmaceutical building block; 2) to separate the excess of amine donors in transamination reactions; 3) to recycle the excess of sugar donors and the waste waters during the biosynthesis of nucleoside drugs.

Nucleoside analogues such as Nelarabine and Rivabirin are important anticancer and antiviral drugs. Their biocatalytic synthesis can be accomplished by purine nucleoside phosphorylases (PNP) through phosphorylation of the sugar donor and transglycosylation to the desired nucleobase. However, a high number of equivalents of the sugar donor were necessary to achieve full conversion during the synthesis of nucleoside drugs in flow reactions (at 10 mM scale, in 2 min). Therefore, we designed a flow set-up that permitted the reuse of the sugar donor while separating the final product as well. To achieve this, a second reactor was filled with a cation exchange resin (Amberlyst® A15) and connected downstream to the biocatalytic reactor (Fig. 3b). The synthesis of a Nelarabine analogue, 84% of the sugar donor was separated from the final product and directly recirculated into the biocatalytic reactor for its reuse. The final product (Nelarabine analogue) that was retained on the cation exchanger could be easily and efficiently recovered (78%) by addition of NaOH. With this set-up, the E-factor (mass of waste/mass of product) was reduced 3.5-fold, therefore reducing the waste generation and increasing the process sustainability.

Finally, Amberlyst® A15 was easily regenerated by flushing with 10% HCl.

**2.4 Spheroplast Biocatalysts: The Peculiar Case of Membrane-bound Enzymes**

Biocatalysts can be applied in the form of purified enzymes or whole cells. Although purified enzymes are preferred to avoid the generation of secondary by-products, whole-cell biocatalysts offer protection from exterior stresses and maintain the enzyme in a more natural environment. Moreover, the (partial) purification of membrane-bound enzymes is not a trivial task and those enzymes are typically used as whole-cell biocatalysts. This is the case of squalene-hopene cyclases (SHCs) which have a great potential for the production of high-value terpenoids such as flavors.
Since SHC is bound to the cytoplasmic membrane of *E. coli* cells, we removed the outer membrane resulting in spheroplast biocatalysts (Fig. 4a). SHC spheroplasts maintained the stability of the enzyme while offering up to 100-fold improved results for the cyclization of squalene, geranyl acetone, and farnesyl acetone (Fig. 4b). Furthermore, we developed a novel immobilized biocatalyst: crosslinked spheroplasts (CLS), by using glutaraldehyde for the crosslinking of the proteins located on the surface of spheroplasts. This enabled the reusability of the SHC spheroplast biocatalysts for at least 4 reaction cycles (96 h).

3. Conclusions and Outlook

Nowadays there is no doubt that biocatalytic reactions have made a meaningful contribution to the catalysis field and they are steadily growing due to their exquisite selectivity, and *a priori*, sustainability. The difficult part comes with the implementation of biocatalysis into industrial set-ups. Waste minimization and reaction efficiency are key points to reach this goal. Herein, four strategies have showcased the advances and the feasibility of greener and more cost-efficient biocatalytic processes for the biosynthesis of pharmaceuticals and fragrance molecules. We have developed versatile technologies that can be further applied to other biocatalytic processes to exploit the full potential that biocatalysis can offer.

**Acknowledgements**

I am very grateful to the SCS and Methrohm for the Best Oral Presentation Award on Sustainable and Catalysis Chemistry. I acknowledge the funding of the SNSF for my Ambizione Grant (216096) and Prof. P. Arosio for hosting us. I am very grateful to my PhD and Postdoc advisors Prof. F. López-Gallego and Prof. F. Paradisi for their support and guidance during the realization of these works. Likewise, I thank all my colleagues who have contributed to the works described herein.

Received: January 24, 2024

---

**Fig. 3.** Biocatalytic synthesis of nucleoside analogues. a) Reaction scheme of the phosphorylation of the sugar donor (inosine) and the transglycosylation of the nucleobase (6-O-methylguanine) for the synthesis of a Nelarabine analogue (6-O-methylguanosine) by an immobilized purine nucleoside phosphorylase (PNP). b) In-line set-up of the flow biocatalysis and downstream steps.

**Fig. 4.** Spheroplast biocatalysts for the cyclization of terpenoids as flavors and fragrance molecules. a) Scheme of the preparation of SHC spheroplasts. b) Cyclization reactions catalyzed by different types of SHC biocatalysts.


License and Terms

This is an Open Access article under the terms of the Creative Commons Attribution License CC BY 4.0. The material may not be used for commercial purposes.

The license is subject to the CHIMIA terms and conditions: (https://chimia.ch/chimia/about).

The definitive version of this article is the electronic one that can be found at https://doi.org/10.2533/chimia.2024.222