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Combination of chemo- and bio-catalysis in flow

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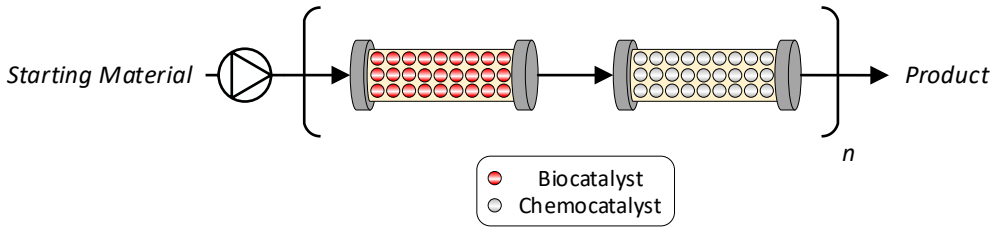
With increased efforts towards enzyme engineering and chemo-catalysis in milder conditions, the combination of chemo- and bio-catalysis has seen a significant rise in interest. Continuous flow methods have also provided advantages to many catalytic processes, which have been increasingly explored by both academics and industry. This review highlights the different approaches for the combination of chemo- and bio-catalysis in continuous flow, together with the main advantages and challenges facing the development of this relatively new research area.

There has been a growing interest in the use of biomimetic strategies to access more complex three-dimensional space. Notably, the utilisation of biocatalytic cascades enables unstable and reactive intermediates to be generated and consumed spontaneously, which can afford interesting bio-active structures in a wider chemical space.[1] Furthermore, combining the high selectivity of enzymes with the diverse applications of chemical catalysts can provide new and improved synthetic routes to a broader variety of molecular structures. Many aspects of these synthetic routes align with the principles and metrics of green chemistry and sustainable development, for example: (i) high selectivity of catalysts reduces the formation of by-products; (ii) catalysts can replace the use of stoichiometric amounts of often more toxic reagents; (iii) heterogeneous catalysts can be easily separated and recycled without the use of distillation or extraction; (iv) integrating reactions avoids separation steps so reduces waste and energy. Although there are many reported examples using either chemo- or bio-catalysis in continuous flow, which have previously been reviewed,[2, 3] there are only a few examples of the combination of them in one system. Therefore, this review is organised by the mode in which the catalysts have been implemented (Figure 1).

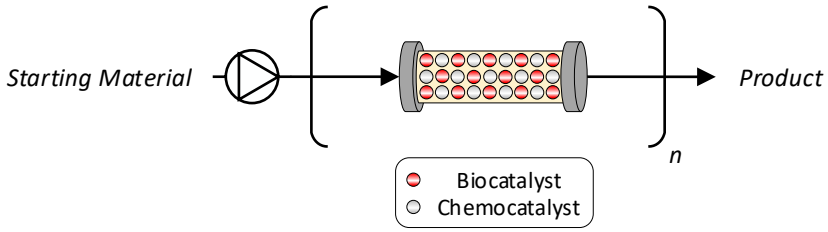
In general, enzymes and chemical catalysts require divergent reaction conditions, such as solvents, temperatures and pH's, which must be overcome to enable the successful combination of processes.[4] When in continuous mode, the use of solid supported catalysts in packed-bed columns enables compartmentalisation, which can circumvent some of the compatibility issues. In some cases, where the kinetics are compatible between chemo- and bio-catalysed steps, supported catalysts can be mixed into one single column. However, supporting a catalyst can be challenging, often hindering its activity, while leaching and deactivation might also occur. An alternative approach is to combine the catalysts in solution. Although still having to overcome compatibility issues, this makes it possible to take

advantage from the increased heat and mass transfer, including other benefits offered by continuous processes (Table 1).

Approach A: Heterogeneous (cascade)



Approach B: Heterogeneous (combined)



Approach C: Homogeneous

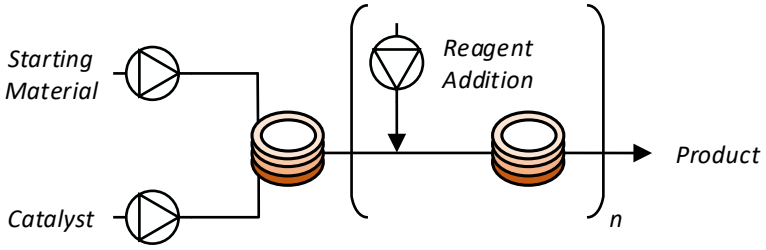


Figure 1. Different approaches for combining chemo- and bio-catalysis in flow: **(A)** heterogeneous enzyme and chemical catalysts compartmentalised in a cascade of packed-bed reactors; **(B)** heterogeneous enzyme and chemical catalysts combined in a single packed-bed reactor; **(C)** homogeneous enzyme and chemical catalysts.

Table 1. Summary of the advantages and disadvantages for each approach used to combine chemo- and bio-catalysis in flow.

Approach	Advantages	Disadvantages
Heterogeneous (cascade)	Easy to separate and recycle catalysts Compartmentalisation can overcome issues with reaction compatibility	Immobilised catalyst required More convoluted and expensive experimental set-ups Unit-number-dependant limitation on conversion

Heterogeneous (combined)	Easy to separate and recycle the combined catalysts No unit-number-dependant limitation on conversion	Immobilised catalyst required Formation of by-products from cross-reactivity Catalysts/reaction conditions are often incompatible Kinetics need to be matched Difficulty in controlling flux
Homogeneous	Homogeneous chemical catalysts are generally more selective Doesn't require immobilisation of catalysts Generally higher catalytic activity in solution	More challenging to separate and recycle catalysts Potential for large waste of expensive materials (e.g. purified enzymes, transition metals)

A) Heterogeneous (cascade)

Chemo-enzymatic dynamic kinetic resolution (DKR) reactions combine enzyme-catalysed enantioselective reactions with chemo-catalytic racemisation to provide enantiopure products. Racemisation generally requires harsh thermal or strong acidic/basic conditions that are incompatible with enzymes, causing denaturation and loss of activity. Furthermore, common metal-based chemo-catalysts often inhibit enzymes themselves, greatly reducing their compatibility.

In 2016, Poppe and collaborators reported the first continuous flow DKR.[5] The work used Subtilisin A to catalyse the enantioselective amidation of the (*S*)-enantiomer with benzylamine from a racemic mixture of *N*-Boc-phenylalanine ethyl thioester. The (*R*)-enantiomer was racemised using a non-nucleophilic base, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) catalyst, then fed directly to the next resolution reaction. The approach was enabled using an immobilised Alcalase® (Subtilisin A) prepared by adsorption onto surface-grafted macroporous silica gels. The optimum support was identified by screening 24 different silica supports looking for temperature, productivity and optical purity. The authors found that Alcalase adsorbed on ethyl-grafted macroporous silica gel was the most suitable, furnishing highly specific activity and enantiomer selectivity (E = >200; c = 41.3% and ee = 98%). Even after one year storage of the columns at 4 °C, the enzyme activity remained high. The racemisation step was also incorporated into a fixed-bed continuous flow operation with the base attached to an ethyl-grafted silica gel (Dv250-Et). To accomplish the dynamic kinetic resolution, columns packed with Alc-Dv250-Et for the KR and Dv250-Et for the racemisation were intercalated in series (11 columns overall, Figure 2). A key aspect was the identification of a common solvent, *tert*-amyl alcohol, for both catalytic steps. Compartmentation of the catalytic reactions allowed for the optimum temperatures to be used for each step, in this case 50 °C and 150 °C respectively. This immobilisation strategy is easier to implement than

covalent bonding, but often suffers declining performance due to catalyst leaching.[6] Nevertheless, in this system both catalysts' performance remained high over 120 hours of continuous operation. The system afforded the desired (*S*)-*N*-Boc-phenylalanine benzylamide in high yield and enantiomeric purity (79%, 98% ee) with a productivity of 8.17 $g.L^{-1}.h^{-1}$, generating almost one kilogram in a working week.

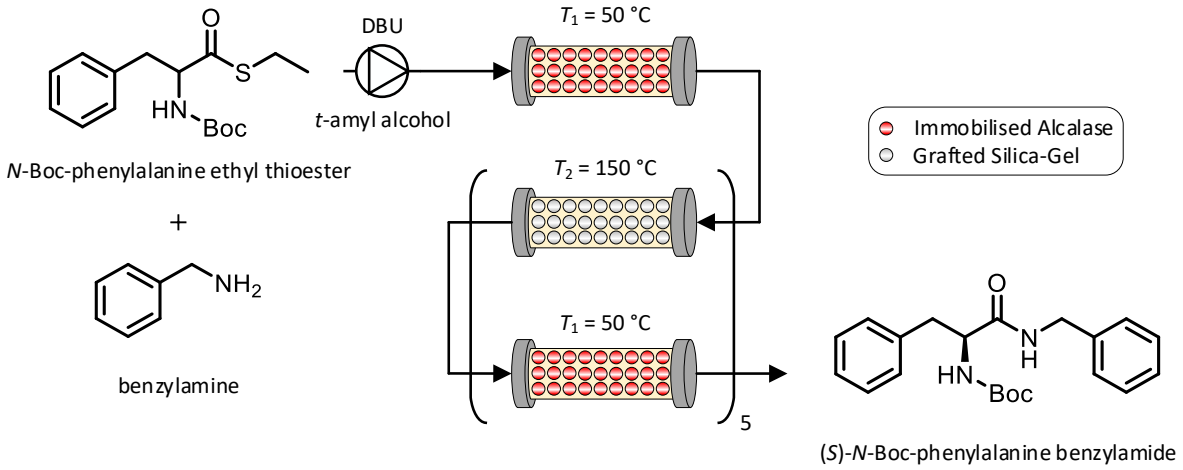


Figure 2. Dynamic kinetic resolution of *N*-Boc-phenylalanine ethyl thioester using an alternating cascade of packed-bed enzyme reactors and racemisation reactors.

A different approach by de Souza *et al* for the continuous flow DKR of *rac*-1-phenylethanol, catalysed by covalently immobilised *Candida antarctica* lipase B (CALB, Novozym-435) and $VOSO_4$, was achieved using a single, site isolated, packed-bed reactor.[7] This was done because $VOSO_4$ promotes the racemisation of benzylic alcohols, but inhibits the activity of CALB.[8] Alternating layers of CALB and $VOSO_4$ were physically separated by thin cotton partitions, such that the single packed-bed reactor had four bio-catalysed reaction stages and three chemo-catalysed racemisation stages. Toluene was identified as the preferred solvent for both reactions, and enabled good selectivity and reaction rates at 70 °C. Using this approach, the authors were able to increase the substrate concentration 10-fold with just a small reduction in yield and ee compared to previous compartmentalised batch reactions (90% ee *cf.* 99% ee; 82% yield *cf.* 93% yield).

Another example of a multi-step continuous flow kinetic resolution (KR) is shown by de Vos in the synthesis of chiral 1,2-amino alcohols from alkenes.[9] In this work, supported ionic liquid-like phases (SILLP), which are polymers functionalised with ionic liquid units, were shown to provide high enzymatic performance and stability. The process was separated into three distinct steps: (i) CALB-SILLP-dec-NTf_2 catalysed formation of organic peroxyacid and subsequent alkene epoxidation; (ii) SIILP-SO_3-$Sc(OTf)_2$ catalysed epoxide ring opening with amine; (iii) Novozyme-435 catalysed kinetic resolution to give the enantio-enriched product. Notably, dimethyl carbonate (DMC) was found to be compatible with both the chemo- and bio-catalysts used, thus providing an environmentally benign solvent and removing the need for convoluted solvent switching between steps. Furthermore, DMC also acted as the precursor to the organic peroxyacid and acylating agent for the kinetic resolution, highlighting its versatility in chemoenzymatic processes. The combination of steps (i) and (ii) was challenging, as the presence of excess H_2O_2 from step (i) was detrimental to step (ii).

Therefore, an in-line water wash and subsequent off-line phase separation was integrated, to give a peroxide-free product from step (i). In contrast, steps (ii) and (iii) were telescoped in a fully continuous manner with excellent enantioselectivity (> 99% ee for the carbonate and 95% ee for the free amino-alcohol) and productivity (step (ii) = 12.2 $g.g_{cat}^{-1}.hL^{-1}$; step (iii) = 1.9 $g.g_{cat}^{-1}.hL^{-1}$).

In cases where enzyme purification is difficult and/or expensive, it can be desirable to immobilise crude cell extracts directly. However, crude cell extracts contain residues which can reduce the sustainability of the process by affecting reaction selectivity and polluting the product. As such, additional downstream purification steps may be required to remove these impurities. Crude cell extracts were used for the biocatalytic production of chiral alcohols, where a rapid method for the immobilisation of HaloTag-*Lb*ADH was developed by Musio *et al*.[10] A packed-bed reactor containing immobilised HaloTag-*Lb*ADH was used for the asymmetric reduction of a wide range of ketones, where 2-propanol was used for cofactor regeneration ($NADP^+ \rightarrow$ NADPH) with remarkably little effect on enzyme activity and stability. The reaction was carried out in pH 7 buffered water, then by feeding base to change the pH to 13, enabled the base mediated ring closure to be telescoped to afford chiral epoxide (*S*)-2-phenyloxirane in a 98% yield with 98% ee. As previously, compartmentalisation of the bio-catalysed and chemo-catalysed steps was crucial to the success of this process, as enzymes are generally denatured under strong basic conditions. Similar flow approaches combining bio-catalysis with base mediated chemical steps have been used for the safe cyanation and subsequent acetylation of aromatic aldehydes.[11]

In a recent study by H. Gruber-Woelfler *et al*, the chemoenzymatic continuous flow synthesis of (*E*)-4-hydroxy-stilbene was enabled by utilising deep eutectic solvents (DES's) and water.[12] Enzymatic decarboxylation of *para*-coumaric acid catalysed with phenolic acid decarboxylase from *Bacillus subtilis* (*Bs*PAD) afforded 4-ethenylphenol, which underwent a subsequent Pd-substituted Ce-Sn-oxide catalysed Heck cross-coupling reaction with iodobenzene to yield (*E*)-4-hydroxy-stilbene. The catalysts were compartmentalised in different packed-bed reactors which allowed the use of 30 °C to increase enzyme stability in the bio-catalysed step and 85 °C to increase the rate of the chemo-catalysed step. Importantly, the use of DES's was critical to overcoming compatibility issues by: maintaining high enzyme activity/stability, reducing hydrolysis sensitivity of the chemo-catalyst and increasing substrate solubility. Furthermore, telescoping these reactions avoided the need to isolate the 4-ethenylphenol intermediate, which is prone to polymerisation.

B) Heterogeneous (combined)

The drawback of packed-bed reactor cascades is the unit-number-dependent limitation on conversion. Therefore, it can be advantageous to identify processes that operate under similar conditions, enabling integration of the catalysts. For example, Poppe *et al* used a packed-bed containing a mixture of CALB-TDP10 and Pd/AMP-KG at 60-70 °C to achieve the dynamic kinetic resolution of amines in continuous flow with excellent enantiomeric purity (> 98.8% ee).[13] The scope of the system was evaluated on 7 different amines, and while most amines required only one column for KR before the DKR combined column, two amines required two KR columns to achieve satisfactory conversion. Remarkably, the system

operated for 48 hours with no major decrease in productivity, whilst achieving a calculated space time yield of 4.29 $g.L^{-1}.h^{-1}$.

C) Homogeneous

In the context of combining chemo- and bio-catalysis, we define homogeneous as any process which uses at least one homogeneous catalyst. Besides one report by Rutjes,[14] where a bio-catalysed formation of cyanohydrins was combined with a non-catalytic chemical acetylation, we could not find any strictly catalytic examples using homogenous bio- or chemo-catalysts or heterogeneous combinations other than a case study from our group published in a PhD thesis.[15]

This example illustrates the complexity of integrating bio- and chemo-catalysts. The immobilised CALB enzyme Novozyme435 was used in fixed bed continuous flow to resolve *rac*-2-phenethylamine by selective acylation using methyl methoxyacetate in toluene at 60 °C (Figure 3). The product (*S*)-amide was produced with 96% ee in 48% conversion at steady-state with a residence time of 30 minutes. Separately to this, the homogenous amine alkylation catalyst $[IrCp^*I_2]_2$, SCRAM,[16] was used to *iso*-propylate the unreacted (*R*)-2-phenethylamine using diisopropylamine in pressurised toluene in batch at 150 °C in 73% yield in one hour, without racemisation and minimal dimerisation. With the aim of integrating the two reactions in continuous flow, the component compatibilities were explored. Initially, the reaction was tested in cascade CSTR's at 100 °C with a residence time of one hour.[17] Addition of methyl methoxyacetate slightly benefitted the alkylation reaction, whilst diisopropylamine and the SCRAM catalyst showed no interference in the enzyme reaction. Since the enzyme was immobilised it was wrongly assumed it would not interfere in the alkylation reaction. Bringing the system together the first stage resolution was successful, but the alkylation failed. It was found that leachate from the enzyme support inhibited the SCRAM catalyst; the identity of the poison is at present unknown.

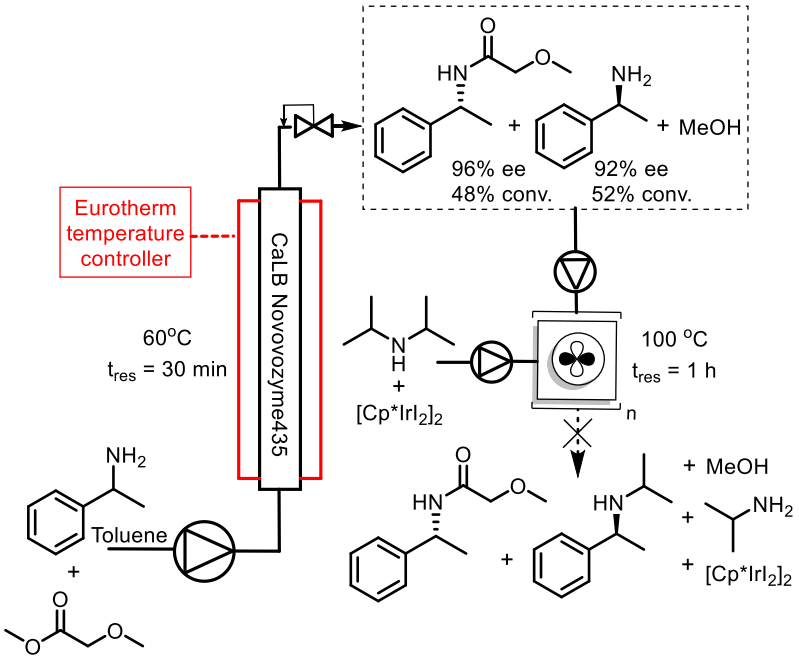


Figure 3. Attempted integration of bio-catalysed resolution and chemo-catalysed *N*-alkylation.

Outlook

There is currently very limited literature describing the combination of chemo- and bio-catalysis in flow, which reflects the infancy of the field and the complexity of integrating these processes. Recent examples have highlighted that continuous flow technology provides a useful tool for circumventing compatibility issues, mainly achieved through physical compartmentalisation and/or solvent switching. Notably, these approaches enable better operating conditions, as different process conditions (e.g. temperature, pH etc.) can be used for each step. However, solvent compatibility remains a significant challenge and severely limits the current scope of possible chemistries. Although solvents such as IL's and DES's help to maintain the activity and stability of both bio- and chemo-catalysts, they do possess drawbacks regarding high toxicity and viscosity. Mixing with water helps to overcome these issues, and therefore the design of chemical catalysts which work well in aqueous environments ('reactions in water') will be crucial for future development in this area.

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