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10 Continuous-flow biocatalysis with enzymes and cells

The advantages of synthetic chemistry performed in continuous flow synergize with the benefits conferred by biocatalysis, including reactions with greener, milder, lower temperatures, and aqueous conditions. Furthermore, the fine control over reaction conditions in continuous flow can solve inherent challenges associated with catalysis by enzymes, such as substrate and product inhibition. Cells and enzymes also benefit from the improved mixing, mass transfer, thermal control, pressurized processing, decreased variation, automation, along with in-line product analysis and purification conferred through continuous flow. Thus, the combination of continuous flow and biocatalysis has emerged as a highly effective approach for creating diverse synthetic targets. Examples include immobilized enzymes and whole cells in continuous flow for the synthesis of pharmaceuticals, chemicals, and materials. Challenges to continuous-flow biocatalysis surveyed in this chapter include immobilization methods, the necessity to regenerate cofactors for cell-free biocatalysis, and optimization of biosynthetic steps with highly divergent conditions in flow.

10.1 Introduction

The use of enzymes in continuous flow, known as flow biocatalysis, is rapidly growing in popularity due to the many advantages, particularly in multistep reactions, with respect to batch conditions. Whether the enzyme(s) is immobilized or used in solution, the mass transfer is greater in flow with respect to batch, generally favoring the reaction rate. When the enzyme is immobilized and compartmentalized in a tube reactor, the catalyst loading accumulated in a small space (ratio of enzyme per substrate molecule at any given time) is exceptionally high, again dramatically increasing the conversion. Of course, the increase in reaction rate has no effect on the equilibrium of the reaction; therefore, if the catalyzed reaction has an unfavorable equilibrium due to a fast reverse reaction, in flow this will be simply reached sooner. In a flow setup, issues linked to product inhibition are also eliminated as the flow itself constantly removes the newly formed product. It is therefore easy to imagine how extremely beneficial flow biocatalysis can be when several enzymes are used in a cascade approach, where the product of the first reaction feeds directly into the second reactor containing the second enzyme, and so on (Fig. 10.1). No negative feedback would take place and, if the system is fully optimized, the conversion rate of each reactor can be synchronized to achieve maximum yields.

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Interestingly, it is also often noted that the (immobilized) biocatalyst used continuously presents a longer working stability when compared to its shelf life under static conditions, with packed enzyme reactors reusable for several weeks.

Fig. 10.1: General sketch of sequential reactors in a flow setup. The substrate is transformed into the product via and enzymatic cascade where the enzymes (E1, E2, etc.) are compartmentalized into separate reactors.

As for any technology, however, the application of enzymes in continuous flow presents some challenges and limitations [1–12]. While a single simple biotransformation (i.e., lipase-mediated ester hydrolysis) is relatively straightforward, as soon as the biotransformation requires, for example, a cofactor, therefore, a cofactor recycling strategy, or when multiple enzymes are assembled in line, the system will require extensive investigation of all variables and careful considerations of the benefits versus drawbacks of such a setup. A key issue is represented by the necessity of establishing a "compatibility window" of the enzymes involved in the cascade. Factors such as pH and buffer would ideally be kept constant throughout the cascade, though pH adjustments are possible with inlets upstream of each reactor. Therefore, it is possible that one or more enzyme could be utilized under suboptimal conditions in the cascade, limiting the overall efficiency of the system. Another potential problem, especially in the case of immobilized biocatalysts, is the solubility of the starting material. While slurries can be utilized (normally with flow reactors equipped with peristaltic pump systems), heterogeneous catalysts are inefficient and conversion yields tend to be poor, often worse than in batch.

The high degree of modularity of a flow apparatus opens up the possibility to utilize biocatalyst-filled reactors in new reaction pathways which are now almost exclusively dominated by standard chemical synthesis. It is important to mention that biocatalysis is new to the flow chemistry scene and has only relatively recently discovered the benefits offered by a continuous-flow setup. With respect to the abundance of systems, including industrial applications, reported for traditional chemical synthesis biocatalytic examples are still very limited. However, researchers have shown that flow biocatalysis enables multigram syntheses of high-value products with an exceptional ability to recover and reuse solvents and water wastes, reducing drastically the environmental impact.

10.2 Considerations for the design of CF biocatalysis procedures

10.2.1 Choice of biocatalysts

When performing a biocatalytic transformation, whether in batch or in flow, the choice of the catalyst is clearly important. Generally, key features such as the substrate scope, stability under operational conditions, and tolerance to suboptimal reaction environments (presence of solvents, shifts in pH, temperature, etc.) are good indicators of the robustness of the selected enzyme. In addition, the ease of expression yield and ease of purification, if needed, should be considered. Depending on the type of catalyst and its chemistry, considerations for the requirement of cofactors (and how to recycle), oxygen-rich or oxygen-free media, reaction equilibrium, and so on are also relevant to establish the preferred form of the enzyme (isolated or in the cell) and to evaluate the overall complexity of the reaction setup. On an analytical scale, for example to establish a proof of concept, the assembly of an elaborate system can be achieved more easily than on larger scale, and often this will impact the real implementation potential of a biocatalytic reaction.

10.2.1.1 Single-enzyme or multienzyme-based processes

When biocatalysis is carried out in flow, the first simple distinction is as to whether the reaction is a single step mediated by a single enzyme, or where a multienzyme system must be employed (Fig. 10.2).

Even a single step reaction may require a multienzyme system. This is the case for example when a cofactor is present and needs a coupled enzyme for its recycling, or if the equilibrium of the reaction is unfavorable and a coupled enzymatic reaction is used to shift it toward the formation of the product.

Clearly a single enzyme reaction, as mentioned before, will require only limited optimization, as the procedure will be simple. When more than one enzyme is needed however, the complexity of the system rapidly increases, with a decision making process that starts from the very basics, such as if the enzymes will be co-located in the same reactor (possibly co-immobilized on the same solid support), or if it is better for the overall process to keep them in different reactors, or whether the individual characteristics of one of the enzymes will yield to a hybrid system with some enzymes compartmentalized and others (generally only one) which may be added in soluble form with other reagents. Commonly, redox systems must be set up with an in situ recycling of the cofactor to minimize costs (this consideration applies to redox batch reactions as well), and while it is possible in some cases to exploit the main redox enzyme to recycle the cofactor as well as driving the intended reaction [13]. In other systems a second

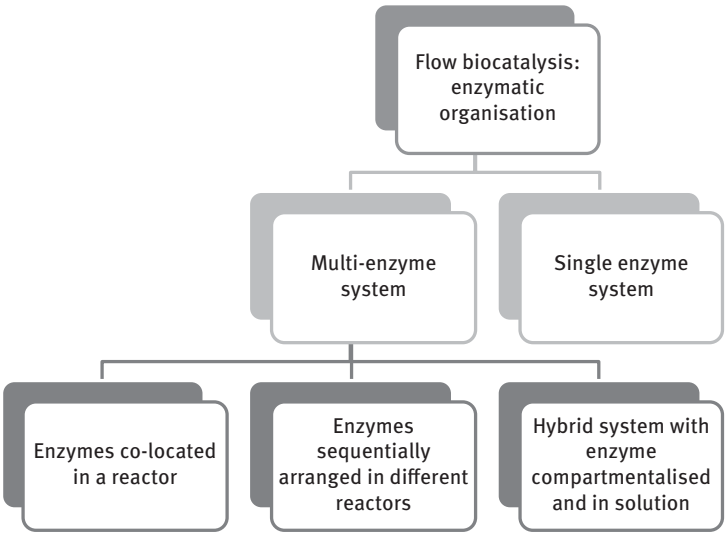

Fig. 10.2: Chart summarizing possible enzyme-based systems in a flow setup.

enzyme–substrate pair must be provided. Intuitively, the second enzyme in this case should be co-located in the same reactor to enable the use of catalytic amounts of co-factor. Co-location of catalysts can also be advantageous in the case of sequential reactions which may benefit from rapid transformation of an intermediate with poor stability, or in the case of two redox reactions which share the same cofactor [14].

10.2.1.2 Cell-free or cell-based: advantages and disadvantages

Both cell-free and cell-based flow systems have been successfully developed [9, 15], with advantages and disadvantages in both cases. These are summarized in Tab. 10.1.

Cells-based systems, normally bacterial or yeast cells, must be set up in such a way that the cells expressing the catalyst must be contained (either in membrane reactors, or immobilized) to enable recovery and simplify product purification. Cell-free systems, on the other hand, allow the catalyst to be added to the reaction bulk, either at the beginning or at some point in the cascade in the case of multiple reactors, but likewise they can be compartmentalized. A more substantial difference among the two systems is the access of the substrate to the biocatalyst. With a cell-free enzyme there is no physical barrier between the bulk of the reaction and the catalyst, in fact, the mass transfer is even facilitated under flow conditions with respect to batch. In whole-cell systems, the integrity of the cell membrane is essential for the retention of the biocatalyst(s) within the cellular space and diffusion across the membrane must be possible for both substrates and products. To enable such diffusion and then the enzymatic

Tab. 10.1: Advantages and disadvantages of cell-free and cell-based flow reactors[a].

Type	Advantages	Disadvantages
Cell free	– Cleaner system – Ready access to the catalyst – Faster flow rate – Higher substrate concentration	– Cofactors (if required) must be added in the media – Possible purification steps needed – Stability of the free form may be limited
Cell based	– Multiple enzymes can be expressed and contained in the same cell system – Cofactors are available within the cell system – Rapid preparation of the catalyst(s)	– Flow rate achievable is generally low – Permeability of the cell membrane/wall may be limited – Integrity of the cell structure and cell viability could be affected over time with the consequent loss of the catalyst(s) – Product recovery may be difficult – Side reactions due to cell metabolism

[a]Adapted from Pinto et al. [15].

reaction to take place, the flow rate that can be achieved is often very low. However, a clear advantage of whole cells is that they can harbor more than one recombinant enzyme, and once good expression levels are achieved, the cells can be simply spun down and utilized. If the reaction is carried out in buffer, this can also be supplemented with small amounts of nutrients to preserve cell viability. Enzymes within a cellular environment are also generally more stable, and this is particularly true for those enzymes that are membrane-bound; therefore, if the cell itself withstands the reaction media, reagents, and so on, it can be greatly advantageous not to extract the catalyst at all. In addition, whole cells are often preferred when the biocatalyst of interest require a cofactor, because these natural products are always endogenously available, and the addition of such expensive reagents can be avoided. In fact, whole cells spontaneously recycle cofactors which again streamline the setup.

In general, however, cell-free systems are more popular among researchers as they offer a higher degree of control with less variables. The quantification of the catalyst, for example, can be very precise, because this is treated like any other reagent, and therefore it is independent of the natural variability which can be observed in a living organism such as whole cells. Through immobilization (see later), the stability of the biocatalyst can be increased, and its retention within a reactor can be almost guaranteed.

10.2.1.3 Immobilized or not?

Immobilization strategies to anchor enzymes or whole cells expressing enzymes to a solid matrix are extensively used in flow biocatalysis because they provide the easiest

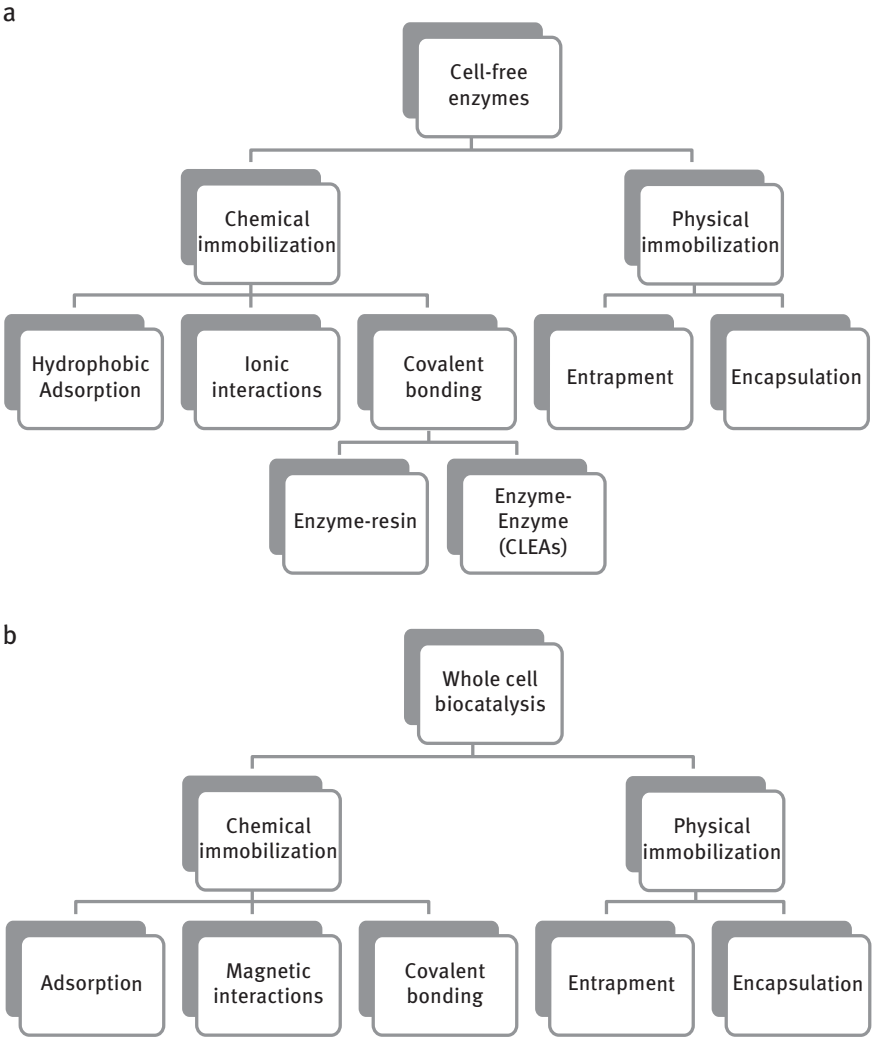

Fig. 10.3: Immobilization strategies for cell-free systems (a) and whole-cell biocatalysts (b).

way to contain a biocatalyst within the defined space of a reactor (Fig. 10.3, panels A and B) [16, 17].

A number of techniques have been developed over the last few years, in particular for cell-free enzyme systems. Enzymes can be covalently or noncovalently attached to a support which is then used to pack a reactor, or on the surface of the reactor itself (normally this approach is suitable for microfluidic systems where the narrow channels would not accommodate a solid resin), or on magnetic nano- and microparticles [16]. Whole cells can also be immobilized, the most common approach is to trap them in hydrogels which preserve their viability and, as it is done for immobilized enzymes,

the matrix can be used in a packed-bed reactor. The ability of whole cells to form biofilms can also be exploited in microfluidic devices where the channel can be coated with a cell layer yielding a large active surface area which favor access to the biocatalyst.

Cell-free biocatalysts greatly benefit from immobilization because the close contact with a matrix (which can be carefully selected to offer a beneficial micro-environment), independently of the immobilization method, confers enhanced stability to the enzyme which can be used for longer period of times, often improving also thermal properties and solvent tolerance [18]. No optimal strategy for immobilization is known however, and each catalyst requires generally a series of trials before a suitable approach, better than alternative protocols, is selected, and this can be quite time consuming. Immobilization of cell-free enzymes has also drawbacks such as the possible obstruction of the active site if the anchoring is nondirectional, reduced activity of the catalyst if excessive rigidification is imposed (typical of covalent immobilization strategies), and possible interaction of the matrix itself with the reagents (often observed between hydrophobic resins and aromatic, hydrophobic reagents). Finally, if the immobilization is not covalent, the possibility of slow leaching of the catalyst, promoted overtime even by the sheer action of the flow itself, and consequent loss of activity in the reactor, must be considered. In whole-cell immobilization, these drawbacks are much less relevant as the catalyst is protected within the cell itself.

Enzyme loading: amount of biocatalyst added to the reactor (for immobilized biocatalysts is better to specify also the matrix loading: $mg_{biocatalyst}\ g_{matrix}^{-1}$).

Enzyme specific activity: U mg_{enzyme}^{-1}, where the enzyme is in solution, normally under its optimal conditions.

Activity of the immobilized enzyme: specific activity of the support following enzyme immobilization, expressed per gram of matrix (U g_{matrix}^{-1}).

Enzyme productivity: g of product synthesized per g of biocatalyst employed in the flow reaction.

Bioreactor stability: biocatalyst activity over time

Depending on the specific requirements of a reaction, it may in fact be preferable to keep the catalyst in solution and recover it (via liquid–liquid extraction or affinity column) downstream of the reactor [9]. Likewise, whole cells, provided they can be efficiently separated at the end of the process, can be used in "soluble" form because this increases the access to the cytoplasm and to the catalyst [19].

10.2.2 Key design criteria

Designing a synthetic process involving biocatalytic steps under continuous-flow conditions require multiple considerations [3, 4, 6, 9, 11, 12]. Typically, a synthetic process targeting a product (TM) involves several steps which might be performed

with the aid of biocatalysts and by other chemical means (Fig. 10.4). Different parts of the whole process can be performed by biocatalysis and classical chemical methods and various (and not necessarily the same) segments of the full synthesis can be carried out under continuous-flow conditions. Consequently, designing and optimization of such a complex system is a demanding task. Even if considering only the biocatalytic parts, the increase in number of the enzymes involved in the process boosts the complexity of the system rapidly [20].

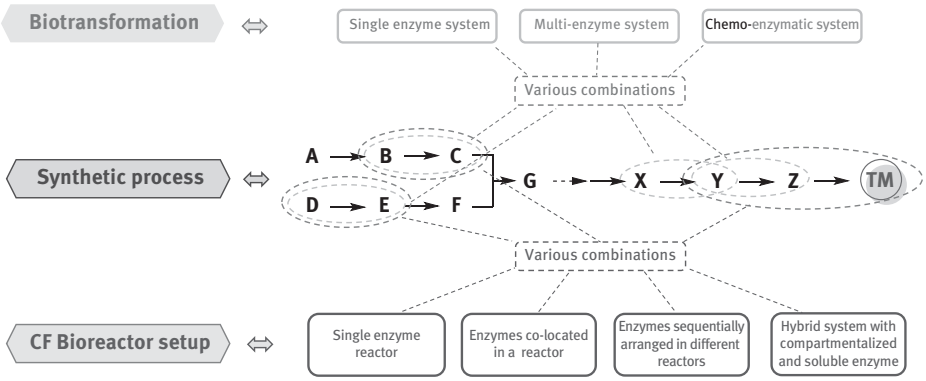

Fig. 10.4: Implementation of biocatalysis and continuous-flow bioreactor systems within a multistep synthetic process.

Even for optimization a single enzyme–based biotransformation, multiple issues should be considered. For example, remarkable differences exist between biotransformation with enzymes requiring no external cofactors (requiring no cofactor or acting with tightly bound and autocatalytically regenerating cofactor) and biotransformation with enzymes requiring external cofactors which should be retained and regenerated [21].

Before discussing the important optimization parameters of continuous-flow biocatalysis in detail, the differences between whole-cell and cell-free enzyme biocatalysis must be understood. Whole-cell biocatalysis uses the entire organism (such as *E. coli*) for biotransformation – even in continuous-flow mode [15] – whereas cell-free enzyme biocatalysis uses partially or fully purified enzyme recovered from the cell in soluble or in immobilized form. Understandably, influence of the operational parameters on the actual biotransformation depends not directly on the inherent properties of the single soluble enzyme but rather on the effective properties (kinetic parameters) of the enzyme in the actual form applied as a biocatalyst (Fig. 10.5) [22, 23].

Logically, optimization of a complex multienzyme system is challenging [24–28], because even in the case of a single-step single-enzyme biotransformation the various immobilization forms or reaction systems result in different effective parameters.

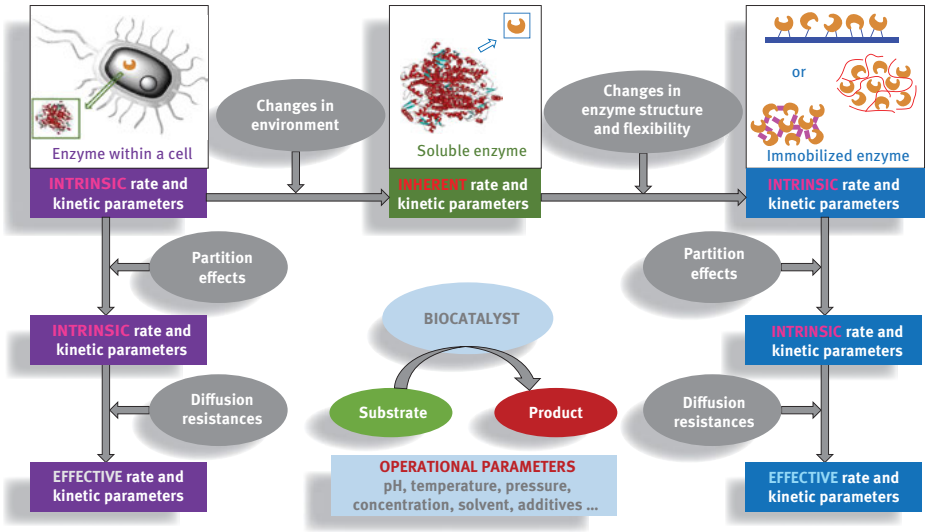

Fig. 10.5: Effect of the actual form of an enzyme applied as biocatalyst on the influence of operational parameters on an enzyme-catalyzed biotransformation.

Thus, when one changes the immobilization or reactor type of single unit in a multi-unit system or changes the usage of an enzyme from a single-enzyme unit to a multi-enzyme or chemoenzymatic unit, redetermination of the effective parameters may be necessary.

Bioprocess engineering is an essential part of biocatalytic processes in the context of sustainable industrial production of chemicals [1, 29]. In cells, enzymes rarely function in isolation, most often in reaction networks rather than in linear sequences. This allows the removal of inhibitory products and the shifting of unfavorable equilibria, and the coupled reactions of enzyme networks can also be used to regenerate cofactors or prepare substrates. Rigorous mathematical descriptions of microbial cells and consortia thereof will enable deeper biological understanding and lead to powerful in silico cellular models [30]. These levels of complexity need to be studied, understood, and modeled to decide when to use isolated enzymes or when to use whole-cell biocatalysis instead.

10.2.2.1 Operational parameters

Among the operational parameters influencing the outcome of biotransformation, pH and temperature are of particular importance. Further important parameters, especially in continuous-flow systems are the composition of solvent, the substrate

concentration, biocatalyst loading, flow rate, pressure. Last but not least, catalytic efficiency, selectivity, and stability of the biocatalyst are also key issues [1–12].

In many enzyme-catalyzed reactions, pH control is an important feature because most of the biocatalytic reactions occur in aqueous solution and the biotransformation consumes or produces acidic or basic species. Additionally, a large fraction of enzymes operates within a narrow pH window and therefore the pH control is of utmost importance. This feature influences strongly the reactor selection, and therefore whole-cell- and soluble enzyme–based processes are best operated in stirred tank reactors or membrane reactors when pH control is required [1].

A study of the continuous-flow kinetic resolution of three different racemic amines by variously immobilized *Candida antarctica* lipase B biocatalysts in the 0–70 °C temperature range on enantiomer selectivity and specific reaction rate indicated, that temperature effect depended significantly both on the substrate and on the mode of immobilization [31]. Alteration of the enantiomer selectivity in the kinetic resolutions of three differently flexible amines a function of temperature was rationalized by the various flexibility of the lipase in its different forms. The results indicated that the optimal method of immobilization depended both on the nature of the substrate and on the reaction conditions.

10.2.2.2 Kinetics

There are seminal reviews on the opportunities and challenges of using microfluidic methods in biocatalyzed processes with isolated enzymes or whole cells for both analytical and chemical synthesis [1–12]. Immobilization of biocatalysts is widely used in microreactor systems because it allows continuous operation, easy separation of product and biocatalyst, and often stabilizes the biocatalyst. However, immobilization of the biocatalyst can alter the activity of the enzyme due to conformational changes, steric hindrances (e.g., overcrowded or non-oriented binding of the enzyme, which causes diffusion restriction of substrates accessing and products egressing the active site), all resulting in changes in reaction kinetics (Fig. 10.5) [32].

Enzyme kinetics: Study of the rate of chemical reactions catalyzed by enzymes (which are usually but not necessarily protein molecules that convert other molecules, called substrates). The substrate molecules bind to the active site of the enzyme and are transformed into product(s) by a series of steps corresponding to the mechanism of the enzyme. Enzyme kinetics investigates the reaction rate as a function of changing reaction conditions (pH, temperature, substrate concentration, etc.).

Enzyme inhibition: When an **inhibitor** interacts with an enzyme, it decreases the enzyme's catalytic efficiency. An irreversible inhibitor binds to the enzyme's active site – most often covalently – producing a permanent loss of catalytic ability even if the inhibitor's concentration is decreased. A reversible inhibitor – usually by forming a noncovalent complex with the enzyme – decreases the

catalytic efficiency temporarily. If the inhibitor is removed, the enzyme's catalytic efficiency returns to its original level.

Substrate/product inhibition: Enzyme inhibition at elevated concentration of the substrate/product.

The immobilized enzyme is often present within porous polymer, ceramic, or silica particles [7, 16]. In such cases, the substrate must also diffuse through the porous medium to reach the enzyme. Thus, the diffusion resistance of the particles must be considered together with the external mass transfer resistance [33].

One of the main obstacles to biotechnological process design and control is the problem of measuring the most important physical, kinetic and biochemical parameters [34, 35]. There exist efficient experimental methods to determine kinetic parameters even within a microreactor [36–38]. Another option is to model and simulate processes within microreactors using state-of-the-art mathematical modeling techniques [39].

For modeling microbioreactors, where the intraparticle and external diffusion resistance are significant, multicompartment models are required to accomplish adequate model accuracy [35]. However, monocompartment models, in which the internal mass transport by diffusion and substrate conversion is considered, are still used in various applications due to the simplicity of the model [40]. In addition, substrate conversion is often studied only when enzyme kinetics approach first- or zero-order kinetics [33, 41].

The simulation approach allows to estimate certain kinetic parameters and to optimize the microfluidic reactor system with significantly decreased time and cost [35, 39, 40, 42].

10.2.2.3 Choice of reactor

Although there are variations in many implementations, size and geometry of the reactors applicable for biotransformations in continuous-flow mode, there exist two basic theoretical types of continuous-flow reactor: continuous stirred tank reactor (CSTR) and continuous plug-flow reactor (CPFR). A systematic study on reactor selection for continuous biocatalytic production of pharmaceuticals with focus on residence time distribution and on the unique mass balance affected by enzyme kinetics for each reactor type revealed that CPFR should generally be the system of choice [43].

In various microfluidics platforms mostly the CPFR-type microreactors are applied in different setups (Fig. 10.6) [44].

However, there are cases, such as the necessity of pH control as mentioned in Section 10.2.1, where they may need to be coupled with a CSTR or replaced entirely by a series of CSTRs, which can approximate plug-flow behavior. In this respect, continuous production in enzyme membrane reactors (EMR), such as in a cascade of continuously operated EMRs, can be considered theoretically as CSTRs [45]. In

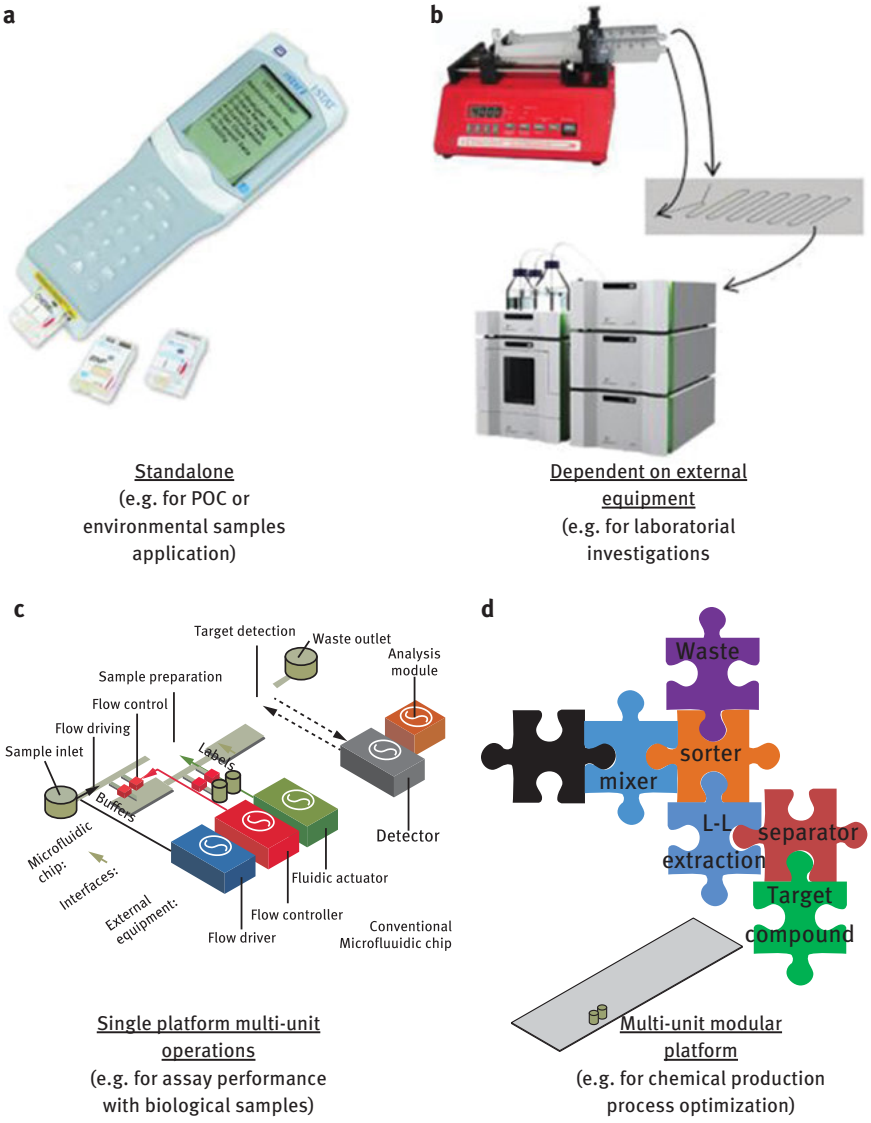

Fig. 10.6: Simplified representation of different types of microfluidic systems or platforms: (a) standalone platform (containing all the components required to carry out the target process or assay); (b) standard microfluidic system (that depends on certain external equipment such as pumps and analytical devices to carry out the target process or assay); (c) conventional approach to microfluidic platforms (miniaturization and integration of all the necessary unit operations in the same chip); and (d) modular approach to microfluidic platforms (integration of all the required unit operations as separate miniaturized unit operations that are interchangeable and replaceable by new units if needed). Adapted from Fernandes et al. [44]. *Copyright © 2018, Elsevier.*

practice, no reactor behaves ideally; instead, it falls within the mixing limits of the ideal CSTR and CPFR.

Analysis of a proposed end-to-end continuous manufacturing process for Sitagliptin, the active pharmaceutical ingredient of the leading dipeptidyl peptidase-4 inhibitor antidiabetic drug by using process modeling and optimization indicated the supremacy of a plug-flow microreactor-based process over the batch implementation [46].

(For related topics please see Volume 1, Chapter 3, Title: Technology overview/Overview of the devices)

10.2.2.4 Analytical methods and in-process control

Many optical, optofluidic [47–50], spectroscopic (such as UV [49], Vis [49], NIR [49], Raman [50], NMR [51, 52]), or MS [53, 54] and electrochemical [55] detection methods are available that allow monitoring a wide range of reaction variables (oxygen, pH, glucose, carbon dioxide, or specific reagents/products) online and real time (Fig. 10.7).

Devices exist for in-line monitoring of reaction conditions such as microfluidic flow rate [56, 57], temperature [58], pH [59], pressure [60], or oxygen [48]. Although monitoring operational parameters and reaction variables in cascade systems and individual reaction rates at different positions in the cascade system can significantly shorten process development time and provide a basis for quality-oriented design approaches, these methods have been used primarily in individual continuous-flow enzymatic reactions, and only very rarely in continuous enzymatic cascade systems [26, 34]. Since many detection methods depend on the appropriate chemical, optical, or spectroscopic properties that allow easy measurement, general detection methods may be of great interest. NMR integrated with continuous-flow systems opens the door to advanced reaction monitoring techniques that have a high level of information content in real time and may be applicable to self-optimize processes using one or more NMR methodologies [51, 52]. Integration of microreactors by chiral analysis and the detection of enantiomers in an unlabeled manner is an important step in the study of stereoselective biocatalytic transformations [61]. Regrettably, there are many methods to detect substrate and product concentrations which cannot currently be performed in-line. The existing in-line monitoring and detection tools can be integrated with various control units enabling highly automated control of the continuous microfluidic system [44].

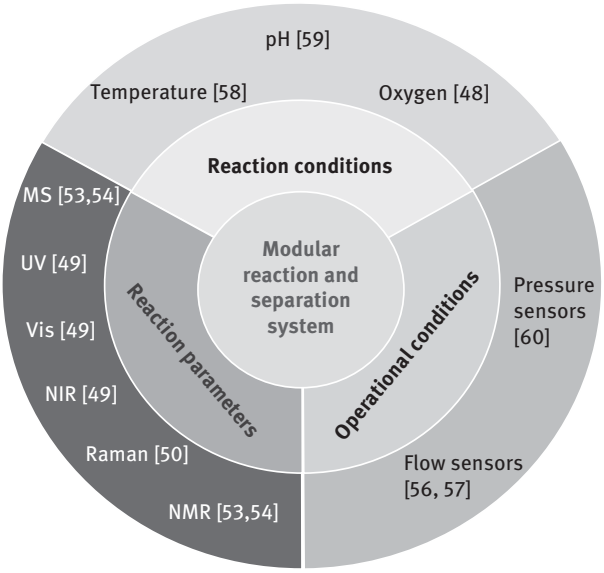

Fig. 10.7: Options for monitoring modular reaction or separation systems to improve control over reactions. The monitoring has to address reaction parameters (e.g., substrates and products), reaction conditions (e.g., pH and temperature), and operational conditions (e.g., flow rates and pressure). Several in-line spectroscopic methods and sensors have been developed and implemented in microfluidic systems which can now address this. Adapted from Gruber et al. [26]. *Copyright © 2017, CY BY 4.0.*

10.2.2.5 Optimization of CF reactors with enzymes

Continuous-flow implementation is an ideal method for efficient execution and computer-controlled optimization of chemical and biocatalyzed reactions due to its inherent advantages such as precise control of reaction time, temperature, and composition. The parameters of the various processes can be pre-programmed, each output can be analyzed automatically, and the protocol can be iteratively repeated or optimized. Selected examples from the vast number of documented optimization procedures indicate several possibilities and strategies of optimizing a single step continuous-flow biotransformation.

Optimization of operating temperature for continuous packed bed immobilized glucose isomerase reactor with pseudo linear kinetics was studied [62]. This optimization problem was based on reversible pseudo linear kinetics and the thermal deactivation of the enzyme and the substrate protection during the reactor operation was considered as well. This method involved the solution of two coupled nonlinear ordinary differential equations of the initial value type during a one-dimensional unconstrained optimization with bounds on the reactor operating temperature.

Design of experiments (DoE) was used to optimize the reaction conditions (pH value and temperature) in batch for optimization of the synthesis of a statin side chain precursor in continuous-flow continuous with immobilized deoxyribose-5-phosphate aldolase (DERA) [63]. In the DoE process the effect of the two crucial process parameters, temperature, and pH value were evaluated on intermediate and product formation in two cycles: (i) a rough, full-factorial lattice was designed for screening the process settings (temperature between 28 and 37 °C and the pH between 6.0 and 8.0); (ii) a fine full-factorial lattice was laid in the optimum of the response surface of the first cycle.

A two-step optimization was performed for continuous-flow hot water extraction and enzymatic hydrolysis using a thermostable β-glucosidase for determination of quercetin in onion raw materials [64]. First, the enzymatic hydrolysis was optimized [a three level central composite design considering temperature (75–95 °C), pH (3–6) and ethanol concentration (5–15%)]; followed by optimization of the hot water extractions from chopped yellow onions [a two-level design considering pH (2.6 and 5.5), ethanol concentration (0% and 5%) and flow rate (1 and 3 mL min^{-1}) at the optimal temperature for hydrolysis]. The optimized continuous-flow method (84 °C, 5% ethanol, pH 5.5, 3 mL min^{-1}) resulted in quercetin from onions in higher yield (e.g., 8.4 ± 0.7 μmol g^{-1} fresh onion) compared to a conventional batch extraction method.

10.3 Practical guide to CF biocatalysis

In this section some practical tips for approaching biocatalysis in flow will be discussed. It is intended as a basic guide for non-specialists to get an insight into the overall process. The fundamental steps to preparing a reactor, the initial setup of a continuous biotransformation, its optimization as well as product work-up will be also outlined.

10.3.1 Production of enzymes and cofactors

Enzyme production has been routinely done in biochemistry labs, but for a scientist with no previous knowledge in this field, even the idea handling a large biomolecule could be off putting. However, the process is relatively simple and in theory any research group, provided they can access to some basic equipment such as an incubator and a centrifuge, could obtain bespoke catalysts to work with. In fact, an ever-growing range of commercial enzymes can be purchased and trialed as any other reagent.

Most biocatalysts can be expressed recombinantly in laboratory strains of *E. coli* (the original gene coding for the target enzyme is cloned into a bacterial cell capable

of translating it into the protein), alternatively, bacterial and yeast cultures spontaneously producing sufficiently high levels of one or more biocatalysts can simply be grown in suitable media. Following successful expression of the enzyme(s), if the intention is to use whole cells for the biotransformation, the cell culture is spun down, rinsed in fresh buffer, spun again and the cell paste is ready for use. The enzyme can also be extracted from the cells (assuming it is not secreted into the media) by cell lysis (mechanical or chemical), separated from the cell debris by centrifugation and purified. Enzyme purifications are generally facilitated by the presence of an affinity tag which allows selective binding to a column, elimination of any other proteins, and elution. The catalyst is now ready for the next step. Cofactors are commercially available, but they can be expensive; therefore, all cofactors that are needed stoichiometrically, for example in a redox reaction, will need to be regenerated within the reaction so that they can be used catalytically, and the overall cost of the process can be contained. Recently, the possibility of co-immobilizing the cofactor with the enzyme or enzyme-pair to create a self-sufficient resin has also been reported and could be particularly advantageous for flow systems [65].

10.3.2 In situ immobilization

In Section 10.2.1.3, a general outline of the different types of immobilization for whole cells and cell-free enzymes was given. One practical aspect of the immobilization process for cell-free enzymes is that no matter the type of immobilization, it generally involves mixing the catalyst with an activated support, rinsing off the unbound enzyme, possibly additional chemical steps to terminate further reactivity, washing again, and final storage of the immobilized catalyst till it is needed. The reactor can then be packed at any time to start the flow reaction. However, it is also possible to pack a reactor with the clean resin and perform all the immobilization steps feeding all reagents sequentially directly through the flow system. This approach offers the advantage that there is no loss of resin through filtration, which is almost inevitable when the immobilization is done in batch mode. On the other hand, true continuous in situ immobilization is not efficient for all steps; normally the time required for the enzyme to bind to the resin ranges from 2 to 16 h (depending on the chemistry); therefore, loading the resin with the enzyme containing solution and stopping the flow for the required time is advisable.

When the cell-free enzyme or whole cells are bound directly to the surface of the reactor channel (wall-coated reactors), this can also be carried out in situ. The aim is to maximize the exposed catalyst surface by creating a monolayer or a micron-thick proteinaceous layer within the microchannel [32] which will favor smooth operation of the reactor afterwards. Cells can also be immobilized in high densities and methods have been developed which ensure that the cells remain stably attached over several days of continuous microreactor operation [66].

The stability of the immobilized catalyst under flow conditions must then be tested (generally with a standard reaction) to estimate the life span of the reactor.

10.3.3 Optimization of reaction under flow conditions

Example:
Batch reaction: 10 mL total volume, 10 mM substrate, 10 mg of enzyme, achieves full conversion in 10h.

Flow starting conditions: the reactor contains 10 mg of enzyme immobilized on 1 g of resin (occupying a volume of 2 mL), reaction volume in the reactor is 0.5 mL, same 10 mM substrate. A residence time of 30 min should give full conversion (likely much shorter residence time will then be found).

When the reactor is assembled, the system is ready to go. In a multienzyme cascade, normally each reactor is optimized separately and sequentially so that the next reactor can be tested with the optimal reaction mix generated by the previous one. It is always important to compare the efficiency of the system with the equivalent batch conditions. The expectation is that in flow, the rate of the reaction is enhanced significantly but to achieve this the correct residence time must be established. A good starting point is to take the batch reaction and translate it into flow considering the fact that the volume of the reaction exposed to the total catalyst is, at any given time, normally a fraction of the batch process.

With immobilized biocatalyst it is possible to further increase the rate of the reaction by increasing the temperature by a few degrees with respect to the optimal temperature established for the free enzyme without significantly affecting its stability. In the absence of in line monitoring equipment, the collected fractions need to be manually checked for product conversion. If the product has high affinity for the resin or the reactor material (more commonly observed with hydrophobic resins and hydrophobic substrates as mentioned), it is common to observe several initial fractions with an apparent lower conversion yield which then stabilizes as the process continues. The resin is at that point saturated with the product and a steady state of production is achieved.

If the substrate is excessively retained in the resin, this could negatively affect the efficiency of the enzyme. The use of a segmented flow could then be explored. Generally, biotransformations are carried out in aqueous buffers, but a second inlet could be used to inject a different solvent at specific intervals. The solvent should be capable of stripping the resin from the trapped product and the true yield of the reaction can then be calculated. The tolerance of the catalyst to the segmented flow should of course be tested in case that the enzyme loses activity more rapidly when in contact with the solvent.

10.3.4 Downstream processing and recycling

Flow technology allows for several downstream processing modules to be integrated in the overall process. This is already well known in flow chemistry and here some of the most used systems will be discussed. As mentioned, many biotransformations are carried out in buffers but it is often possible through acidification/basification inlets to change the protonation state of a product or an unreacted starting material so that it can be extracted in a solvent.

Liquid–liquid extraction module. Several processes have been reported which include a liquid–liquid extraction system. Normally, an inlet with a non-water miscible solvent is position right after the (last) reactor. The mixing of the two phases takes place within the junction and the separation follows (Fig. 10.8).

Depending on the size of the separator, different volumes can be handled. If a segmented flow is used, with an inlet of solvent upstream of a reactor, the relative volumes of the two phases may be sufficient downstream to enable efficient separation. In such case, no additional solvent inlet is required.

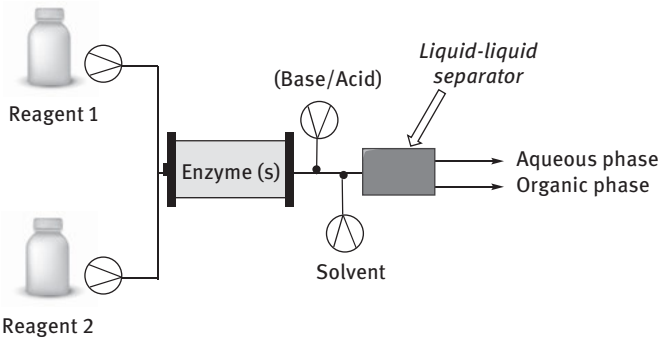

Fig. 10.8: Scheme showing the positioning of a liquid–liquid separator in a flow. apparatus.

Purification and scavenger modules. A range of columns containing differently derivatized resins (sulfonic, aminated, etc.) could be used downstream of a flow process, or even intercalated between reactors, to purify the product from unwanted by-products. The process is very simple and again routinely used in flow chemistry, the downside is that these columns not always can be regenerated and can be quite costly, but they allow complete automation of the system.

Recycling. Under flow conditions, the amount of reagents added in excess which is then lost downstream can be significant. This is particularly true in biocatalytic processes, especially those which require cofactors that are generally added in the mobile phase. It is possible, however, to recycle the water and/or organic phases, by simply feeding them back into the system, provided they can be "cleaned up" from unwanted molecules exploiting the same technology mentioned above. Cofactors are

water soluble and it is conceivable that they will remain in the aqueous phase following a liquid–liquid extraction. This phase can be fed back into the system, supplemented with fresh starting material and reused several times [67].

10.4 Examples of CF biosynthetic syntheses

Below a selection of different examples of biosynthetic reactions in flow will be presented. While this is by no means exhaustive, it should give an overview of the various approaches that have been developed at large scale in the past and at lower scale in the last few years.

10.4.1 Large-scale biocatalysis in CF

Biocatalysis has been applied in many applications as an alternative to chemical catalysis in various fields. The most striking examples of using enzymes in organic syntheses are the preparation of pure enantiomeric forms of various chiral drugs and the synthesis of important agents for the taste, food, and fragrance industries [68]. Biocatalysts are also used on a large scale to produce special and even bulk chemicals. Applicability is greatly influenced by the scalability of processes using enzymes in the production of industrial chemicals.

The very high activity of glycohydrolases (such as amylases, cellulases, and pectinases) has long been used for industrial purposes. Carbohydrate manipulating enzymes are widely used in the hydrolysis of various polysaccharides and in glucose production, now completely replacing acid catalysis. The use of glucose isomerase to produce fructose can be considered as a real biotransformation. This method using immobilized glucose isomerase (IGI) for production of high-fructose corn syrup (HFCS; 42–55% fructose, residual glucose and <4% oligosaccharides) at ~14 million t a^{-1} scale (dry weight) [69] is by far the largest scale synthetic biotransformation. The low catalyst consuming process (~0.05 g IGI kg^{-1} HFCS) is operated continuously in packed bed reactors at 60 °C with high efficiency [STY ~ 1 kg L^{-1} h^{-1} (calculated on dry HFCS)] [70, 71].

By using lipase biocatalysts in combination with proper acylating agents, kinetic resolution of racemic amines can be performed with high efficiency (Fig. 10.9). An example is the enantiomer selective acylation of racemic 1-phenyl-2-ethanamine according to the process patented by BASF [72].

The kinetic resolution of the racemic amine performed in continuous-flow mode using a column packed with immobilized lipase B from *Candida antarctica* and isopropyl 2-methoxyacetate as acylating agent resulted in one step a mixture of almost enantiomerically pure (*S*)-1-phenyl-2-ethanamine and the (*R*)-amide (Fig. 10.9). Large-scale

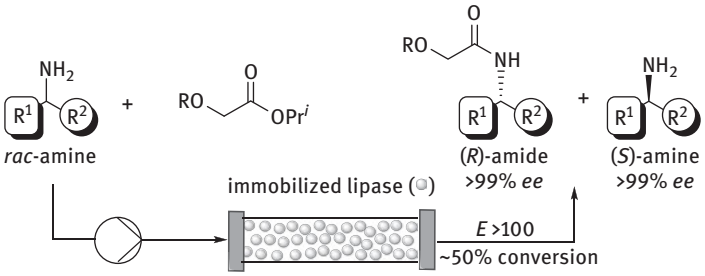

Fig. 10.9: Large-scale kinetic resolution of racemic amines with isopropyl 2-alkoxyacetates in continuous-flow mode.

production of the (*S*)-amine has been carried out in a cGMP-compliant plant (cGMP: current good manufacturing practices) of BASF on a scale of more than 1,000 t a^{-1}. In another BASF plant, 2,500 t a^{-1} (*S*)-(1-methoxy)-2-propylamine – being a synthesis intermediate of an herbicide – can be produced by this process [73]. Recently, the isopropyl 2-propoxyacetate among a series of isopropyl 2-alkoxyacetates was found to be the most efficient acylating agent in kinetic resolution of several racemic amines including racemic 1-phenyl-2-ethanamine under continuous-flow conditions [74].

Researchers of Pfizer developed a continuous enzymatic process for an efficient synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid at multikilogram scale using soluble D-lactate dehydrogenase from *Leuconostoc mesenteroides* (D-LDH) and formate dehydrogenase from *Candida boidinii* (FDH) (Fig. 10.10) [75]. (*R*)-3-(4-Fluorophenyl)-2-hydroxy propionic acid is a building block for the synthesis of Rupintrivir, a rhinovirus protease inhibitor. The production was performed in a 2.2 L continuously operated EMR (CEMR) using flow rate ratio of ~20 for recirculation versus filtration resulting in a residence time of 3 h leading to >90% conversion and high space–time yield (560 g L^{-1} day^{-1}). In a period of 9 days without adding fresh D-LDH and FDH, the enzymes lose their activity at a rate of only about 1% per day. The product was obtained with excellent enantiomeric excess (ee > 99.9%) and good overall yield (68–72%).

10.4.2 Examples of continuous-flow biotransformations

Whole cells in flow: early days. One of the first examples of biotransformations in flow was reported in 1990 by Lee and Chang [76] for the continuous production of acrylamide (Fig. 10.11). The immobilization of *Brevibacterium* sp. CH_2 whole cells, which displayed high nitrile hydratase activity and high tolerance to acrlylonitrile, was carried out in polyacrylamide gel and used to pack a jacked glass column. To maintain the enzymatic activity stable over time, the whole reactor and feeding reservoirs were kept at 4 °C. Due to the tolerance to adapted cell strain, the concertation of acrylonitrile starting material could be increased from 2% (used in biotransformation up until

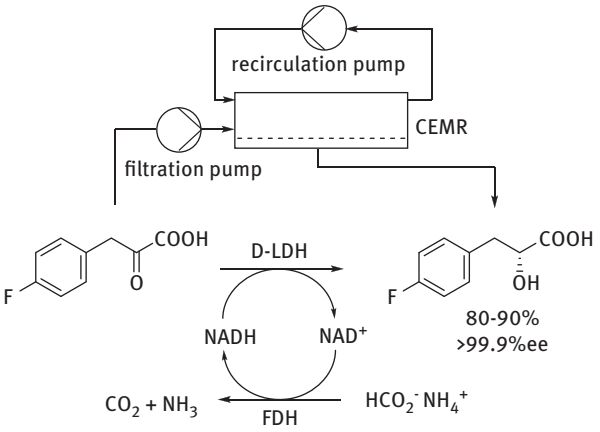

Fig. 10.10: Kilogram-scale production of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid by bioreduction in continuously operated enzyme membrane reactor (CEMR).

then) to 6%. To further increase the productivity of the system beyond the state of the art, the authors run two reactors in series with an additional feed of pure acrylonitrile before the second reactor. These allowed yields up to 55% with respect to the average 20% conversion achieved to date.

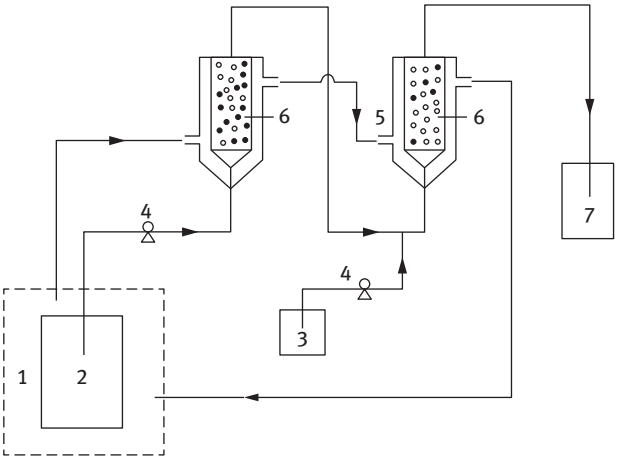

Fig. 10.11: Original scheme displaying a reactor assembly for acrylamide production in 1990. (1) Water bath kept at 4 °C, (2) 6% acrylonitrile solution, (3) pure acrylonitrile, (4) pump, (5) water jacket, (6) packed bed reactor, and (7) product collection. Adapted from Lee and Chang [76]. *Copyright © 1990, Springer.*

Many things have changed and evolved in the 30 years from this original paper with significant and unthinkable innovation at the time.

Self-sustainable flow biocatalysis with the recycling of "waste waters." Contente and Paradisi developed an original closed-loop platform of packed-bed flow reactors for the biocatalytic cascade synthesis of alcohols from amines based on enzyme covalent immobilization [67]. A transaminase for the haloadapted bacterium *Halomonas elongata* (HEWT) and a suitable reductase (horse liver alcohol dehydrogenase – HLADH, or a ketoreductase from *Pichia glucozyma* – KRED) were covalently immobilized on suitable supports using, to set up two packed-bed reactors. While the HLADH could be used also to recycle the cofactor by adding ethanol to the reaction mix, the KRED required a coupled enzyme system (a glucose dehydrogenase from *Bacillus megaterium* – *Bm*GDH) which was immobilized separately and a mix-bed reactor containing both KRED and *Bm*GDH was assembled (Fig. 10.12). A continuous-flow serial connection of these reactors was used for the synthesis of high-value primary and secondary alcohols from a variety of commercial amines via a carbonyl intermediate. A liquid–liquid extraction followed by purification of the product in the organic phase allowed the isolation of the alcohol product in excellent yield and purity.

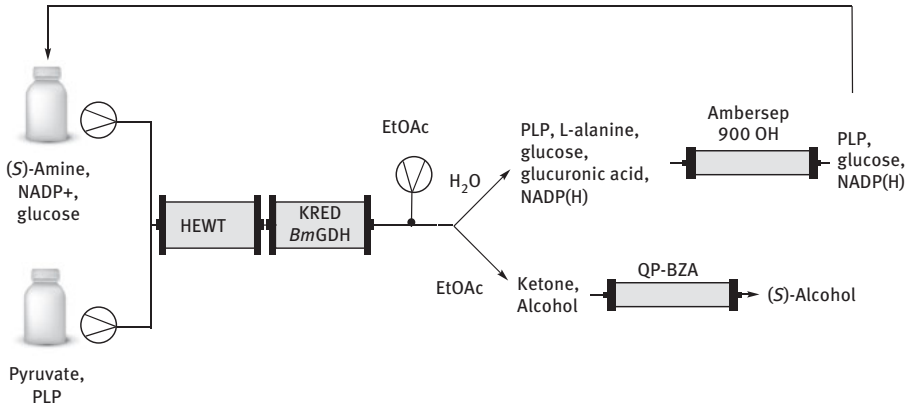

Fig. 10.12: Scheme showing multienzyme synthesis of chiral alcohols from amine with a transaminase followed by a redox reactor, with the in situ recycling of the cofactor, and reuse of the waste waters.

Of industrial relevance, the conversion of dopamine to hydroxytyrosol was achieved with 45 min residence time using a biphasic stream toluene/buffer and yielding 75% product recovery. Similarly, tryptophol and histaminol were synthesized from the corresponding biogenic amines tryptamine and histamine yielding 70% and 68% product recovery, respectively.

An additional feature of this work was the effort of recovering and reutilizing the wastewater stream which contained the cofactor. A scavenger column was introduced in line to partially purify the waters which could be recirculated for 20 times without further addition of the cofactor and virtually no loss of activity of either reactor.

Large-scale enzyme-based synthesis of melatonin. An example which shows the capacity to scale up the production of an enzyme-mediated reaction in flow was developed by Contente et al [77]. Here a highly productive biocatalytic reaction on a multigram scale for the continuous-flow synthesis of melatonin in an ultra-efficient closed-loop strategy was reported. The acetyltransferase from *Mycobacterium smegmatis* (*Ms*AcT) was covalently immobilized on glyoxyl activated agarose with a ratio of 1 mg $g^{-1}_{support}$ enzymatic loading, resulting in 73% recovered activity.

A packed-bed flow reactor was set up with just 1.6 g of charged resin and used initially for the continuous synthesis of *N*-(1-phenylethyl)acetamide from 1 M 1-phenylethanamine (120 g L^{-1}) in a biphasic flow system (5:15 aqueous phase/EtOAc), with 5 min residence time, allowing *N*-acetylation with 90% conversion. This covalent immobilization strategy significantly increased the enzyme stability in the presence of different esters and high temperatures. In addition, this covalent immobilized biocatalyst showed excellent operational stability, which allowed for an extensive utilization of the packed-bed flow reactor with an unprecedent amide production of 56 g day^{-1} using only 1.6 mg of MsAcT. This methodology was then successfully applied to the synthesis of melatonin from 5-methoxytryptamine (Fig. 10.13), as well as melatonin analogues, on a 0.5 M scale yielding very high molar conversions (up to 37 g day^{-1}).

As the acyl donor was EtOAc, this could be recovered following the isolation of the product and reused, once again closing minimizing the environmental impact of the process.

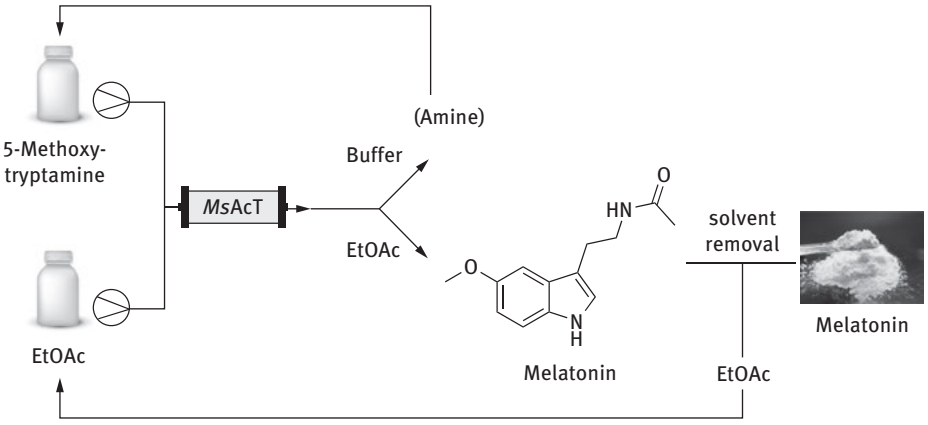

Fig. 10.13: Scheme of the flow setup for the synthesis of melatonin mediated by an acetyl transferase.

Chemoenzymatic continuous-flow dynamic kinetic resolution (DKR) *of amines by lipase catalysis.* Farkas et al. described a new chemoenzymatic system that allows the DKR of racemic benzylamines (*rac*-**1a–f**) in a completely continuous-flow manner (Fig. 10.14) [78].

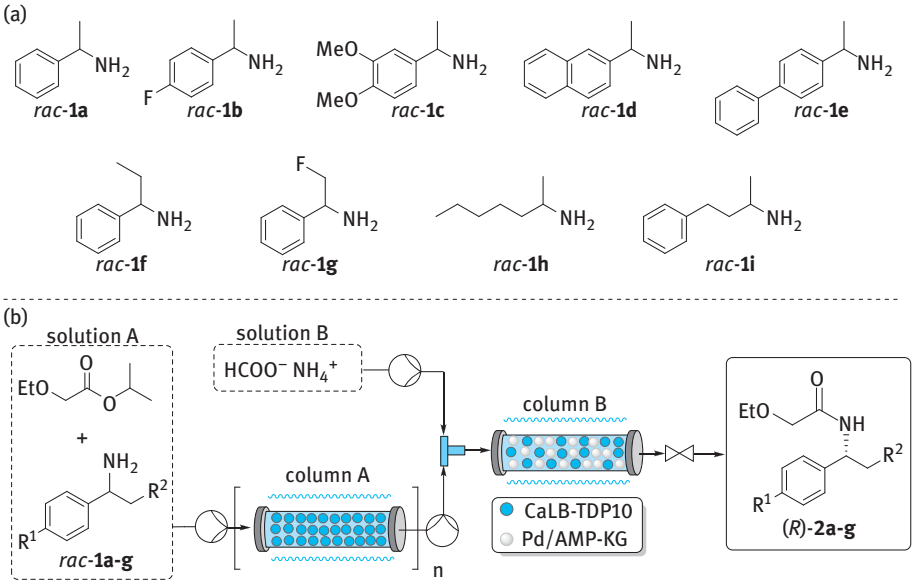

Fig. 10.14: Continuous-flow dynamic kinetic resolution of racemic amines by lipase-catalyzed acylation with isopropyl 2-ethoxyacetates. Adapted with permission from Farkas et al. [78]. *Copyright © 2018, American Chemical Society.*

For the DKR process, kinetic resolution with a robust, sol–gel matrix-immobilized form of *Candida antarctica* lipase B (CaLB-TDP10) was combined with palladium fixed on aminopropyl-functionalized silica (Pd/AMP-KG) and ammonium formate hydrogen source performing mild racemization. This DKR system, which contains ammonium formate completely dissolved in 2-methyl-2-butanol, could be operated at medium–high temperatures (60–70 °C). The study showed the racemization process only be compatible with benzylamines that do not have an easily reducible function without a reduction side reaction. The optimized DKR system consisting a purely KR unit (columns ($n = 1$ or 2) packed with CaLB-TDP10) and a mixed bed DKR unit (one Pd/AMP-KG + column packed with CaLB-TDP10) could be successfully applied to convert six valuable benzylamines (*rac*-**1a–f**) to (*R*)-amide [(*R*)-**2a–f**] in medium or high isolated yields (57–96%) with excellent enantiomeric purity (>98.8%).

Continuous-flow system comprising a capillary microreactor coupled to an extractor for kinetic resolution of acylated amino acids. An example of systems integrated with

downstream units is the full kinetic resolution *N*-acetylamino acids in a capillary PFR – microextractor system containing cross-linked enzyme aggregate (CLEA) of aminoacylase co-cross-linked with polylysine (Fig. 10.15) [79].

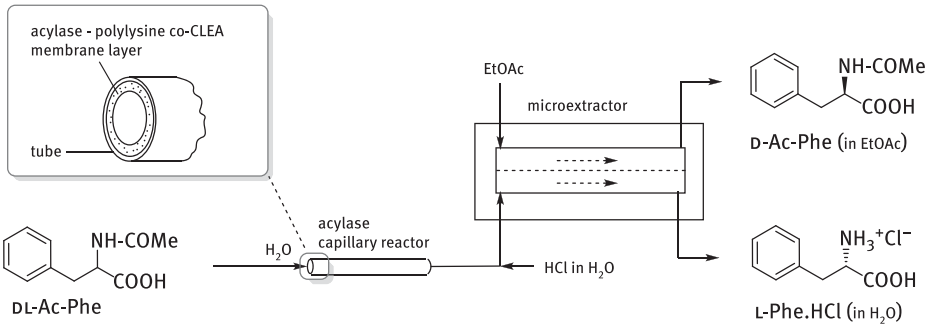

Fig. 10.15: Continuous-flow system for kinetic resolution of racemic amino acids (Honda et al. [79]). First, the L-enantiomer in the racemic substrate (4 mM) is converted by enantiomer selective hydrolysis into an amino acid through the tubing acylase-reactor. After acidifying with 0.2 M HCl the acetyl-D-Phe in the acidic aqueous phase is extracted selectively to the ethyl acetate phase flowing along the silicon-side in a microextractor.

The performance of the acylase-capillary PFR was evaluated with racemic *N*-acetyl phenylalanine (Ac- D,L-Phe, 1 mM) at a flow rate of 1.0 µL min^{-1} yielding the L-isomer in high enantiomeric purity (99.2–99.9% ee). The full KR of Ac-D,L-Phe and six further *N*-acetylamino acids were performed in this integrated system at a flow rate of 0.5 µL min^{-1} in the capillary PFR giving the L-isomers in 92.9–99.7% ee and 38–48.6% yield.

Biotransformation of propargylglycine by PAL in continuous-flow magnetic nanoparticle-based system with in-line UV detection. In microfluidic systems, protein-coated magnetic nanoparticles (MNPs) can optionally either co-flow with the fluid or even anchor with a magnet at defined site(s) letting to pass the free-flowing fluid through the regions of the anchored MNPs. Enzyme-coated MNPs can be fixed at certain positions in a lab-on-a-chip system using a permanent magnet (Fig. 10.16). In this microfluidic system called "Magne-Chip," multiple magnetic cells can be used for biotransformation, analysis, or protein biochemistry studies. Weiser et al. applied this microfluidic reactor equipped with in-line UV detection to study the mechanism of phenylalanine ammonia-lyase (PAL) immobilized on MNPs [80]. With this "Magne-Chip" system, it was first demonstrated that the acyclic, nonaromatic L-propargylglycine can be converted to (*E*)-pent-2-en-4-ynoate by PAL catalysis. This reaction strongly questioned the idea of a mechanism for the PAL reaction assuming a Friedel–Crafts-type attack on the aromatic ring of the substrate.

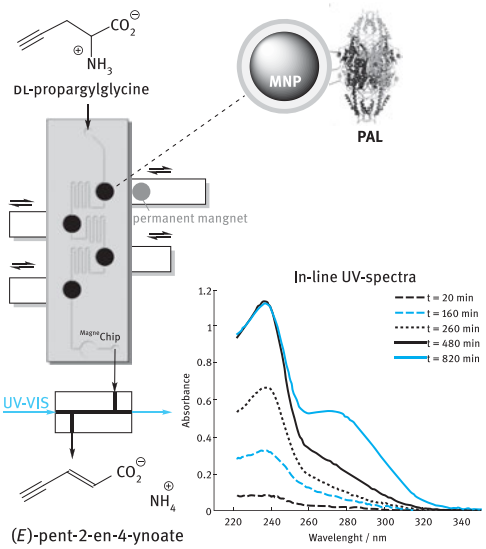

Fig. 10.16: Ammonia elimination from DL-propargylglycine in a light-protected microfluidic reactor with multiple magnetic cells filled with PAL immobilized on MNPs and equipped with an in-line UV/vis detector (reaction in D_2O at pD 8.8, 37 °C). Adapted with permission from Weiser et al. [80]. *Copyright © 2015, Wiley-VCH Verlag GmbH&Co. KGaA.*

10.4.3 More illustrative examples

Huffman and coworkers (Merck & Co.) reported in 2019 an outstanding approach to the synthesis of Islatravir, a nucleoside analogue used in the treatment of HIV [81]. which traditionally required between 12 and 18 steps, and can now be achieved exclusively enzymatically in fewer than half the steps with just five key enzymes (plus an additional four auxiliary ones) (Fig. 10.17). The enzymes were heavily engineered to ensure a compatibility window throughout the process, and some had been also immobilized to facilitate filtration from the reaction. The steps were run sequentially which maximized the yield.

This cascade would be an ideal candidate to be moved into flow, many of the challenges have been already addressed and flow could offer a further level of automation.

The possibility of using a multienzyme system of comparable complexity under flow conditions is indicated by an early report on cascade biocatalysis. Seven enzymes involved in the biosynthetic pathway were immobilized via His-tags to Ni-NTA agarose resin for the continuous production of uridine diphosphate galactose (UDP-galactose) from galactose and uracil monophosphate [82]. The multienzyme

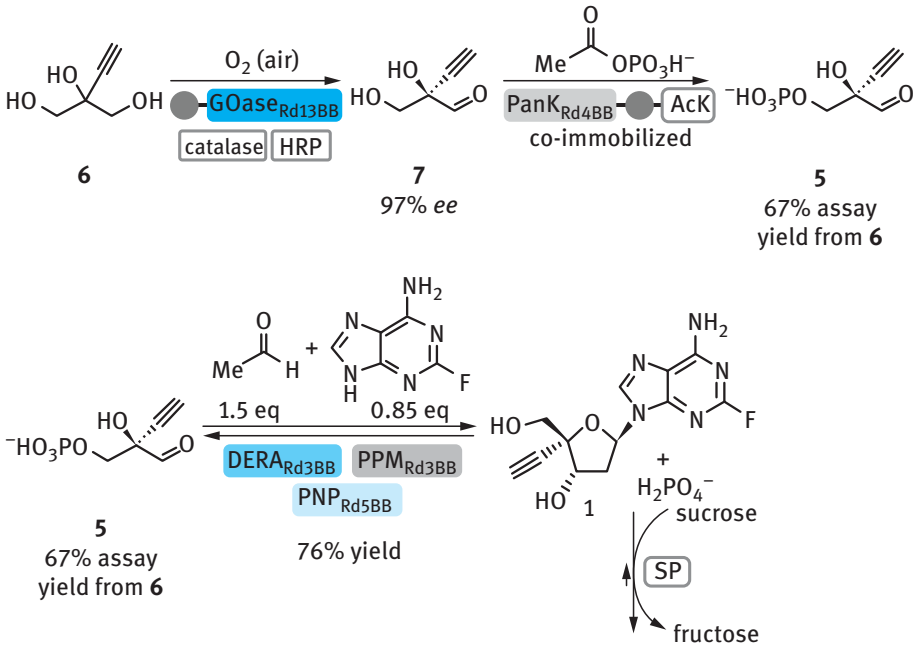

Fig. 10.17: Fully assembled biocatalytic pathway of Islatravir. *Copyright © 2019 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works* [81].

resin was packed into a column to perform the bioconversion from galactose to UDP-galactose with an overall conversion of 50% by recirculation of the reaction mixture through the column over a period of 48 h.

10.5 Challenges and future opportunities

The challenges in continuous-flow biocatalysis and future opportunities of microfluidics-based enzyme-catalyzed applications are well reviewed [4–6, 11, 83, 84].

Performing biocatalytic transformations in continuous flow has the potential to assist in bioprocess intensification in many areas from academia to industry. Biocatalytic process development comprising enzyme/cell engineering and screening, substrate, medium, and operational parameter optimization, as well as reactor engineering and further process integration significantly benefit from the implementation of microflow processing. However, the scope and efficiency of current examples leave room for significant improvements, particularly with respect to catalytic efficiency and stability of the biocatalysts, ISPR, and implementation of multistep reactions.

Increasingly efficient and smart enzyme immobilization strategies continue to be developed, along with the expanding number and kind of available biocatalysts. Eventually, the realization of biocatalysts in flow with immobilized enzymes needs to surpass the trial-and-error approaches and evolve toward immobilization engineering [85] applying rational designs that fulfill the process requirements [16]. Importantly, the use of immobilized enzymes in microfluidic bioreactors further extends the applicability of biocatalysis by enabling creation of "plug-and-play" biocatalyst systems. The multiple "off-the-shelf" enzymic units can be assembled into cascade systems and be integrated with other enabling technologies [26].

Implementation of multienzyme cascade systems benefit from automatization of microfluidic devices, integration of in-line analytical tools, and emerging use of mathematical tools such as DoE, data analysis, and process simulations. This is expected to significantly improve the impact of biocatalysis on industry and medical applications. Creation of compartmentalized cascade reactions in continuous flow enabling end-to-end processing will have a significant impact on sustainable manufacturing of biobased products.

10.5.1 Upscaling and integration

Instrumentation of the continuous-flow processes depends on the number of molecules to be handled/synthesized and on the amount of the required compound(s). The examples in Section 10.4.1 and the literature survey for biocatalytic processes implemented so far indicated that instrumentation pattern of the biocatalytic processes implemented in continuous flow still follows the general trend for continuous-flow syntheses in pharma industry as summarized by Wheeler et al. in 2007 (Fig. 10.18) [86].

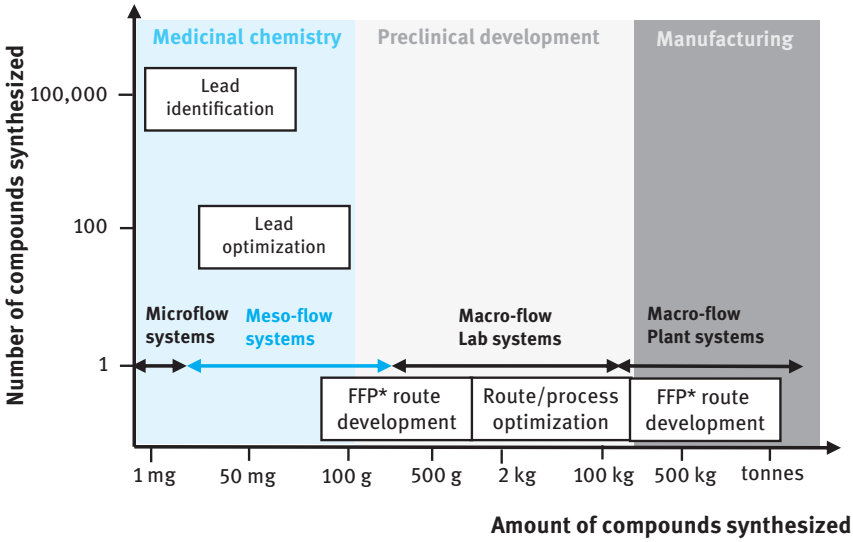

Fig. 10.18: Instrumentation of continuous-flow processes involved in the development of a drug (*FFP: Fit For Purpose). Adapted with permission from Wheeler et al. [86]. *Copyright © 2007, American Chemical Society.*

The typical tasks for biosensing, high throughput screening of enzymes or substrates, and bioprocess development experiments (information driven applications) can be best addressed in small-scale/high compound number cases by using microfluidic systems or in smaller number of compounds cases by applying larger-scale meso-flow systems. When the task is to develop an industrially relevant technology for production of a single target compound (product-driven applications) one of the two possible upscaling strategies should be selected [87]. Most often the *scale-out* concept (the extension of product collection time or increase of the length or size of the micro-/meso-flow reactor) is applied for scale-up. Alternatively, the *numbering up* principle (parallel operation of many identical micro-/meso-flow reactor units) can be envisaged. However, from a process engineering point of view this strategy is far from ideal because identical flow properties should be provided for each individual flow system, the system requires highly complex online monitoring and replacement of the exhausted biocatalyst is more complicated.

This is especially obvious, when one considers integration of a single enzyme/single step unit with other units, such as with a premixing unit, an in situ product removal unit, an in-line detection unit, or other chemical or enzymic reactor units [26]. In the case of the parallelized solution multiple connections should be made between the numerous small reactors and the additional units which rapidly and unnecessarily increase the complexity of the system. All examples of continuous-

flow implementation of biocatalyzed processes at large scale (Section 10.4.1) utilized the *scale-out* concept and were implemented in macroflow plant systems.

The challenges and solutions of integration to chemoenzymatic or multienzyme systems were already discussed in the preceding sections.

10.5.2 On-demand fabrication of bioreactors

Recently, 3D printing in microfluidics has fascinated substantial interest due to the rapid development of commercial 3D printers [88–90]. A possible use of 3D printing in continuous-flow microfluidics is to create on-demand reactors, often mentioned as "reactionware." While 3D printing became common for continuous flow synthesis, the first 3D-printed continuous-flow biocatalytic reactor manufactured from Nylon Taulman 618 appeared only in 2017 [91]. The 3D-printed reactor channels were activated with glutaraldehyde for immobilization of an (*R*)-selective ω-transaminase (TA). The TA reactor was applied for kinetic resolution of racemic methylbenzylamine yielding 49% of the (*S*)-amine with >99% ee. Importantly, reactor stability was tested, and little to no effect on performance was observed over 48 h storage.

3D-printed "labware" has been used for high-throughput immobilization of enzymes [92], for cell immobilization applicable for propionic acid fermentation [93], or for enzyme immobilization after proper chemical modifications [94].

Another possibility for 3D-printing-based biocatalytic applications when a "bioink"- containing thermostable enzymes are used to create hydrogel-based scaffolds for various continuous-flow applications [95–97].

10.5.3 Machine learning in continuous monitoring and control

In recent years, there has been a growing interest in computer-aided syntheses. Achieving this is a serious challenge due to the large dimensions of the chemical and reaction space, even if we consider only processes involving purely chemical steps. It is an even greater challenge if the syntheses are to be performed in a continuous-flow mode [98, 99]. Although continuous flow offers a number of potential benefits, not all reactions are capable of operating continuously. Much of the consideration raised for computer-aided syntheses by purely chemical methods are also valid for biocatalyzed processes, and these computational methods are starting to enter the field of general and flow biocatalysis [1, 84, 100].

Further readings

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Study questions

10.1 What are the advantages and disadvantages of using cell-free enzymes or whole cells in flow systems?
10.2 What are the challenges of a multienzyme cascade in flow?
10.3 In redox system requiring a cofactor, how can we ensure that the cost of the process is contained?
10.4 What is the use of scavenger columns in a flow setup?
10.5 What are the differences between inherent rate and kinetics of an enzyme and effective rate and kinetics of a biocatalyst using this enzyme?
10.6 Which are the most important operational parameters of an enzyme-based continuous-flow reactor?
10.7 Which are the most important kinetic considerations for the biocatalyst in a continuous-flow reactor?
10.8 Which are the mostly used reactor types for biocatalytic reaction in a continuous-flow mode?

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