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Process intensification in continuous flow biocatalysis by up and downstream processing strategies

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In this review, we focus on the holistic continuous enzymatic production and put special emphasis on process intensification by up- and downstream processing in continuous flow biocatalysis. After a brief introduction, we provide an overview of current examples of enzyme immobilization as an upstream process for flow biocatalysis. Thereafter, we provide an overview of unit operations as downstream processing strategies, namely continuous (i) liquid–liquid extraction, (ii) adsorptive downstream processing, and (iii) crystallization and precipitation. Eventually, we present our perspectives on future trends in this research field.

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Introduction

Biocatalysis has emerged as a powerful tool and greener basis as an alternative for 'classical' industrial chemical processes in recent decades [1–10]. However, when enzymes are used on larger scales, bioprocesses should be designed and handled holistically from the beginning (upstream) to the very end (downstream), to fulfill the requirements of the manufacturing market (Figure 1). Enzymatic reactions may suffer from (i) stability issues of the biocatalysts, (ii) deactivation due to substrate or product inhibition, (iii) solubility challenges of the substrate(s), (iv) the use of expensive cofactors, or (v) a challenge with the downstream processing (DSP) due to low product titers that need to be solved through process intensification [11–14].

'Flow biocatalysis' has gained great momentum in recent years both in research and in the industry [15]. Under the aspect of biocatalytic process intensification, (i) high surface-to-volume ratios, (ii) improved mass transfer, (iii) superior temperature control, and (iv) small volumes requiring significantly reduced amounts of reagents are some major arguments for transferring biocatalytic processes into flow applications. However, converting a substrate enzymatically to a product is only half of the story since the product must be eventually isolated from its reaction mixture, for example, removing the catalyst and the solvent.

Therefore, in this review, we focus on downstream processing techniques to be applied for biocatalyzed reactions being carried out under continuous operation. To the best of our knowledge, this is the first review focusing on this topic. In addition, we review very recent examples of upstream processing for the formulation of enzymes for their use in flow. We narrow the applications of the up and downstream processing techniques on enzymatic (cascade) reactions using cell-free biocatalysis or microbial biocatalysis with non-growing cells. That means, we herein exclude up- and downstream methodologies on fermentative processes.

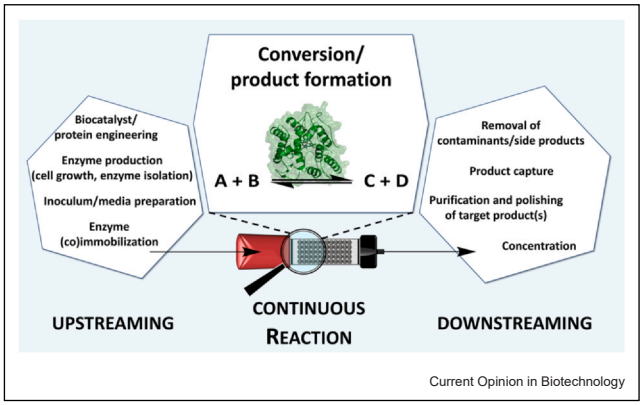
Enzyme immobilization as upstream processing for flow biocatalysis

The immobilization of enzymes and whole cells facilitates their use in continuous operations and is therefore a key upstream process in flow biocatalysis [15,16]. To obtain these heterogeneous biocatalysts, various methods are available and a selection of those is discussed in this paper.

Carrier-bound immobilization strategies

An exemplary process for carrier-bound covalently immobilized enzymes was reported by Padrosa et al. with a transaminase system to produce the nonproteinogenic amino acid L-pipecolic acid [17]. Lysine dehydrogenase

Figure 1



Process steps in a typical (continuous) bioprocess, including (i) upstreaming, (ii) reaction, and (iii) downstreaming.

from *GeoBacillus stearothermophilus* (Gs-Lys6DH) was co-immobilized with pyrroline-5-carboxylate reductase from *Halomonas elongata* (He-P5C) for cofactor regeneration. In a carrier screening, epoxy-functionalized agarose microbeads were selected as solid support. While the recovered activity of Gs-Lys6DH was already high with 91%, the one for He-P5C was initially lacking but could be improved to 21% by a polyethyleneimine coating applied during the immobilization. In this way, a high space–time yield (STY) of 2.5 $g \cdot L^{-1} \cdot h^{-1}$ could be achieved, and the system was further refined with a scavenger column for product and cofactor recovery (see also below).

A particular case of covalent immobilization was published by the group of Paradisi [18••], where a purine nucleoside phosphorylase from *Halomonas elongate* (HePNP) was bound to thiol-activated agarose beads. Notably, the bonding by disulfide bridges between enzyme and carrier was reversible by the addition of 50 mM dithiothreitol allowing the reuse of expensive carrier materials after the biocatalyst is inevitably inactivated. Since the enzyme had no free Cys residues on its surface, a (6x)Cystag was introduced to directionally immobilize HePNP. The immobilization increased the enzyme's stability toward temperature and cosolvents and facilitated its use in continuous operation to produce a Nelarabine analog in a packed-bed reactor (PBR). With this approach, > 99% conversion of 10 mM substrate over 100 column times with a STY of 89.1 $g \cdot L^{-1} \cdot h^{-1}$ was achieved. In this PBR, the reuse of the carrier material with fresh enzyme was conducted directly in the flow setup with similar results as the initial immobilization.

Beyond these examples, several further continuous applications of covalently immobilized enzymes were reported [19–23].

Carrier-free immobilization strategies

One method for carrier-free immobilization of biocatalysts is gel entrapment, as demonstrated by Kruschitz et al. for the continuous production of 2-α-D-glucosylglycerol from glucose and glycerol [24•]. For that purpose, whole-cell *Escherichia coli* expressing sucrose phosphorylase from *Bifidobacterium adolescentis* was immobilized in polyacrylamide particles that were shredded to a size of 0.25–2 mm. The immobilized biocatalyst exhibited a high activity of 60–70% of the cell extract. Applying the particles in a PBR, a stable production over 40 days with a STY of 45.8 $g \cdot L^{-1} \cdot h^{-1}$ was obtained under optimized conditions.

Beyond this example, several other continuous applications of entrapped biocatalysts were reported [25–28].

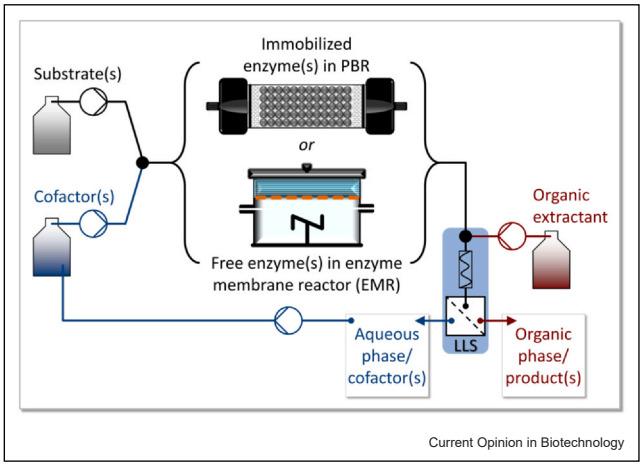
A valid goal for continuous biocatalysis is to possibly avoid any additional material for immobilization. One such attempt using so-called *in vivo* immobilization for continuous biocatalysis was reported by Ölçücü et al. [29]. The formation of catalytically active inclusion bodies (CatIB) of alcohol dehydrogenase from *Ralstonia sp.* (RADH) and lipase A48 from *Bacillus subtilis* (BsLA) was investigated. In a comprehensive screening of four different aggregation-inducing tags and C- or N-terminus as fusion site, the CatIB formation was optimized in *E. coli*. The most active heterogeneous RADH formulation was chosen for implementing a flow process with closed-loop cofactor regeneration. However, due to low particle size, the CatIBs had to be mixed with silica to avoid clogging the flow system. This way, with 15 mM substrate, a conversion of 92% and STY of 3.55 $g \cdot L^{-1} \cdot h^{-1}$ for (*R*)-2-chloro-1-phenylethanol-1-ol production was achieved.

In summary, there are a variety of immobilization methods available for flow biocatalysis, and much more research will certainly follow, broadening the selection and improving performance even further. However, there are few truly integrated approaches to biocatalyst immobilization, and a future focus on these could yield major advances in the technology.

Downstream processing for continuous biocatalytic systems

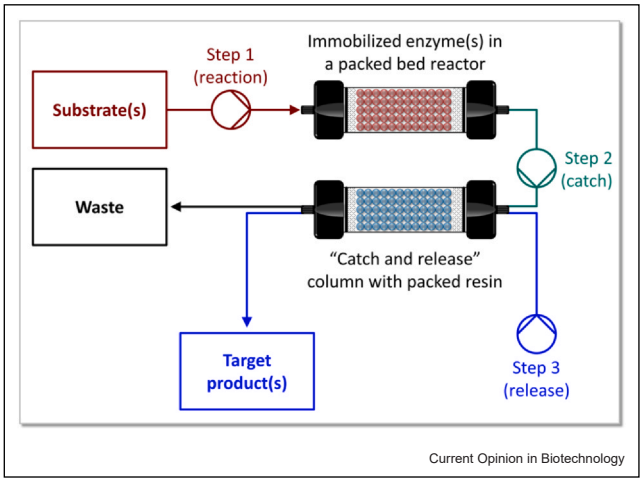
In continuous enzymatic synthesis, the resulting target product is eventually released at the end of the reactor. However, by-products and/or unconverted substrate may be eluted at the same time. In addition, the product stream also contains the solvent in which the former substrate was diluted so that the effluent must be subjected to a work-up process. In this context, we also want to highlight important literature for DSP methods in general such as the review by Seidel-Morgenstern and coworkers dealing with processes to separate

Figure 2



General example of a continuous liquid–liquid extraction setup with an organic extractant. LLS: commercially available liquid–iquid separator either from Zaiput (e.g. [36]), or from Syrris (e.g. [37••]). Optional recycling of cost-intensive redox cofactors is also possible (blue recycling loop, e.g. [37••]).

Figure 3



General example for a continuous adsorptive downstream processing method directly after a continuous production in a sequenced flow setup.

enantiomers [30], Nidetzky and coworkers about DSP technologies in the biocatalytic production of oligosaccharides [31], or articles by Hori and Unno [32], or Wohlgemuth [33].

The most common DSP unit operations for enzymatic reactions performed in the continuous mode are listed below. Hereby, we paid special emphasis on the general setting of the integration of the DSP units in continuous enzymatic operations. In the literature, this is also referred to as an 'in-line' DSP units. In this section, not only examples for flow biocatalysis are selected as use cases but also continuous enzymatic synthesis in stirred-tank reactors equipped with a membrane (so-called enzyme membrane reactor, EMR) is introduced.

Continuous liquid–liquid extraction

In many cases, enzymatic reactions are performed in liquid media. Most often, this liquid phase is composed of (i) aqueous buffer solutions, (ii) aqueous solutions with water-immiscible organic solvents, (iii) aqueous solutions with special additives such as ionic liquids or deep eutectic solvents, or (iv) the substrate is used purely as 'neat substrate' [34]. However, it follows from this consideration that the product must be separated from the liquid phase. Here, liquid–liquid extraction can be the suitable unit operation [35], either in an additional, noncontinuous operation mode or directly after the product stream exits the reactor [36]. In addition, such a setup concept may also allow the continuous recycling of cost-intensive redox cofactors [37••] (Figure 2).

Similar downstream extraction procedures were also studied by Paradisi/Tamborini and coworkers [38–42], Žnidaršič-Plazl and coworkers [43], and Pietruszka and coworkers [44].

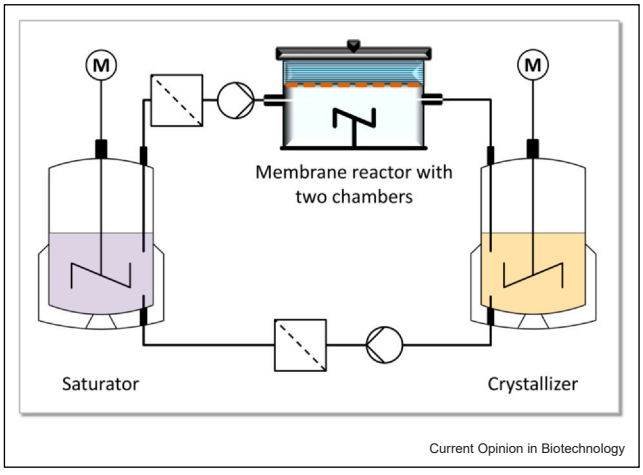
Continuous adsorptive downstream processing

To obtain the desired product and preferably in high purity, sorbent materials being packed in columns offer a great opportunity to recover both the product and the unreacted substrate from the solvent to be recycled (Figure 3). In addition, if a product-selective carrier is chosen, it can serve as an *in situ* product adsorption unit operation (as a special case of *in situ* product removal (ISPR)) to shift the equilibrium to the product's side. However, before implementation in a continuous system, screening of the various (commercially available) sorbents should be performed [45,46].

A DSP approach integrated into continuous biocatalytic synthesis was presented by Semproli et al. [47]. Here, a packed-bed reactor (PBR) for transamination reaction was used followed by a subsequent adsorption column packed with ion-exchange resin Dowex Marathon C. The product, by-product, and unreacted substrate were first continuously adsorbed, then the resin was washed with water to remove these impurities, followed by a desorption step with 1 mol L^{-1} solution of NH_4OH. Eventually, the pure amine was recovered with 35% isolated yield after evaporation of the solvent.

Similar DSP approaches were also achieved with zeolites packed in a column as subsequent continuous adsorption for laminaribiose production [48]. Likewise, an integrated purification step was applied by De Vitis et al.

Figure 4



Transaminase-catalyzed reaction in a triple-vessel approach by Hülsewede et al. [58•]. M: motor as an agitator.

using a column equipped with ion-exchange Ambersep 900 OH^- resin to recover 2-hydroxymethylalkanoic acid products after enzymatic oxidation of achiral 2-alkyl-1,3-diols using immobilized *Acetobacter aceti* [49]. In addition, comparable procedures were also studied by Paradisi and coworkers [17], Jamison and coworkers [50], Tamborini and coworkers [51–53], and Conti and coworkers [54].

Continuous crystallization and precipitation

Generally, the unit operation of continuous (enantioselective) crystallization enables the DSP of many produced substances, also integrated directly after continuous production [55]. More information on *in situ* product crystallization and related techniques applied in biocatalytic processes, in general, can be found in the review of von Langermann and coworkers [56]. Generally, continuous crystallization may occur in two different fundamental ISPR modes: (i) either the crystallization of the product takes place inside the reactor (internal crystallization), or (ii) an external loop is used to separate the actual reaction chamber from the crystallization compartment (external crystallization). In the literature, sometimes *in situ* product recovery is used for case (i), and in-stream product recovery for case (ii) [57].

As an example for an in-stream product-recovery application in continuous crystallization, Hülsewede et al. used a special membrane reactor for the continuous synthesis of an amine via transaminases. The reactor was coupled to one vessel for the coproduct (amine donor in the 'saturator') and to one vessel for precipitation ('crystallizer', Figure 4) [58•]. With this setup, the unfavorable chemical reaction equilibrium was shifted to the product's side and a fully stoichiometric reaction was achieved with a STY of 1.2 $g{\cdot}L^{-1}{\cdot}d^{-1}$. The process integration demonstrated in triple-vessel setups is not limited to stirred systems and can be transferred to flow applications.

Another integrated crystallization strategy was shown by Luis and coworkers where product crystallization was forced with a cooling unit [59].

Other unit operations integrated into the continuous processes

Other unit operations within biocatalytic synthesis involve continuous filtration within modular microfluidic reactors [60], or continuous gas stripping/distillation [61,62]. In the latter, substances with high volatility may be removed from continuous production via reduced pressure. This may either apply to directly gain the product of interest or to remove unwanted side products or toxic components that could deactivate the biocatalyst, for example, acetaldehyde.

Conclusion and future perspectives

The design and implementation of biocatalytic systems need a holistic methodology in which the formulation of biocatalysts for their use in continuous conditions and the isolation of target products should be thought out and planned in an integrated fashion. Recent examples within the biocatalysis community demonstrate that a toolbox of enzyme-formulation methods, reactor setups, and unit operations is available. Owing to the several advantages of flow biocatalysis, more focus has been and will surely be dedicated to using heterogeneous enzyme preparations packed in columns of different sizes (from µLs to mLs).

Future perspectives for process intensification in continuous flow biocatalysis might incorporate thermomorphic multiphasic extraction systems in which the number of unit operations can be decreased by simply changing the operation temperature. We recently studied thermomorphic multiphasic systems using deep eutectic solvents [63] or ionic liquids [64]. However, continuous in-line use has not yet been reported. In addition, we believe that theoretical calculations such as quantum chemistry-based equilibrium thermodynamics methods (e.g. COSMO-RS) will support the future development of optimized DSP strategies [65]. Beyond that, the role of enzyme engineering can be expected to increase even further, firmly establishing early-stage biocatalyst design as an integrated development step, both regarding the actual enzyme and the immobilization [66,67]. Furthermore, the possible incorporation of process analytics in flow biocatalysis may play an important role. While sensors are already widely used to control biocatalytic processes [68,69], high automation in the sense of 'industry 4.0' methods and concepts such as

smart reactors are to be explored in flow biocatalysis [70]. Together with the integrated process steps discussed within this review, these upcoming advances can be expected to play a major role in the future development of flow biocatalysis.

Conflict of interest statement

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the paper and there is no financial interest to report.

Data availability

No data were used for the research described in the article.

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