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Unlocking the Potential of Flow Biocatalysis with Enzyme Immobilization

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Abstract: Flow biocatalysis combines the superior selectivity and sustainability of enzymes with the flexibility, automation potential, and enhanced productivity of continuous manufacturing. However, to apply a biocatalytic step in flow, some intrinsic limitations of biocatalysts must be addressed, especially their stability and reusability. Thus, enzyme immobilization is a key enabling technology and remains a critical step and one of the main bottlenecks. Immobilizing enzymes on solid supports improves their stability, reusability, and compatibility with flow conditions, but it is limited by the trial-and-error approach at the development stages. In this short perspective, we discuss recent innovations in enzyme immobilization, including *in silico* design, the combination with 3D printing and high-throughput screening, and present selected examples of applications in flow of immobilized enzymes, with a particular focus on process flexibility and their combination into chemoenzymatic cascades.

Keywords: Biocatalysis · Biotransformation · Enzyme immobilization · Flow

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1. Introduction

Continuous flow strategies have grown immensely in recent years in all fields of chemistry. From the academic point of view, flow chemistry initially gained attention within the field of organometallic chemistry for two main reasons: (1) its' inherent safety, as reactions are confined within a closed system, enabling the safe handling of air-sensitive molecules; and (2) its superior temperature control, made possible by the higher surface area and enhanced heat dissipation. Beyond exothermic and air-sensitive reactions, flow chemistry offers other advantages that are of utmost importance in any chemical reaction, such as improved mixing and tight control of the reaction conditions. These features make flow chemistry a highly attractive tool for modern synthetic strategies.[1]

In industry, flow chemistry started to gain momentum in the petrochemical and bulk chemical industries, due to its higher economic efficiency compared to batch. Other applications have been flourishing in the last few years, especially in the synthesis of natural products and APIs (active pharmaceutical ingredients).[2,3] Flow chemistry's broad adoption in industry is driven by its ease of automation, reproducibility, safety, and process reliability. Its modularity and flexibility allow for diverse reactor configurations and the integration of multiple modules in a plug-and-play fashion,[4] and unlike traditional batch systems, continuous flow reactors enable precise control over temperature, pressure, and reaction times.

From the sustainability perspective, flow also has major upsides. Several reviews exist on the comparison of batch and flow processes, and in most, continuous production is synonymous with higher efficiency, better product quality and a smaller footprint.[5,6] And from the sustainability angle, there is a second technology that is gaining momentum in the fine chemical industry to design and apply more sustainable synthetic routes: **biocatalysis**.

Biocatalysis, the use of cells or their components to catalyze chemical reactions, has a broad range of applications - from natural products to key intermediates,[7,8] and the use of enzymes, in their pure or semi-purified form, has grown significantly in the last decades. Biocatalysts exhibit exquisite selectivity (chemo, regio, and enantioselectivity), operate at mild temperatures and pressures and use water as the main solvent.[8–10] So, the question naturally arises: can biocatalysis and flow chemistry be combined in a synergistic way?

The answer is yes, but it requires addressing key challenges regarding the biocatalyst itself. Particularly to integrate biocatalysts in flow in an efficient manner we must address their stability in intensified conditions, their capacity to be separated from the reaction bulk and reused, and the compatibility of enzymes with

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chemical steps up or downstream. To address these challenges, enzyme immobilization is a key enabling technology (Fig. 1).

Enzyme immobilization was first described in 1916 and the field has grown immensely since the 1990s.[11–13] The process of immobilization, simply attaching the enzyme to a bigger particle and creating an insoluble material which retains the biocatalytic activity, offers a robust solution to easily integrate biocatalytic steps into existing syntheses and in continuous packed bed reactors. However, immobilization involves a trade-off: some enzymatic activity is typically lost due to reduced flexibility after immobilization, at the expense of a long lasting and reusable biocatalyst. Therefore, the first thing that needs to be considered when trying to apply biocatalytic steps in a multistep synthesis or in continuous flow is the optimization of the immobilization protocol, as it is essential to design robust biocatalysts that maximize productivity and seamlessly integrate into the desired synthetic route.

2. Innovations in Enzyme Immobilization

Over the past century, enzyme immobilization has evolved into a multidisciplinary field, incorporating structural biology, biotechnology, material science, and process development.[14,15] For simplicity, in this publication we will focus on the immobilization onto supports through chemical linkages (covalent or not). Supported immobilizations tend to create biocatalysts that withstand harsh reaction conditions to a greater degree, such as organic solvents or high substrate concentrations. While other forms of immobilization exist, these are less suited for continuous flow applications.[16] While other reviews detail the variety of immobilization techniques and their specific applications,[15,17–19] supported immobilization remains the preferred choice for biocatalysis in continuous flow processes.

In supported immobilization, there are three main components that need to be taken into consideration: the enzyme to be immobilized, the material selected as the support, and the chemistry used to create the linkage. Despite advancements, immobilization screenings often still rely on trial-and-error approaches to navigate the vast number of possible combinations. This mirrors the challenges that we faced in other biotechnological fields, where large combinatorial problems initially limited our progress. For example, in enzyme engineering, the immense number of potential modification sites and their combinations was initially addressed through random mutagenesis and high-throughput screening.[20,21] However, the field experienced a significant transformation with the adoption of data-driven approaches, such as *in silico* design and computational modeling, which greatly accelerated development.[22,23] Some advances have also been made in recent years in the field of enzyme immobilization, incorporating data-driven approaches and high-throughput screening techniques.

2.1 *In silico Approaches for Enzyme Immobilization Design*

The integration of data-driven approaches, and *in silico* modeling to immobilization, is relatively new and it remains an anecdote rather than a general trend. A recent review highlighted the use of different computational tools in immobilization studies.[24] Focused on the immobilization of hydrolases and oxidoreductases, it found that from the 3873 records, only 60 had mentioned the use of bioinformatic tools – that is only 1.5% of the studies. Compared to protein engineering, bioinformatic and data-driven approaches are extremely scarce in immobilization protocols and from those that use *in silico* approaches, most only perform analyses of the protein surface and molecular dynamic simulations to analyze the flexibility or conformational changes of the desired proteins in the solution. Only a few examples considered the different reactivity of the residues on the surface and the effect of both material and immobilization conditions (Fig 2). In addition, in none of the cases there is a unified protocol that is followed or available for such analysis. In 2021, inspired by the previous attempts at the incorporation of bioinformatic tools for enzyme immobilization,[25,26] and with

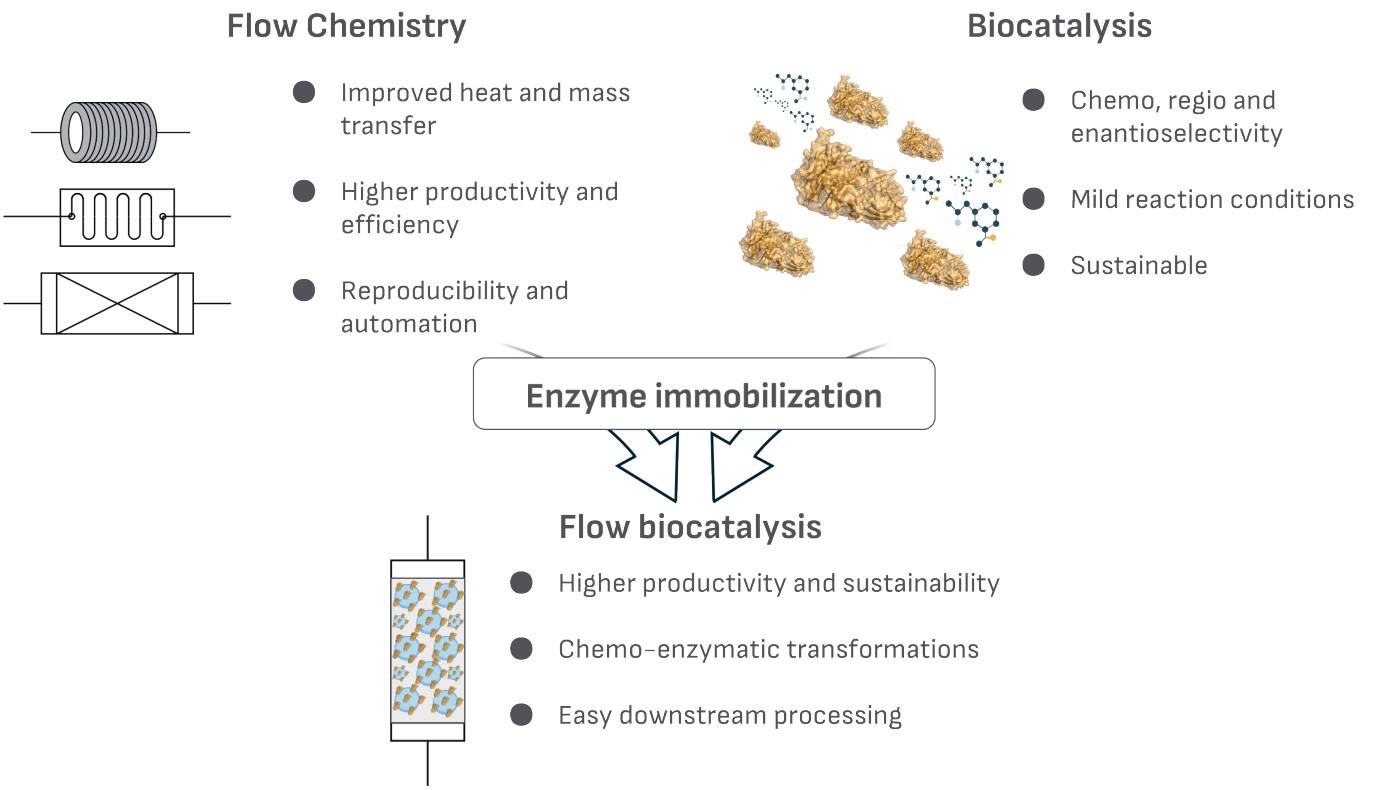

Fig. 1. Schematic representation of the main advantages of flow and biocatalysis, and the potential of combining both in a synergistic way through enzyme immobilization.

the aim to standardize and democratize these analyses, we published a set of easy-to-use tools to apply rational planning to immobilization – CapiPy.[27] CapiPy (Computer Assisted Protein Immobilization using Python) allows researchers with experience in enzyme immobilization to perform simple bioinformatic analysis to inform their decisions. CapiPy can be used both at the initial steps, for immobilization planning,[28–32] and *a posteriori*, to explain and rationalize the main forces affecting a protein after immobilization.[33]

CapiPy, though, only covers one of the aspects that needs to be addressed for efficient immobilization – the analysis of the protein of interest – but more advanced tools are still needed to explore the effect of the materials and chemistries of immobilization. The simulation of material-protein complexes has been reviewed before, but most of the discussed examples only focus on the adsorption phenomena and give little information on the effect of the binding chemistry.[34] In this same publication, it is stated that a limited number of efforts have been made in the use of *in silico* analysis for immobilization planning and the field is indeed in its infancy. Thus, since the release of CapiPy, we have been expanding the capabilities of our bioinformatic workflow for tailored immobilization design. In this sense, we have incorporated material-protein simulations for different types of supports to consider the interaction of the protein with different materials and have developed a scoring algorithm to characterize the reactivity of the different residues on the protein surface. These new tools are now routinely used for *in silico* screenings, to predict the immobilization behavior of a protein, and therefore, reduce the experimental load to get to the optimized solution.

2.2 *Automation and (Semi) High-throughput Screening of Immobilized Enzymes*

Despite the applications of *in silico* tools to enzyme immobilization, the combinatorial testing problem still remains. The selection of the right support, chemistry, and conditions for immobilization is a process that can be time-consuming and costly, but like in other fields, high-throughput screening (HTS) can be applied to ease these burdens (Fig 2).

One persistent bottleneck in the application of HTS to enzyme immobilization is the challenge of handling both solids and liquids, which can significantly slow down screening efforts. A clever solution to the handling of problems was proposed by Spano *et al.*[35] who developed a 3D-printed labware capable of delivering precise and accurate aliquots of granular solid material. The design of the multichannel weighing plate (MWP) allows uniform resin aliquots of the solid resins to be dispensed without the need of a balance and can be directly combined with a 96-well plate analogue. To prove the utility, the authors indeed performed a screening of up to 7 resins in parallel, and in one single experiment they were able to optimize various parameters of immobilization (protein loading and activity of the immobilized enzyme). According to the authors, this reduces the time and effort required for immobilization screening by over 95%.

A similar principle has been more recently applied by López *et al.*[36] who leveraged both data-driven immobilization planning and microtiter plate (MTP) technology. In their work, they tested multiple supports and chemistries simultaneously, with the ambitious goal to assemble an enzymatic cascade of four enzymes to produce (*S*)-3-hydroxybutyric acid. Cascade immobilization indeed requires lengthy experiments to find the optimal condition for each enzyme ensuring that the rest are not negatively affected. In this work, the MTP protocol was applied to measure different parameters at once – activity, stability, and final productivity. After optimization, the immobilized multi-enzymatic system achieved a 70% higher efficiency compared to using free enzymes, underscoring the importance of integrating immobilization screening of enzymatic cascade development systems, combined with data-driven tools like CapiPy.

Another interesting example of HTS for enzyme immobilization and its application, is the Immobilized Biocatalyst Engineer-

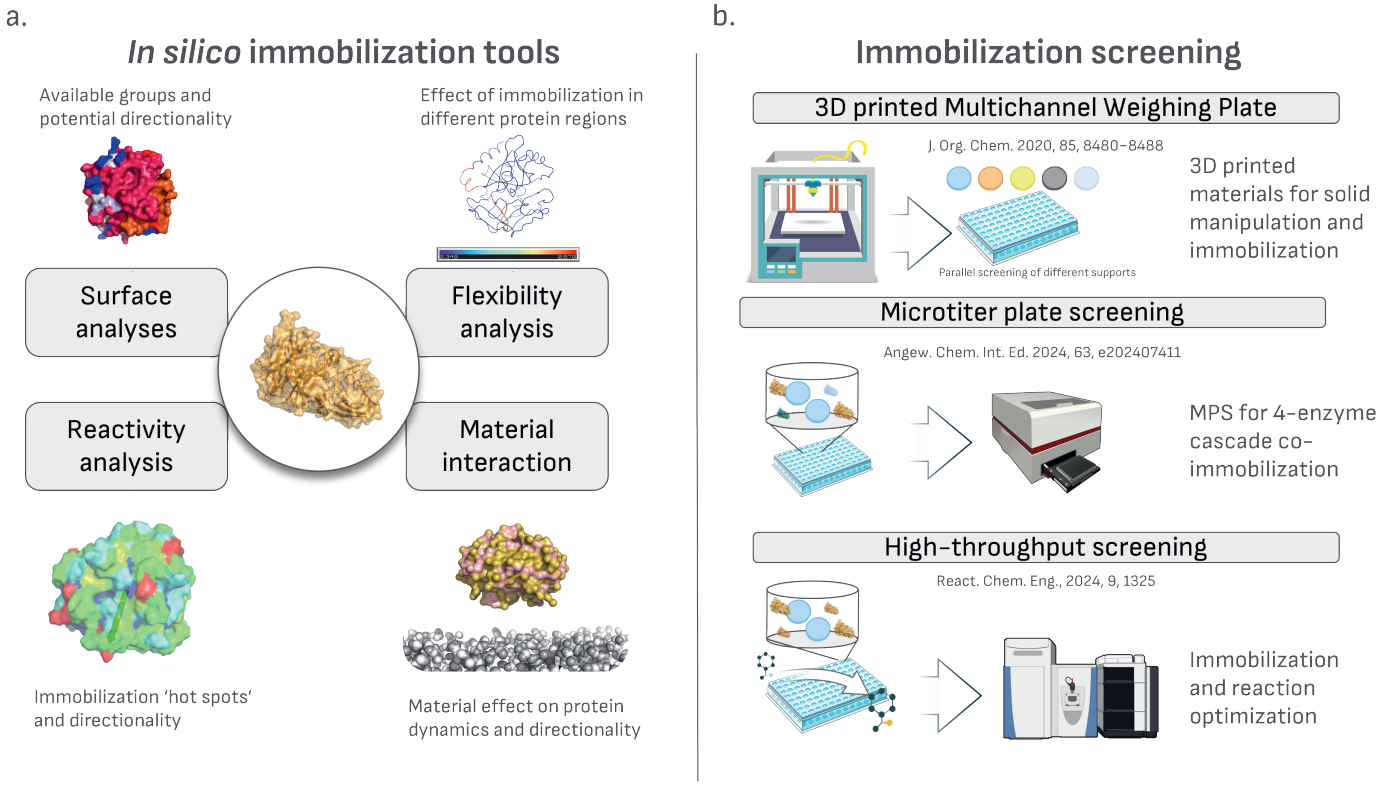

Fig. 2. Overview of the main recent advances in the field of enzyme immobilization. *In silico* approaches (left) and selected examples of the emerging technologies (right) are highlighted.

ing (IBE) platform, developed to integrate protein engineering and enzyme immobilization into a single HTS platform.[37] Unlike traditional sequential methods – where enzyme variants are optimized in their soluble form and immobilized – the IBE platform integrates these steps into a unified workflow. This approach directly evaluates enzyme performance in its immobilized state, identifying variants with enhanced stability and activity that might otherwise be overlooked during conventional screening.

A similar approach was recently published by Schober *et al*.[38] at Novartis. They addressed the time-intensive and costly development process, presenting an automated HTS workflow using a liquid handling robot integrated with supercritical fluid chromatography coupled with UV/vis detection, enabling parallel µL-scale experiments in 96 well plates. Their workflow goes from enzyme screening (identifying the best enzyme for a specific reaction) to resin and chemistry selection with parallel optimization of the immobilization conditions. But going even further, the same setup was also used to select the optimal catalyst, the best reaction conditions, such as the equivalents of the sacrificial substrate and the buffer used, for example. This setup led to significant cost and sustainability improvements, an 11-fold decrease in process mass intensity (PMI) and a 19-fold reduction in reagent costs.

Together, these innovations illustrate how HTS methods, when combined with automation and data-driven tools, are transforming the development of immobilized enzymes, and easing their developmental burden. By optimizing immobilization protocols efficiently and in a case-specific manner, we are one step closer to the fast development of more robust, scalable, and cost-effective biocatalysts for industrial applications.

3. Application of Immobilized Enzymes in Continuous Operations

With immobilized enzymes in hand, integrating and implementing biocatalysis in flow processes becomes a natural step forward. Readily available solutions, in the form of immobilized enzymes, ease up the application of flow biocatalysis and offer opportunities for process intensification, enhanced productivity, and developing more sustainable methodologies. There are several reviews available discussing the latest examples and innovations in the field of flow biocatalysis,[39–41] the assembly of multi-enzymatic cascades in flow[42,43] and even the combination of biocatalytic steps with chemical steps in continuous mode.[44,45] Nonetheless, the transformations that are the most common are still biocatalytic ester and amide formation, which have been extensively applied in industry for almost 40 years now, especially using lipases and amidases.[46,47]

These hydrolytic reactions hold immense potential to access, with excellent enantiopurity, chiral building blocks and their broad implementation is due to two simple factors: (1) the excellent selectivity and stability they offer in their immobilized form and more importantly, (2) their commercial availability at scale and in optimized form. But while these enzymes can certainly perform interesting reactions, the potential of biocatalysis is in the enzymatic diversity that exists and can be engineered. To unlock this potential, at inSEIT we focus on the development of novel immobilized biocatalysts to expand the possibilities beyond what is currently commercially available. For example, the immobilization of the commercially available Flavourzyme main aminopeptidase: LAP2. In a collaborative study, we attempted the immobilization of a semi-purified version of LAP2. We achieved an efficient immobilization at more than 20 $mg_{protein}/g_{support}$ and with excellent recovered activity (48–67%), which allowed for its integration into a packed bed reactor for the efficient resolution of piperazine-2-carboximade to enantiopure (*S*)-piperazine-2-carboxylic acid, a precursor to Linvencorvir (Fig 3a). Compared to batch operations, the flow system increased the productivity 4-fold (3.67 $g_{product}/g_{enzyme}$/hour) while reducing waste formation by a factor of 4, demonstrating the efficiency and sustainability benefits of continuous flow biocatalysis.[48]

While lipases and amidases are the most common enzymes, other hydrolytic enzymes with different characteristics are also gaining momentum – for example acyl transferases. Some acyl transferases can catalyze the condensation of a carboxylic acid and an alcohol or amine to form the corresponding ester or amide in the presence of water. One of the most flexible acyl transferases characterized so far is the one from *Mycobacterium smegmatis* (MsACT).[49] This enzyme has been extensively applied for the synthesis of multiple esters, amides and even thioesters in water as a free enzyme.[50–53] To further expand its applications, MsACT has been immobilized and exploited in a continuous flow reactor for the multi-gram production of several different amides, such as melatonin and analogues (Fig 3.b).[54] In this system, immobilization greatly enhances the stability of the enzyme and allows its reusability for the synthesis of different tryptamine derivatives with excellent productivity (up to 36.9 g/day of melatonin). In this example, the modularity and flexibility of the flow system is also exploited. The acyl donor – vinyl acetate- is used both as a substrate for the acetylation and for the extraction of the product, avoiding any downstream processing to obtain the pure products.

Building on the success of these more common enzymes in continuous processes, chiral transformations are taking the stage. Ketoreductases, for example, have started to find their niche for the formation of chiral alcohols and transaminases are established catalysts for the formation of chiral amines.[55–58] These single step transformations are key steps in the integration of biocatalysis into flow platforms, but the combination of these steps with up or downstream chemical steps performed also in flow is what will confirm flow biocatalysis as a key technology.

Chemoenzymatic systems offer an additional dimension of versatility and efficiency. By combining the selectivity of enzymatic transformations with the robustness of chemical catalysis, these systems enable the development of hybrid workflows that streamline complex synthetic pathways. However, linking each module can be challenging. A compelling example that addresses these challenges is the telescoped synthesis of chiral, pyridine-containing amines through a combination of enzymatic transamination, Boc-protection, and Suzuki-Miyaura coupling in continuous flow (Fig 3.c). The process begins with the biocatalytic amination of a pyridine substrate in a biphasic system using an immobilized transaminase, achieving a modest space-time yield of up to 68 mg/L/h but an excellent >99% enantiomeric excess. Following phase separation and solvent switching, inline BOC-protection and Suzuki coupling deliver the final biarylamine product with a 57% overall yield. This example highlights the potential of flow chemistry to integrate and telescope multiple steps with high efficiency, scalability, and safety.[59] But it also shows that the limitations still exist in the integration of biocatalytic steps into full scale production.

4. Future Outlook and Potential for Impact

Continuous flow processing and biocatalysis represents two significant advancements in modern chemistry, offering enhanced efficiency and sustainability. Integrating these technologies in an effective way requires enzyme immobilization to unlock these new possibilities for sustainable chemical synthesis - we should not only address the limitations of traditional batch processes but think on how we can align our synthetic strategies with the principles of green chemistry, and flow biocatalysis is an excellent tool for it.

Like other fields in biotechnology, the evolution of enzyme immobilization will be driven by high-throughput experimentation, automation, and data-driven strategies. The application of these technologies promises a future where biocatalysis can realize its full potential in the chemical industry. The integration of

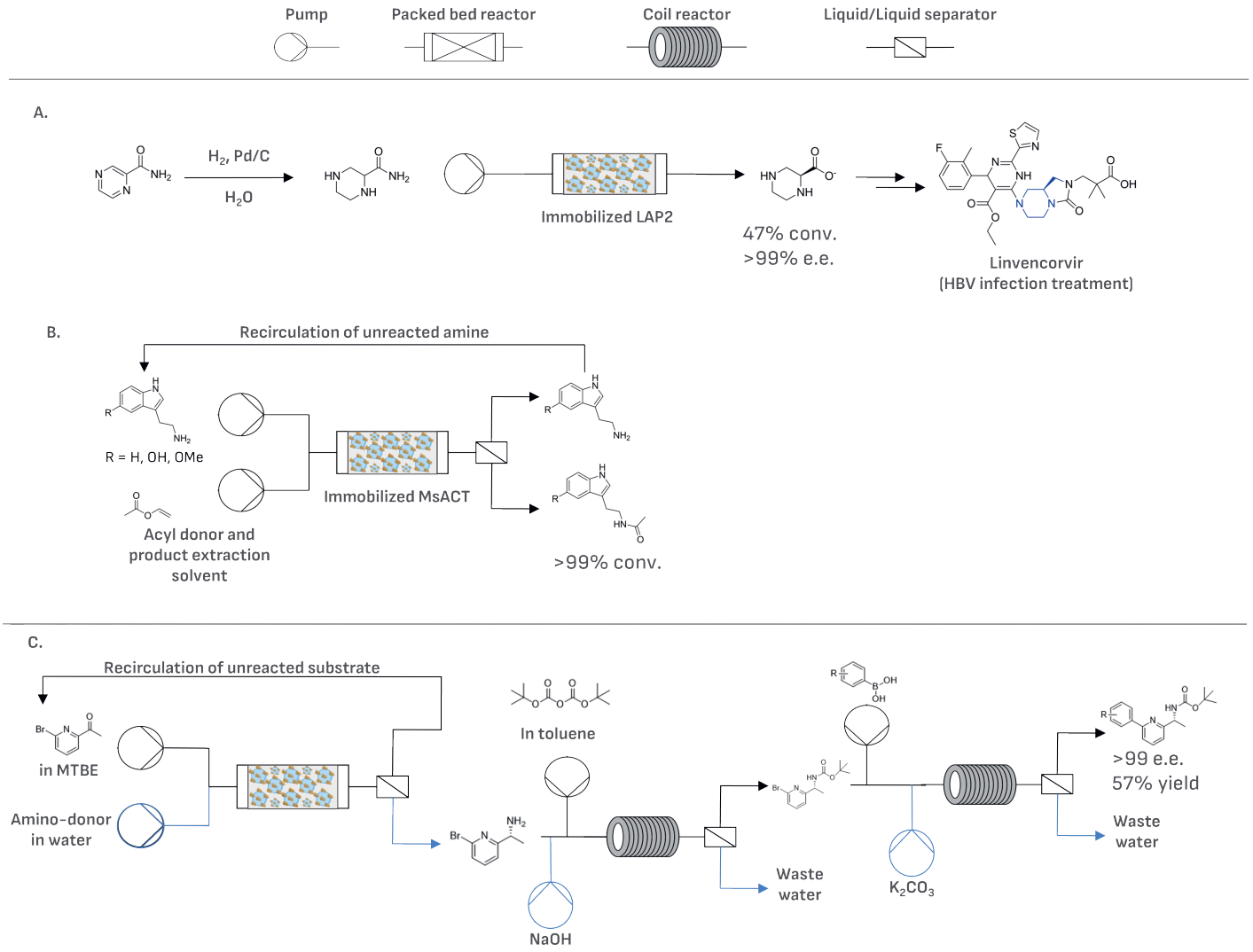

Fig. 3. Selected examples of the application of immobilized enzymes in flow. From single step transformations (A and B) to the combination of enzymes in chemoenzymatic cascades (C). A small legend of the main flow components is shown on top

data-driven approaches in biocatalysis is already enabling the fast and reliable implementation of biocatalytic steps, and an efficient development of immobilized enzymes tailored for specific applications has the potential to expand the scope of biocatalytic processes across various sectors. As industries increasingly adopt sustainable practices, the demand for biocatalytic solutions will grow. In this context, inSEIT aims to contribute in facilitating this transition, through the development and implementation of sustainable biocatalytic processes, fostering a positive impact on diverse industries and promoting a greener approach to chemical synthesis.

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