doi:10.2533/chimia.2023.307 Chimia 77 (2023) 307–311

Flow Chemistry Set-up Enables Integration of Chemo- and Biocatalysis

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Abstract: The move towards sustainable syntheses is a widespread effort which sees academia and industry developing new strategies and solutions. Flow chemistry, and in general the flow set-up, with the compartmentalization of different steps in dedicated reactors, offers new possibilities to integrate biocatalytic steps within a chemical cascade, often without the need to redesign the whole pathway. Here we report key examples in the field over the past few years.

Keywords: Biocatalysis · Enzymes · Flow chemistry · Sustainability

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Introduction

In the last few decades biocatalysis and flow chemistry have seen an increased interest both in academia and industry: on the one hand flow biocatalysis aligns with and respects the 12 principles of green chemistry,[1] and on the other hand flow chemistry is the technique that makes it possible.

The aim of this article is to showcase the significant potential of the combination of both traditional organic chemistry and biocatalysis in a continuous manner.

It is clear to the whole chemistry community that nature is an excellent chemist. The complexity and diversity of many natural products (see Fig. 1), such as paclitaxel (taxol) (**1a**), penicillin (**1b**), lovastatin (**1c**), among many others, would not be possible without an extreme control of the reaction conditions and the selectivity. Since the beginning of life itself, Nature has evolved a diverse, complex and highly specific set of tools: enzymes, the catalysts of all biological processes.

Inspired by the elegance and efficiency of nature, a major effort has been put into developing biocatalytic methodologies[2] to access increasingly complex molecules by means of enzymatic cascades.

Although there are several examples of one-pot enzymatic cascades,[3–5] in many cases it is impossible to combine multiple enzymes at the same time, unless they share (at least to some extent) precise reaction conditions dependent on pH, temperature, ionic strength, or concentration of the starting materials. How does nature handle these challenges so efficiently? Taking a step back to nature and looking at the eukaryotic cell one would soon realize that it is the perfect manufacturing site. Everything happens in the same enclosed space but in different microenvironments. For example, in the endoplasmic reticulum amide bond formation takes place, in the lysosomes multiple types of degradation reactions occur, the mitochondrion is responsible for regulating a plethora of redox reactions in a precisely controlled fashion. All those reactions require different conditions to be successful, without a physical differentiation they would be impossible to perform.

In a similar fashion, flow chemistry and flow biocatalysis are proving to be very useful since each reaction can be performed in an isolated reactor mimicking an artificial metabolism. Recently Mattey and coworkers[6] showcased the potential of flow set-ups to unlock a multi-enzymatic cascade to access a wide range of amines.

In a similar way, the combination of traditional organic synthesis and biocatalysis is subjected to the same incompatibility challenges that can be potentially tackled by means of compartmentalization. It may well be that even if there is a possible biocatalytic route towards the desired product, it is still more efficient to stick to the classical chemical route, for example if substrate concentration in water is very low (mM-microM scale), or when the biocatalyst suffers from fast deactivation during the reaction.

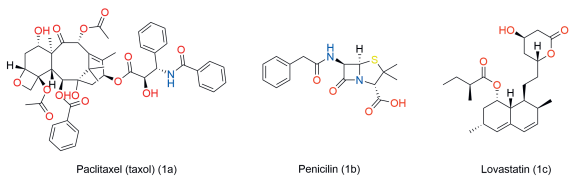

Fig. 1. Relevant natural products with biologically active functions.

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In these cases, it is very unlikely that industry will embrace this technology. If a traditional chemical approach is efficient and sustainable, one should stick to it.

Even though flow chemo-enzymatic approaches are rising as alternatives in terms of efficiency and sustainability, there are still a number of challenges to overcome, in common with any other telescoped process. One challenge, and probably the most frequent, is the incompatibility of the reaction conditions between steps in terms of solvent, temperature, pH, salts, *etc*. Even if flow chemistry solves some of those problems, the truth is that few examples of efficient and commercially available continuous unit operators have seen the light.[7]

Relevant Examples in Continuous Chemo-enzymatic Synthesis

Probably one of the earliest examples of continuous chemo-enzymatic synthesis is the one reported in 2005 by Spain and coworkers[8] where they exploited the reactivity of zinc to obtain hydroxyl aminobenzene followed by the action of hydroxyl aminobenzene mutase[9] (HAB) that converts it to *ortho*-aminophenol. The system consisted of two columns: the first one was filled with zinc and the second one with immobilized HAB mutase on biomimetically-derived silica (Fig. 2). The system proved very efficient for the transformation of a 1 mM solution of nitrobenzene to *ortho*-aminophenol for over 5 h, achieving a final conversion of 89%. The authors further demonstrated the applicability of the system for the synthesis of a chloramphenicol derivative (**2c**) (see Fig. 2). The synthesis ran without interruption for 24 h achieving 100% conversion. This early example clearly showcased not only the benefits of combining classical chemistry with biocatalysis but the enabling role of continuous flow set-ups. One of the advantages of the continuous system compared to the batch process is that the unstable HAB (**2b**) intermediate is rapidly converted into the *ortho*-aminophenol (**2c**), the same reaction in batch is less efficient due to rapid degradation of **2b**. One of the highlights of this work is that the biosynthesis of antibiotics using bacterial cells is limited due to the biocidal properties of the product.

Three years later the same group published a continuous chemo-multienzymatic cascade[10] exploiting the system described above but in this case with an additional module in which a silica-immobilized soybean peroxidase performs the final synthesis of 2-aminophenoxazin-3-one (APO). In this case, a microfluidic device was used. Despite the low overall yield of 18% the system provided a rapid and versatile method for screening the conversion of nitroarenes.

With the focus on rapid reaction optimization, the Ley group developed a fully continuous automated platform for the synthesis of lignamides, which are antimicrobial molecules biosynthesized by plants.[11] Several reactors were packed with immobilized reagents, one of which was a horseradish peroxidase. This set-up proved to be very efficient for the automatic synthesis of multiple lignamides such as the natural product grossamide. With this the authors set the foundation for modern and innovative synthetic chemistry by merging three different fields: synthetic chemistry, biocatalysis and automation.

The continuous chemo-enzymatic synthesis of captopril (**3f**), a drug used for the treatment of hypertension, is another example.[12] Here an overall yield of 50% was achieved over four synthetic steps (Fig. 3), where a biocatalytic regio- and stereoselective oxidation of the prochiral alcohol (**3a**) to the enantiopure carboxylic acid (**3b**), is followed by chlorination, amide coupling and finally a nucleophilic substitution to obtain captopril (**3f**). This work highlights the advantages of using enzymes when high stereoselectivity is needed but also the limitations in productivity that still present a bottleneck in the scale-up of biocatalytic transformations.

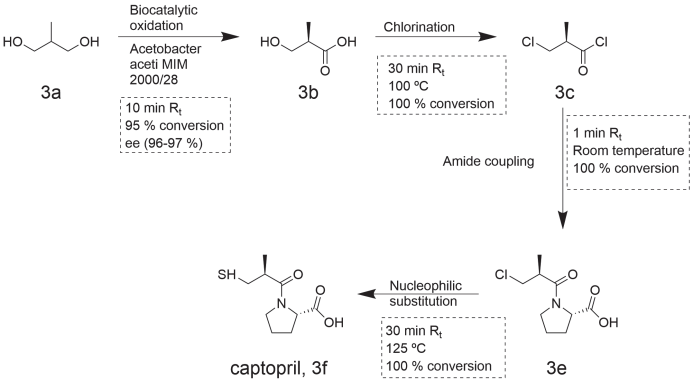

Fig. 3. Chemo-enzymatic synthesis of captopril (**3e**).[12]

With a special focus on sustainability and safety, the work of Brahma *et. al*.[13] clearly exemplifies the enabling power that the combination of flow chemistry with biocatalysis offers. They reported the multistep-synthesis of chiral cyanohydrins in a continuous manner, where the direct handling of HCN is avoided by using an immobilized lipase (CalB), that in a first step hydrolyses a less toxic cyanide source, ethylcyano formate (ECF), liberating HCN that is subsequentially reacted with an aldehyde in the presence of hydroxylnitrile lyase from *Arabidopsis thaliana* (AtHNL) to afford the final chiral cyanohydrin. Although this system was efficiently applied to the synthesis of *(R)*-mandelonitrile with an excellent enantiomeric excess (ee) of 99% and 97% of conversion, the instability of the produced cyanohydrins needed to be addressed. For this reason, an additional in-line protection module using acetic anhydride and pyridine was added. The final coupled system (Fig. 4) ran smoothly, affording the O-acteylcyanohydrins in very good conversions and ee values (75–99% conversion; 40-98% ee). This set-up not only shows an innovative way of synthesizing cyanohydrins but also stresses how the combination of a chemoenzymatic cascade in continuous flow can enable the safe handling of a hazardous reagent such as HCN. In flow, the HCN never accumulates inside the system, since it is consumed straight after its generation. Another remarkable feature that has important relevance in terms of process intensification is the concentration of the starting materials ranging from 0.5 to 1.0 M, simplifying a future scale-up of the process.

The excellent stereoselectivity of enzymes has also been exploited in dynamic kinetic resolutions (DKRs). The principle exploits on the one hand the enzymatic stereoselective acylation (for example), and on the other hand the continuous racemization of the starting material, that can be done either by a chemical catalyst

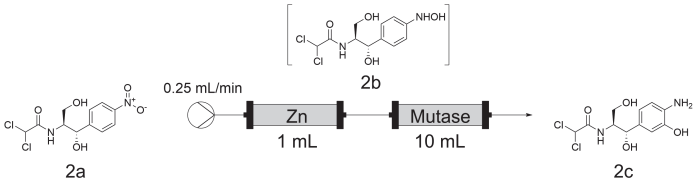

Fig. 2. Transformation of chloramphenicol (**2a**) (1 mM) into *ortho*-aminophenol derivative (**2c**).[8]

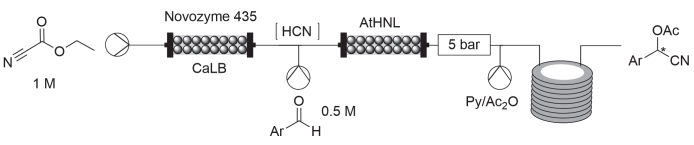

Fig. 4. Three-step continuous chemoenzymatic cascade towards chiral O-acetylcyanohydrins.

or heat. As seen in Fig. 5, the enantiomeric enrichment occurs because of the different equilibrium constants, which push the whole equilibrium towards the favored enantiomer.

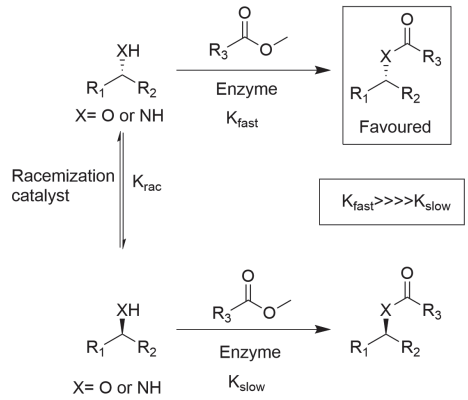

Fig. 5. Dynamic kinetic resolution scheme (DKR).

By virtue of their robustness and stability, commercially available immobilized lipases are probably the most featured enzymes in a chemoenzymatic system. When it comes to selecting the reaction solvent, these enzymes work excellently in a wide range of organic solvents. Their thermostability also favors them for this approach. Thus, by exploiting the enantioselectivity typical of biocatalysts, there are several examples of chemoenzymatic processes that combine the use of lipases and chemical steps to produce chiral products. A particularly inspiring work is that of Farkas *et al.*,[14] on the dynamic chemoenzymatic kinetic resolution of amines in continuous flow mode. The system uses a robust lipase (CaLB-TDP10) for the kinetic resolution step and a palladium catalyst for the racemization step (Pd/AMP-KG) as shown in Fig. 6.

This strategy yielded almost complete conversion of the starting racemic mixtures and presented an innovative mixed-bed unit where the racemization catalyst (Pd/AMP-KG) works in the presence of the lipase. The heterogeneous combined mode requires perfect compatibility between the reaction conditions of the single biological and chemical catalysts, and it is impressive how the DKR system, operated at 60 °C for 48 h, revealing an excellent stability for at least 2 days, producing enantiopure (*R*)-**4a** ($ee_{(S)\text{-}\mathbf{1a}}$ >99.8%) with a space time yield of 4.3 g L^{-1} h^{-1}.

In our recently published paper,[15] we focused on a new type of biocatalysts, traditionally not yet exploited in continuous flow. In this work, a bacterial decarboxylase is innovatively integrated into a chemoenzymatic system (Fig. 7) for the production of the high-value product, hordenine (**5c**), operating in continuous conditions. The excellent compatibility between the enzymatic and chemical steps allowed the realization of the system also in the absence of any organic solvent. The relevant benefit of this approach is the revaluation of L-tyrosine (**5a**), a conveniently abundant raw material, readily available in nature. The amino acid was decarboxylated in-line by the tyrosine decarboxylase from *Lactobacillus brevis* (*Lb*TDC)[16] immobilized here for the first time on a methacrylic resin (Fig. 8).

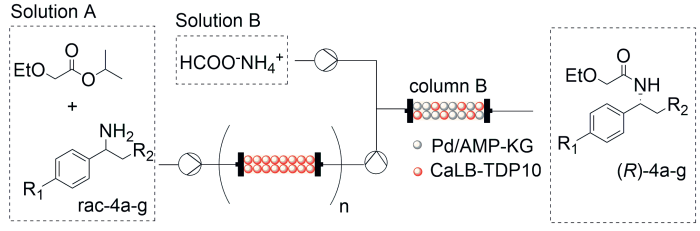

Fig. 6. Lipase/Pd-mediate dynamic kinetic resolution (DKR) of various benzylic amines in continuous-flow operations.

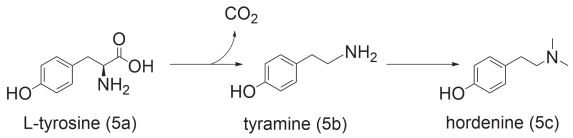

Fig. 7. Hordenine production *via* biocatalytic decarboxylation (step 1) and reductive amination (step 2).

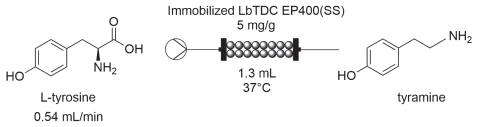

Fig. 8. Continuous decarboxylation by immobilized *Lb*TDC.

Compared to chemical decarboxylation, where organocatalysts[17] are employed at temperatures as high as 150 °C, the equivalent enzymatic reaction with *Lb*TDC occurs at 37 °C, in aqueous buffer, achieving complete conversion in only 2.5 minutes residence time. The *in situ* production of the resulting biogenic amine, tyramine (**5b**), is advantageous as it reduces the biosafety hazards.[18] Typically, tyramine is the starting material in the synthesis of hordenine and it involves high temperature and pressure, as well as precious metal catalysts for the amine methylation.[19–22] In barley, hordenine is biosynthesized in the plants' young roots by sequential N-methylation of tyramine.[23] It was shown that two distinct SAM (S-adenosyl-L-methionine) dependent enzymes, working at different pHs, and characterized by distinct stabilities, are responsible for these methylation reactions. Such enzymes are notoriously challenging to work with and SAM itself is very expensive, therefore an *ex vivo* fully biocatalytical hordenine synthesis could have compromised the success of the project, leading to a costly and over-complicated system. As we searched for an approach that could be intensified for potential scale-up, a more sustainable chemical reductive amination of tyramine was envisaged. We explored the application of two different reducing agents, sodium triacetoxyborohydride (STAB) and picoline borane (pic-BH_3), as the greenest alternative to the classically used, toxic, cyanoborohydride.[24]

In the preliminary phase of the project, working in batch, we sought to identify the minimum amount of reducing agent and formaldehyde required for the complete conversion of tyramine to hordenine. Here, the stability of the two compounds under aqueous conditions was key. STAB requires the use of an aprotic solvent in order to prevent its hydrolytic degradation, whereas with pic-BH_3, the system consists of a heterogeneous phase, as pic-BH_3 is stable but mostly insoluble in water.[25] In batch, STAB required at least 50% of the aprotic solvent acetonitrile (MeCN), to reduce the water effective concentration and suppress its hydrolysis. Seven equivalents of reducing agent and 30 equivalents of formaldehyde yielded a good conversion (88%) in buffer sodium acetate pH 5, after 1 h reaction time.

As an alternative, even though pic-BH_3 is more expensive compared to STAB, its application was considered to investigate the possibility of performing the reductive alkylation of tyramine in an organic-solvent free reaction environment.[26–29] In sodium

acetate buffer pH 5, within 1 h, the amount of pic-BH_3 required to reach a conversion, comparable to STAB, was a large excess (48 equivalents), with 60 equivalents of formaldehyde. Under basic conditions though, at pH 9, the yield of the reaction improved significantly, achieving complete conversion with 24 equivalents of pic-BH_3 and 30 equivalents of formaldehyde.

This difference in optimum pH for the reaction was not the only one considered for an in-flow set-up with STAB and pic-BH_3. Exploring both the alternatives in terms of reducing agents, we developed two different in-flow chemo-biocatalytic systems significantly different in terms of process design (Fig. 9). Working with STAB, a homogeneous mode was employed to perform the reductive amination in continuous operation, by virtue of its solubility in the optimized solvent system with acetonitrile 75% v/v. With 12.5 eq. formaldehyde and 12 eq. STAB, at room temperature, almost complete conversion (96%) was achieved, within a R_t (residence time) of 4.62 min, without the requirement of a back pressure regulator or temperature control.

This set-up was rather simple and straightforward but limited by the organic solvent waste generated at the end of the process. Nevertheless, the equivalents ratio adopted with this first alternative reducing agent was used as a reference condition for the reductive amination performed with pic-BH_3. Investigating the possibility to avoid organic solvents, pumping pic-BH_3 continuously in line was not an option because the reducing agent is insoluble in the aqueous buffer. Therefore, we packed pic-BH_3 in a PBR and operated the system in heterogeneous mode, performing a cascade in line with the enzymatic step for the L-tyrosine decarboxylation.

Without immobilizing the reducing agent on any support, a 1.3 mL column was filled with a mixture of Celite and pic-BH_3 and fed with 130 mL of 5 mM feedstock solution, for 4 hours, achieving almost complete conversion with only 7.5 eq. of formaldehyde and 2.5 min residence time.

To perform the reductive amination reaction at pH 9, 250 mM sodium carbonate (pH 11.5) was used as medium for the formaldehyde feedstock solution.

Notably, working with pic-BH_3 in heterogeneous mode not only improved the sustainability of the process, eliminating the use of organic solvents and avoiding further dilution of the final product hordenine, it also shortened by half the process time, from 5 to 2.5 minutes. As a result, a higher space-time yield (STY) of 11.4 g hordenine $L^{-1}h^{-1}$ was obtained, improving by 4-fold the STY reported with STAB (2.66 g hordenine $L^{-1}h^{-1}$).

These synthetic examples clearly showed the unlocking potential that the combination of traditional organic synthesis and biocatalysis has when dealing with continuous multi-step synthesis. Depending on the compatibility and the stability of the catalysts, heterogeneous conditions, with mixed-units or single catalyst PBR reactors, or homogeneous conditions, with in-line coil reactors, can be applied in the process design.

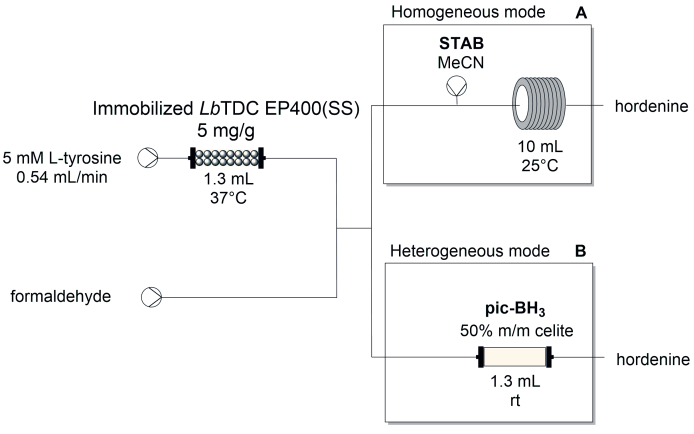

Fig. 9. Alkylation of tyramine with formaldehyde and reducing agent. A) STAB is introduced as a slurry (homogeneous mode) in the coil reactor. B) pic-BH_3 is packed in a PBR (heterogenous mode) mixed with celite.

Conclusions and Outlook

Even if in the last decade several examples of flow chemoenzymatic synthesis have been reported,[30–32] this field is still in its infancy mostly because traditionally there has been an almost dogmatic approach in proving that biocatalysis was the final solution to all environmental concerns and almost 'demonizing' traditional organic synthesis. Over the past few years we have seen a wave of publications revealing the potential of chemo enzymatic approaches,[33–35] proving that a synergistic approach between traditional organic chemistry and biocatalysis could act as a key linker between fundamental research and industrial application.

To succeed, the scientific community needs qualified and specifically trained chemists with a solid background in fundamental biology, process engineering and traditional organic synthesis. Flow chemistry is rarely part of undergraduate programs, but some universities have started to implement it in undergraduate curricula, such as T. Noël in the Netherlands[36] and N. Hartrampf at the University of Zurich. This year the University of Bern has also implemented the first flow-biocatalysis course as a part of the undergraduate practical labs.

Acknowledgements

The authors acknowledge the Swiss National Science Foundation (SNSF) (grantnumber200021_192274).

Received: November 30, 2022

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