Feature article

Let the Biocatalyst Flow

Polona Žnidaršič-Plazl[1,2,*]

[1] *University of Ljubljana, Faculty of Chemistry and Chemical Technology, SI-1000 Ljubljana, Slovenia*

[2] *University of Ljubljana, Chair of Microprocess Engineering and Technology -COMPETE, SI-1000 Ljubljana, Slovenia*

** Corresponding author: E-mail: polona.znidarsic@fkkt.uni-lj.si*

Received: 11-04-2020

Abstract

Industrial biocatalysis has been identified as one of the key enabling technologies that, together with the transition to continuous processing, offers prospects for the development of cost-efficient manufacturing with high-quality products and low waste generation. This feature article highlights the role of miniaturized flow reactors with free enzymes and cells in the success of this endeavor with recent examples of their use in single or multiphase reactions. Microfluidics-based droplets enable ultrahigh-throughput screening and rapid biocatalytic process development. The use of unique microreactor configurations ensures highly efficient contacting of multiphase systems, resulting in process intensification and avoiding problems encountered in conventional batch processing. Further integration of downstream units offers the possibility of biocatalyst recycling, contributing to the cost-efficiency of the process. The use of environmentally friendly solvents supports effective reaction engineering, and thus paves the way for these highly selective catalysts to drive sustainable production.

Keywords: Microreactor, enzyme, flow biocatalysis, continuous process, process intensification, process integration

1. Introduction

The introduction of so-called "flow chemistry" in synthetic organic chemistry laboratories and also in industrial chemical production at the beginning of the 21st century brought a new paradigm in chemical processing. The availability of miniaturized flow reactors enabled the synthesis of complex molecules under controlled reaction conditions that yielded products of better quality and with fewer undesirable side reactions, as well as the ability to perform chemical reactions that are not possible in traditional batch operations.[1] Over the past two decades, microflow technology has matured from early devices and concepts to today's wide range of commercial devices and a variety of applications that also enable very efficient process analytics and control.[2] Microflow systems are now important tools in chemical processes, from single-step to end-to-end processing, from (photo)catalytic to separation processes, from (nano)materials synthesis to pharmaceutical and fine chemicals production, and in environmental applications. Recent guidelines in the production of fine chemicals and the pharmaceutical industry to replace batch by continuous processes have further spurred interest in the implementation of "flow chemistry".[3–5] Moreover, the quest to reduce the environmental factor, *i.e.* the E-factor (mass of waste per mass of product), which reaches the highest values in the fine chemicals and pharma industries, requires profound changes in production systems.[6]

Biocatalytic processes, along with continuous processing, have been identified as one of the crucial key areas of green engineering research for sustainable production in these sectors. They also play an important role in biomass valorization and circular economy.[7,8] Biocatalysts were already known decades ago as environmentally friendly catalysts operating under mild conditions and with very high regio-, stereo-, and reaction selectivity, making them ideal catalysts for green chemistry.[9] Nevertheless, it has been a major challenge to use these sensitive biomolecules and cells in harsh industrial environments, as they often need to convert non-natural substrates at concentrations several orders of magnitude higher than under natural conditions, and also require non-aqueous media for their solubilization. In addition, the frequently observed substrate or product inhibition, poor operational stability, and short shelf-life of biocatalysts prevented a wider application of biocatalytic processes in industrial production. However, in the last two decades, the understanding of protein and cell structure and function has improved tremendously. Genetic manipulations, metabolic

flux analysis, and the application of new techniques and materials for biocatalyst immobilization have led to unprecedented opportunities to develop more efficient and robust biocatalysts. Moreover, the use of novel solvents such as ionic liquids and deep eutectic solvents has led to efficient medium engineering that allows for more environmentally friendly production and high substrate availability.[10–12]

However, to achieve successful biocatalyst application, enzyme/cell, substrate, and medium engineering need to be complemented by reaction, reactor, and process engineering based on a thorough understanding of the reaction system and the specifics of the biological catalyst.[10–11,13] In this regard, new concepts of reactor and unit operation design that incorporate continuous operation and miniaturization also provide new opportunities for efficient use of novel biocatalysts.[14]

The traditional use of large stirred tank reactors operated in a batch mode, or in some cases packed bed or fluidized bed reactors with biocatalysts retained in the particles appears to be *sine qua non* of industrial biotransformations.[15] The implementation of microflow reactors in biocatalytic process development and operation has been much slower than for their chemical process counterparts.[14,16] Even the term "flow biocatalysis" was introduced in the scientific literature only a few years ago. The first review paper devoted to biotransformations in microstructured reactors written by Bolivar, Wiesbauer, and Nidetzky in 2011, reported a relatively small number of published studies in this field, mostly using dissolved enzymes, and the challenges of biocatalyst reversible immobilization.[17] In the last decade, flow biocatalysis with a special focus on micro- and mesoscale devices has gained increasing attention in the academic and slowly in the industrial community, as evidenced by several comprehensive review articles,[14,16,18–25] special issues of scientific journals, book chapters,[26–29] special sections at scientific conferences with industry participation (Biotrans, Flow Chemistry Europe, Implementation of Microreactor Technology in Biotechnology -IMTB, *etc*.), and specialized webinars such as the "Flow Biocatalysis" organized by European Society of Applied Biocatalysis in October 2020.

Although biocatalyst immobilization is gaining momentum by the application of novel materials and techniques,[23,24,26–30] the majority of industrial biotransformations are carried out in aqueous environments with dissolved enzymes or free cells, which is also reflected in a modest market share of immobilized enzymes in the overall enzyme market.[30,31] This is usually associated with additional immobilization costs and an often perceived decrease in biocatalyst activity related to either additional mass transfer limitations or biocatalyst deactivation.

Due to the typically low solubility of organic substrates in water, the natural and most common environment for biocatalysis, substrates are either engineered by varying the substrate structure, by adding an immiscible liquid phase (typically organic solvent), or in the case of a single-liquid phase, by applying organic co-solvents. To lower the E-factor, the reduced use of organic solvents has been considered in the last two decades, as well as the application of neoteric solvents, such as ionic liquids (IL) and deep eutectic solvents (DES).[6,10] Besides, non-conventional media typically used in environmentally friendly separation processes, such as supercritical CO_2 ($scCO_2$) and aqueous two-phase systems (ATPSs), are also gaining attention in biocatalytic processes and offer new opportunities for green biochemical production.

This feature article addresses the advantages of continuous microflow-based processes for the efficient utilization of non-immobilized biocatalysts, and for rapid biocatalytic process development. Recent achievements in microreaction technology involving dissolved enzymes or suspended cells in the presence of one or more fluids are discussed with an emphasis on the implementation of green solvents for more sustainable production. Process integration enabling the recycling of biocatalysts, as well as opportunities for analytics integration and capacity expansion will also be considered.

2. Microreactors With Biocatalysts in a Single Liquid Phase

The use of continuous operation in microfluidic devices offers several advantages over batch processing, especially when tuning of process variables can prevent biocatalyst deactivation. Most commonly used are simple tubes with diameters ranging from submillimeter to a few mm, typically used in analytical devices such as high-performance liquid chromatography (HPLC), or more sophisticated meander chips (Figure 1a), which are microfabricated from glass or various polymer materials. The intense mixing in stirred tank reactors required to transport substrates and products to and from the active site can lead to interfacial effects that can damage the biocatalyst,[32] while a high flow rate required for the same purpose in conventional plug flow reactors can lead to insufficient residence time for completion of the reaction.[33] This can be circumvented by the use of microflow systems, where μm-scale diffusion paths allow for very efficient mass and heat transport, the latter also allowing for very precise temperature control, which is very important for processes involving thermo-sensitive biocatalysts. Diffusion efficiency can be visualized by the dye distribution at the Y-shaped outlet of the microchannel of 12.5 mm length, 205 μm width, and 100 μm height, where the dyed and pure water were pumped separately into the Y-shaped inlets. As shown in Figure 1b,[34] laminar flow of aqueous methylene blue solution and water in the channel for 0.3 s (flow rate of 50 μL/min) resulted in moderate diffusion of the dye into the water (and *vice versa*), while 3 s residence time (flow rate of 5 μL/min) allowed diffusion throughout the entire channel.

The definition of process parameters such as fluid flow rate, enzyme and substrate inlet concentration, reactor geometry, *etc*. could be established based on mathematical modeling comprising transport phenomena and reaction kinetics.[35]

The advantages of moving from batch to continuous production using, among other devices, a microreactor with dissolved enzyme have been demonstrated for the production of the antidiabetic drug sitagliptin.[33] This chemo-enzymatic production, developed a decade ago by Merck and Codexis, is one of the flagship industrial uses of engineered enzymes, in which a highly efficient and solvent-tolerant amine transaminase was developed based on substrate walking, modeling, and mutation approach followed by directed evolution.[36] Replacing the environmentally problematic rhodium-catalyzed asymmetric enamine hydrogenation with a biocatalytic step resulted in a product with 99.5 % enantiomeric excess, a 10 to 13% increase in overall yield, a 53% increase in productivity, a 19% reduction in overall waste, elimination of all heavy metals, and a reduction in overall manufacturing costs. In addition, the enzymatic reaction could be carried out in multipurpose vessels, eliminating the need for dedicated high-pressure hydrogenation equipment. [33] The study on the multistep synthesis of sitagliptin monophosphate from chloropyrazine encompassed the design of a continuous end-to-end manufacturing process comprising microreactors and microseparators, and optimization of the biocatalytic step with dissolved transaminase based on a steady-state plug-flow model, taking into account enzyme recycling. Based on the evaluated optimized productivity and a comprehensive techno-economic analysis of this process, a net present value of $150 million over 20 years was calculated. Besides, an assessment of the environmental impact of the process demonstrated its sustainability with an E-factor of 53, which outperforms conventional pharma batch processes with a typical E-factor of 200.[33]

Another obstacle that is very often perceived in biocatalysis is the alteration of enzyme microenvironment by the reaction, which can lead to its deactivation. The product may inhibit enzymes or be toxic to cells, while the pH change affects not only the activity and stability of enzymes, but also ionization and stability of substrates, products, and other components in the reaction mixture. Besides, high substrate concentrations can inhibit the biocatalyst, which can be prevented by using a continuous stirred tank reactor with low steady-state substrate concentration. To address this problem in tubular reactors, Szita's group developed a "side-entry reactor" (Figure 1c) in which the principle of a fed-batch substrate feed strategy was efficiently introduced into a microflow reactor. When tested for the transketolase-catalyzed reaction of lithium hydroxypyruvate and glycolaldehyde to L-erythrulose, a 4.5-fold increase in outlet product concentration and a 5-fold increase in throughput were achieved compared to a single-input reactor.[37] Un upgraded version with the integrated optical pH sensors enabled not only monitoring of pH but also adjustment of this parameter *via* the side entries. As a result, the pH drop in the penicillin G acylase-catalyzed synthesis of 6-aminopenicillanic acid was significantly attenuated and the product yield was increased by up to 29% compared to the process without pH adjustment. This contribution represents a further step towards fully instrumented and controlled microfluidic reactors for biocatalytic process development.[38]

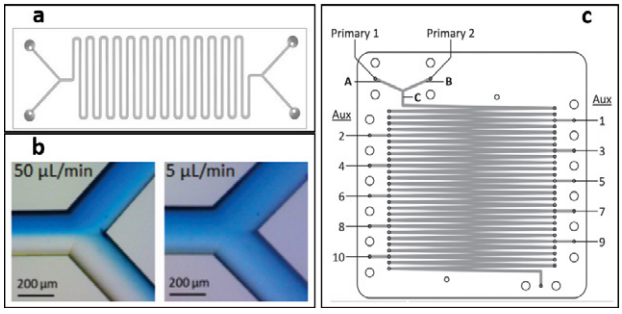

Figure 1: a) A Y-Y-shaped meander chip with 2 inlets and outlets; b) the outlet of the Y-Y channel presented in a) into which stained and pure water were pumped separately; the residence time in the channel was 0.3 and 3 s at the indicated flow rates of 50 µL/min and 5 µL/min, respectively; reproduced with permission from Miložič *et al.*, *Chem. Biochem. Eng. Q.,* 2014 28, 215–223;[34] c) a microfluidic side-entry reactor scheme with the indicated inlets (A, B), and side entries (1–10); all channels in the reactor had a cross section of 1 mm × 0.5 mm, and the length of individual sections of the main channel was 60 mm; reproduced with permission from Lawrence *et al.*, *Biotechnol. J.* 2017, 12, 1600475.[37]

3. Microreactors With Biocatalysts in the Multi-Liquid Phase System

The introduction of another liquid phase into the (typically aqueous) phase containing the biocatalyst opens, among others, the possibility of preventing its inactivation by compartmentalizing the inhibitor (substrate, product) from the biocatalyst, as well as shifting the reaction equilibrium toward product synthesis by *in situ* product removal.[19] Another important result of controlled flow in the microflow systems is the prevention of stable emulsion formation, which often hinders product isolation after multi-liquid phase processing in conventional stirred tank reactors.[39]

Liquid-liquid two-phase flow in microflow devices can be efficiently controlled, resulting in a variety of fluid flow regimes (Figure 2) and efficient transport between compartments. Since diffusion times are proportional to the square of the characteristic length, typical mixing times in microfluidic devices based on diffusion are in the range of milliseconds, which is several orders of magnitude better than in conventional reactors. The flow pattern in microscale channels is a function of operating conditions, such as flow rates, phase ratio and fluid properties. In addition, the flow is influenced by the roughness and

wettability of the channel wall, as well as by the geometry of the inlet channels (Y-, X-, or T-shaped) and the main channel diameter or aspect ratio for cylindrical or rectangular channels, respectively.[19]

Biocatalytic reactions within microflow systems typically involve various alkanes in addition to the aqueous phase. Among non-aqueous media, ionic liquids and recently also deep eutectic solvents (DES), both liquids consisting of ions with melting temperatures below 100 ˚C, are gaining increased attention in biocatalysis because they offer very high solubility of organic substrates. DESs are considered to be the fourth generation of ILs, although they do not consist entirely of ionic species.[40] While the synthesis of ILs requires chemical synthesis, often performed efficiently in microflow reactors,[41] DESs are prepared by simply mixing at least two inexpensive, nontoxic, and readily available components that are capable of self-associating in a specific molar ratio to form a new eutectic phase. The most typical compounds that constitute DESs are choline and urea, although amines, sugars, alcohols, polyols and organic acids are also used.[42] Both solvent classes are nonvolatile, nonflammable, highly viscous, and can be prepared in a plethora of variations, resulting in properties that can be tailored as needed, which also makes them attractive for application in biocatalytic processes.[40,42]

Aqueous two-phase systems (ATPSs) are another green solvents that are gaining importance in biotransformations. They are mostly used in bioseparations to integrate solid phase removal and extraction of the biomolecule of interest based on selective partitioning between phases. Typically, they are formed from two polymers such as polyethylene glycol (PEG) and dextran (Dex) or a polymer and an inorganic salt, e.g. phosphate and sulfate, dissolved in water, although some hydrophilic ILs are also capable of forming IL-ATPSs when mixed with aqueous solutions of inorganic salts.[43] They provide a benign environment for the biocatalyst along with the possibility of reducing substrate and/or product inhibition by compartmentalization in the other of the two phases. The industrial use of ATPSs is still hampered by their drawbacks such as slow diffusive mass transfer, long settling time for phase separation, and batch processing,[44] so processing in microfluidic systems present a promising tool for wider use of this green technology.[45] Recently, we reported the use of the microfluidics for the generation of a temperature-dependent aqueous micellar two-phase system (AMTPS) containing a surfactant in the time frame of a few seconds (Figure 2 d). The ability to change temperature almost instantaneously, and further integration with a microsettler and micro-ultrafiltration unit enabled sustainable and efficient purification of a high value-added value protein from algal biomass extract.[46]

The introduction of an additional liquid phase offers a wealth of flow patterns and attractive features that greatly expand the applications of liquid–liquid two-phase microfluidics. Three-liquid phase systems are widely used for various purposes, such as kinetic studies, microparticle synthesis, sample purification, and pharmaceutical crystallization. In addition to the parallel flow of all three phases (Figure 3 a2) and the generation of double emulsions (Figure 3 b2), which are of interest for the sorting of biocatalyst and other applications, a novel hybrid slug flow-laminar flow system (Figure 3 c2) was reported, where one layer is the laminar aqueous flow and the other layer is the slug flow. This flow was successfully stabilized by installing a partition wall between the two channels.[47]

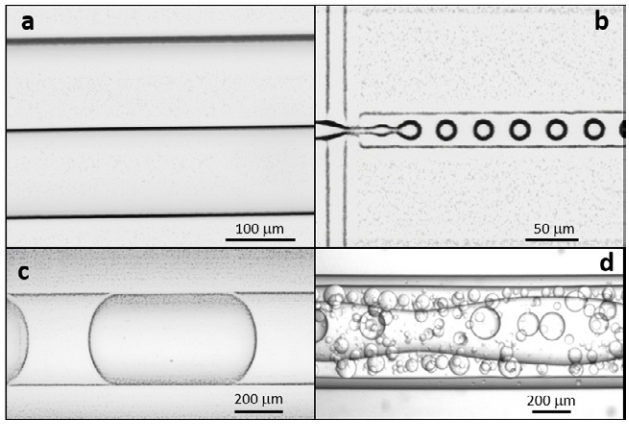

Figure 2: Typical liquid-liquid two-phase flow in microchannels: a) parallel flow of aqueous and organic phase, b) formation of water-in-oil droplets, c) slug or Taylor flow of the hydrophobic ionic liquid in aqueous phase, d) mixed flow of the aqueous micellar two-phase system described by Seručnik *et al.*[46] with core-annular flow and annular flow in the centre of the channel surrounded by droplets.

In the following chapters, the application of multi-liquid systems in biocatalytic processes will be highlighted.

3. 1. Microreactors With a Parallel Flow of Immiscible Liquids With Biocatalysts

Due to their small dimensions and low applied flow rates, laminar flows are typical of microflow systems in which immiscible liquid phases flow in parallel to form a stable and continuous interface through which mass transfer occurs.[47] The use of parallel flows has been achieved in microchannels with 2 Y-Y-shaped (Fig. 1a) or three Ψ- Ψ-shaped inlets and outlets. The main advantage of such processing is the possibility to separate the phases at the output of the microchips with multiple inlets and outlets presented also in Figs. 1a, 1b, so that no further phase separator is required. To achieve this, precise tuning of the flow rates of both phases is required so that the interface can be positioned in the middle of the channel, while exiting channels can be chemically modified to become more or less hydrophobic.

In a comprehensive review on enzymatic reactions utilizing non-aqueous media, several examples of enzymatic reactions with liquid-liquid (Fig. 2a and 3 a1) and liquid-liquid-liquid (Fig. 3 a2) parallel flow were given.[19] A pioneering work by Maruyama *et al.* on the environ-

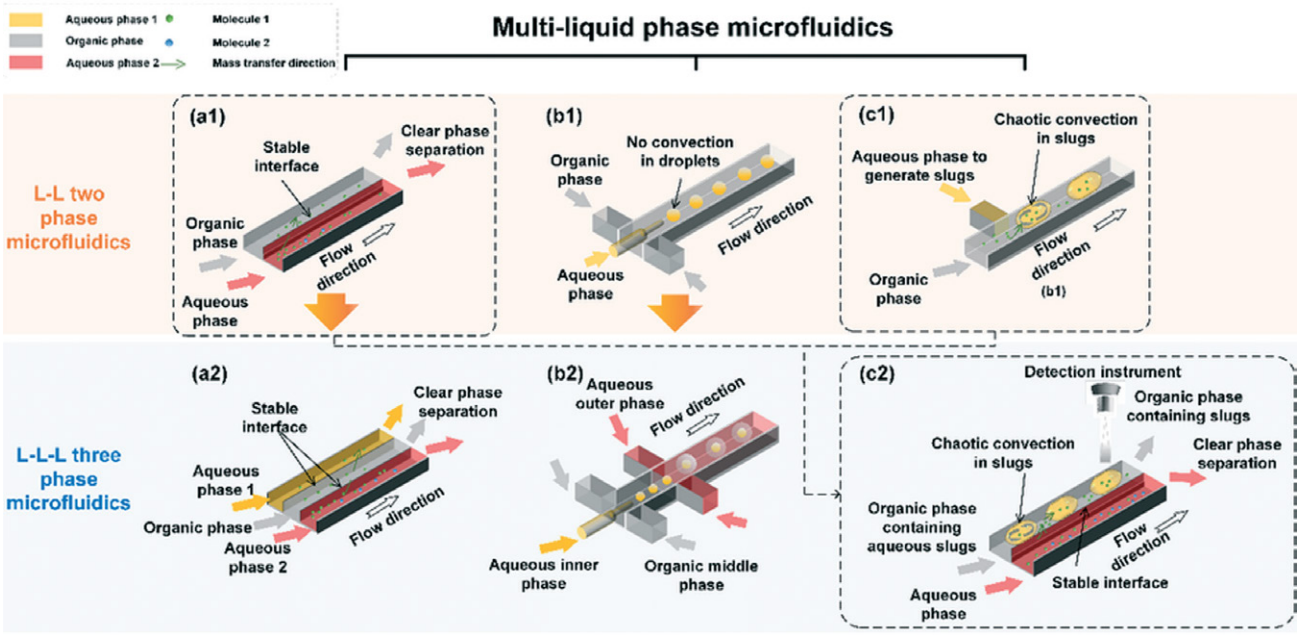

Figure 3: Schematic diagram and characteristics of multi-liquid microfluidics comprising liquid-liquid (L-L) or liquid-liquid-liquid (L-L-L) flow: (a1) L–L: laminar flow shown also in Fig. 1a (a2) L–L–L: three-layer laminar flow, (b1) L–L: droplet flow shown also in Fig.1b, (b2) L–L–L: double emulsions, (c1) L–L: slug flow shown also in Fig. 1c, and (c2) L–L–L: hybrid slug flow-laminar flow. Reproduced with permission from Wang *et al., Lab Chip* 2020, 20, 1891-1897, published by The Royal Society of Chemistry.[47]

mentally relevant laccase-catalyzed dechlorination of *p*-chlorophenol revealed 50-fold better specific productivity than in a laboratory-scale vessel with gentle shaking, achieving nearly 70% dehalogenation of the toxic substrate in 2 s.[48] In later studies, the most frequently used enzyme was *Candida antarctica* lipase B (CaLB), which acts at the interface of the two phases, so that in a parallel flow the reaction surface is well defined. This enabled very accurate modelling and reactor performance prediction for the esterification of isoamyl alcohol and acetic anhydride with substrate and product convection in the flow direction, diffusion in all directions, and reaction at the interface of the Y-Y- shaped microchannel.[49]

Along with aqueous buffers, alkanes are most commonly used as the second liquid phase in parallel flow.[19] The use of an ionic liquid as the second phase was demonstrated in the enantioselective separation of *(S)*-ibuprofen from a racemic mixture based on an enzymatic reaction. A thin film of ionic liquid between two aqueous phases with different lipases in each flow within the Ψ-Ψ-shaped microchannel provided a high interfacial area and processing time of only 30–60 s to achieve efficient enantioselective transport of this drug, which exhibits different pharmaceutical and/or toxicological effects depending on its optical purity.[50]

Urease-catalyzed hydrolysis in an aqueous two-phase system of PEG and Dex using parallel laminar flow in a Y-Y-shaped microfluidic device, schematically shown in Figure 4, showed a 500-fold increase in the apparent reaction rate compared to conventional ATPS in a beaker under gentle stirring. The very short residence time in the channel was increased by 4 consecutive reaction cycles, resulting in a 4-fold increase in conversion.[44]

A theoretical study of the enzymatic production of cephalexin, an important β-lactam antibiotic, using an ATPS based on PEG and phosphate in a microscale device comprising a thin dialysis membrane that provides flow stabilization and prevents transport of the enzyme and enzyme–substrate complex from the salt phase to the PEG phase. In the synthesis catalyzed by penicillin acylase, the effect of counter-current and co-current arrangements on cephalexin yield in microreactors with parallel flow of ATPS was discussed, as well as the possibility of transport enhancement by a direct-current (DC) electric field applied perpendicular to the interface. Based on the mathematical model comprising also mass transport across the membrane induced by an imposed electric field, the counter-current arrangement within the microreactor-separator was found to be suitable for cephalexin synthesis under most of the conditions studied.[51.]

3. 2. Microfluidics-Based Droplets With Biocatalysts

Droplet-based microfluidic systems, which use passive microfluidic structures to rapidly generate and manipulate subnanoliter-volume droplets in microchannel environments, have changed the paradigm of biochemical experimentation.[52] Compartmentalization of liquids into droplets within an immiscible carrier liquid, usually stabilized with a surfactant molecule, has been successfully ap-

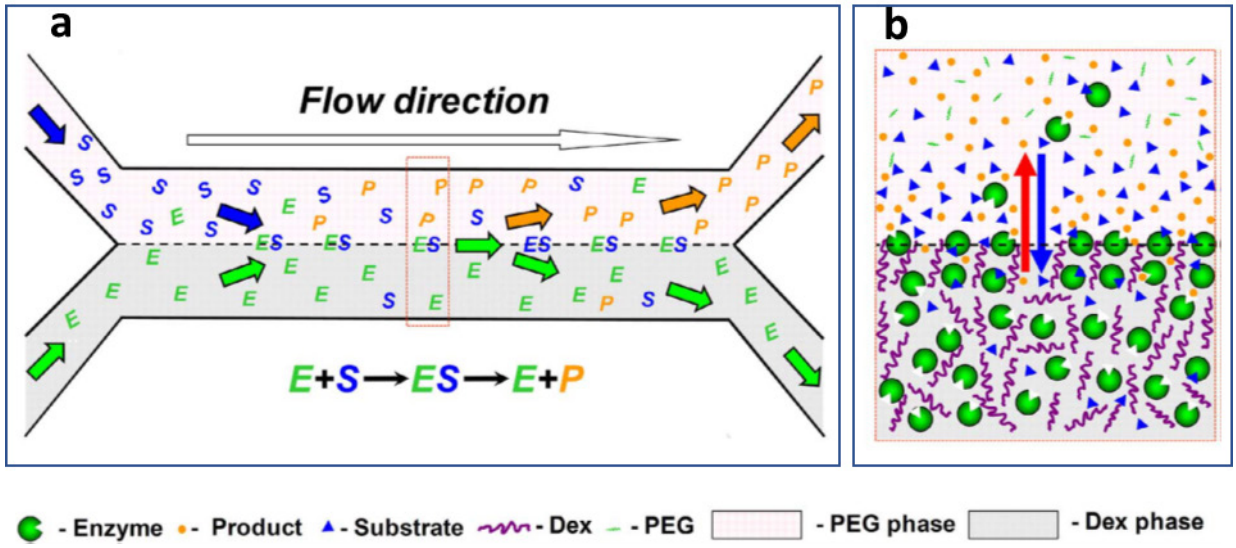

Figure 4. Schematic illustration of the ATPS enzymatic reaction and product separation in microchannel a) and at interface b) with a simple double Y-branched microfluidic device. Reproduced with permission from Meng *et al., Chem. Eng. J.* 2018, 335, 392–400.[44]

plied in numerous fields including single-cell and biomolecule analysis, diagnostics, drug delivery, protein crystallization, and chemical reactions.[22,52,53] Discussed herein are their applications in biocatalytic process development phases, as well as for process intensification.

3. 2. 1. Droplets in Biocatalyst Screening and Characterization

Selecting the most promising among the plethora of mutants generated by genetic manipulation or random mutagenesis is often the rate-limiting step in modern approaches to industrially relevant biocatalysts. Microfluidics-based ultrahigh-throughput screening of native or engineered enzymes and cells using droplets currently represents the most powerful tool for very rapid biocatalyst discovery and evolution at remarkably low cost.[22,52–55] Furthermore, microfluidic platforms developed for directed evolution of enzymes in droplets, allowing screening of 10^7 mutants per round of evolution, have revolutionized the area of enzyme engineering.[56]

Briefly, aqueous droplets in oil generated, as shown in Figs. 2b and 3 b1, at frequencies up to 2 kHz are capable of encapsulating a single enzyme or cell together with the substrate, which is often barcoded. After the reaction, which is performed during on- or off-chip incubation, the droplets are typically re-emulsified into water-in-oil-in-water droplets, as shown in Fig. 3 b2, and re-injected into the sorter and dispersed in an oil stream leading to the Y-shaped junction (Fig. 1b). Here, droplets are flowed into one of the channels, while those containing an active biocatalyst are selected by a detector and directed into the other channel. Most commonly, fluorescence-activated droplet sorting (FADS) based on laser activation is used.[52–57] As an example, a reliable and convenient ultrahigh-throughput screening platform based on flow cytometric droplet sorting (FCDS), shown in Figure 5, was demonstrated to efficiently isolate novel esterases from metagenomic libraries by processing 10^8 single cells per day.[58]

Further encapsulation of single cells producing an enzyme of interest in microfluidic-based droplets along with a fluorogenic substrate and optionally lysing agents ensures that product formation occurs in the same compartment as the catalyst-encoding gene. The fluorescent product-containing droplets can then be sorted using FADS enabling ultrahigh-throughput directed evolution.[59–61] As an example, a droplet-microfluidic screening platform was used to improve a previously optimized artificial aldolase by an additional factor of 30, resulting in a rate increase of over 10^9-fold .[59] Evolutionary units in the form of monodisperse double emulsions or gel-shell beads (GSBs) containing a protein mutant and its coding DNA represent further step towards extremely fast biocatalyst engineering.[62] Another ultra-high throughput protein screening platform called Split-and-Mix Library on Beads (SpliMLiB) was presented by Hollfelder's group. Directed evolution workflows were accelerated by DNA libraries constructed on the surface of microbeads suitable for direct functional screening in water-in-oil emulsion droplets with cell-free expression.[63]

To expand the application of this technique beyond non-fluorogenic substrates/products, assays based on absorbance are being investigated.[64] Future detection modes will include fluorescence-based approaches (anisotropy, Förster resonance energy transfer, lifetime) and label-free approaches based on light scattering (including Raman scattering) or droplet morphology.[55] Reports on the application of positive dielectrophoresis-based Raman-activated droplet sorting for culture-free and label-free screening of enzyme function *in vivo*,[65] and droplet sorting by inter-

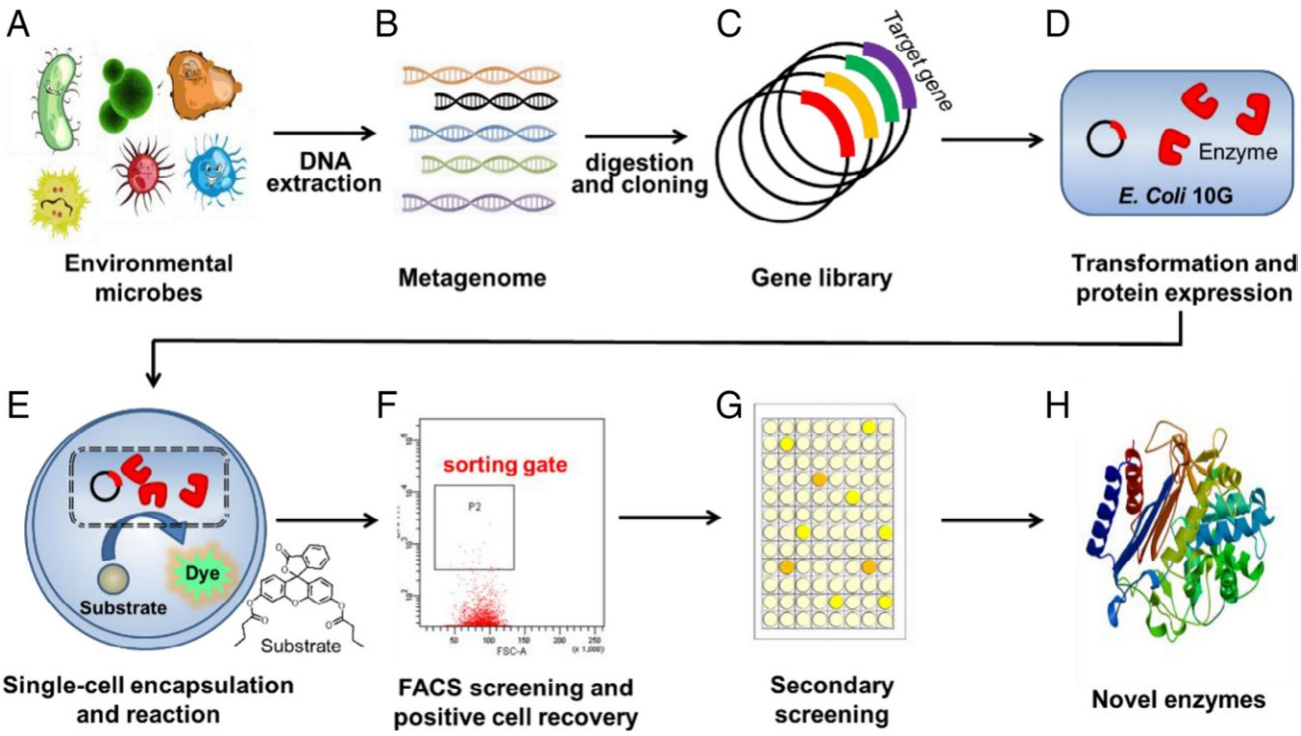

Figure 5 Workflow of the ultrahigh-throughput screening platform based on flow cytometric droplet sorting to mine novel enzymes from metagenomic libraries. A. Collection of environmental microbes. B. Extraction of metagenomic DNA. C. Digestion and cloning of metagenomic DNA into an expression vector. D. Transformation of recombinant plasmids into a host strain for encoded protein expression. E. Encapsulation of single cells into water-in-oil-in-water double emulsion droplets, along with the screening substrate. F. Flow cytometric analysis and sorting of positive droplets. G. Secondary screening based on 96-well plate assays. H. Identification of novel enzymes. Reproduced with permission from Ma *et al., Environ. Microbiol.* 2020, DOI**:** 10.1111/1462-2920.15257.[58]

facial tension[66] confirm these expectations. A sophisticated Raman-activated droplet sorting device uses periodically applied positive dielectrophoresis force to capture fast-moving cells, followed by simultaneous microdroplet encapsulation and sorting. The label-free method of sorting droplets by pH requires no active components and provides a robust platform for enzyme sorting in high-throughput applications.[65] Another promising approach in this regard is the coupling of droplet microfluidics with electrospray ionization – mass spectroscopy (ESI-MS), which provides a label-free high-throughput screening platform. The system also enabled effective *in vitro* transcription-translation within the droplets analyzed directly by MS, demonstrating opportunities to greatly accelerate the screening of enzyme evolution libraries.[67]

Few nL or even pL surfactant-stabilized monodisperse droplets can be regarded as moving reactors that allow an extraordinarily large number of experiments to be performed simultaneously. As they move along channels, the reaction in the droplets can be monitored e.g. *via* laser-induced fluorescence measurements of product concentration that provide a time-dependence of the reaction.[54] Because they consume minute amounts of reagents to provide the necessary information on reaction kinetics and biocatalyst inhibition, they significantly outperform conventional microtiter plates in terms of cost and time.[52,53] In addition, droplet microfluidics offers the potential to generate and analyze enormous amounts of kinetic data through a high degree of integration with detection modalities. Some excellent reviews of droplet applications comprising examples of controlled microfluidic systems used for e.g. automated analysis of enzyme kinetics, screening of protein crystallization conditions and protein solubility can be consulted for further information on this topic.[52,53,57]

However, the requirements for advanced droplet dispensing control and accurate sequential addition of samples or reagents to droplets at a high volumetric flow rate remain a challenging task. To address this, droplet array technologies have begun to offer a pathway to high-throughput screening.[52] After many years of intensive research, and despite the enormous potential for industrial use, few commercial applications have been developed, and significant development in the field is still needed to make them reliable and widely applicable.[68] There is a strong belief that high-throughput, high-sensitivity droplet-based microfluidics will become the gold standard for optimizing computationally engineered enzymes.[61] Exploration of 3-D printing technologies, robotics, and artificial intelligence is paving the way for smart platforms that could change the paradigm and drive the development of industrial biocatalytic processes.[22,52]

3. 2. 2. Droplets in Biocatalytic Processing

Microfluidics-based droplets are characterized by a very high surface-to-volume ratio that allows high mass transfer between the phase containing the biocatalyst and the phase containing substrate and/or product, so their use can lead to process intensification when the reaction is limited by mass transfer. The benefits of using microfluidics-based droplets were demonstrated in the bio-hydration of acrylonitrile to acrylamide using *Rhodococcus ruber* whole cells containing nitrile hydratase, which is one of the important large-scale biotransformations. Conventional processing in stirred tank reactors is hindered by the low aqueous solubility of acrylonitrile, the low concentration of free cells, limitations on external mass transfer resulting in reduced apparent reaction rates, and by the limited ability to increase impeller speed and thus mass transfer due to potential interfacial effects leading to cell damage.[32] To circumvent these problems, acrylonitrile was dispersed into small droplets of 25 to 35 μm using a specially designed membrane dispersion microreactor. This enabled approximately 30% higher product yield in 5-times less time and also proved to extend the life of the free cells.[69,70]

The very high surface-to-volume-ratio of the 190 μm droplets generated in an X-junction microchannel (Fig. 1b) was also advantageous for the *Candida antarctica* lipase B (CaLB)-catalyzed synthesis of isoamyl acetate, allowing the "natural" production of this important aroma. The amphiphilic enzyme positioned together with the substrate in the hydrophilic ionic liquid tends to attach to the surface of the organic phase forming droplets. The high interfacial area as well as the *in-situ* product removal into the organic liquid droplets allowed much higher volumetric productivities than reported in the literature for this esterification. Furthermore, the incorporation of a hydrophobic membrane-based separator allowed separation of the enzyme from the product in the organic phase and several successful recyclations of the biocatalyst.[71]

The same reaction has been studied in flow reactors developed by Corning®, which allow efficient mixing of two-phase systems without the need for high energy or high pressure drop devices. The key component of the system is a fluidic module made of special glass, which consists of a chain of identical cells with variable cross-sections and internal elements. The fluid is forced to split and then recombine in each cell, leading to the renewal of the interface in two-phase systems such as liquid-liquid (Figure 6 a). The ease of their scalability from laboratory to production scale and customization to meet specific requirements provides a cost-effective solution for a broad portfolio of reactions in organic synthesis as well as for extraction.[72,73] Application of the low-flow module to lipase-catalyzed esterification in an aqueous- *n*-heptane two-phase system enabled efficient interfacial mass transfer and *in situ* product removal, resulting in unprecedented volumetric productivities.[74] Further scale-up of the process in a 70-mL modular reactor demonstrated excellent process scalability.[22]

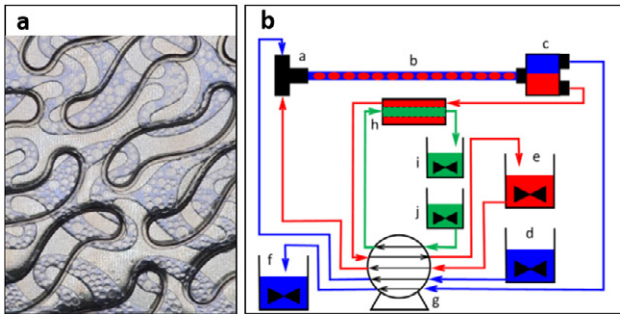

Figure 6: Reactors with two-phase flow: a) a close-up of a liquid-liquid flow regime obtained in a Low Flow Advanced Flow™ Reactor developed by Corning®, and b) a scheme of the microfluidic system with an enzyme recycle: a – a T-shaped element, b – reaction microcapillary, c – settler, d– reservoir of the top phase with dissolved reactants, e – reservoir of the recycle stream, f – product reservoir, g – peristaltic pump, h – dialysis micromodule, i – waste, j – dialysate solution, k – microdialyzer ports. Reproduced with permission from Vobecká *et al., Chem. Eng. J.* 2020, 396, 125236.[75]

The droplets generated by microfluidics were also used for the preparation of semipermeable silica microparticles that allowed compartmentalization of enzymes. The porous shell allowed selective diffusion of substrate and product while protecting the enzymes from degradation by proteinases and maintaining their functionality over multiple reaction cycles. The system was tested for β-glucosidase encapsulation and for the combined compartmentalization of glucose oxidase and horseradish peroxidase, which form a controlled reaction cascade for the glucose detector. The microparticles were trapped in a microfluidic array device in which the enzyme activity could be tested in a single microparticle, which also provided information on reaction kinetic parameters and stability.[76]

3. 3. Segmented-Flow Microreactors With Biocatalysts

Segmented flow with alternating fluid segments (e.g., slug flow in Fig. 1c and Fig 2c) is much easier to achieve than stable parallel flow in a long channel that allows complete conversion, so this type of flow prevails in biocatalytic processing in liquid-liquid systems.[19] It also allows very efficient mass transfer between phases, based on convective motion in each segment that renews the interface, which increases the concentration gradient of the product and facilitates diffusive penetration through the interface.[77] A typical setup consists of the mixing unit (T- or Y-shaped mixer), tubes with lengths that provide the appropriate residence times, and phase separators based on gravity, membranes, *etc.* Compared to batch processes, where the intensive mixing of several phases required for efficient mass transfer regularly leads to emulsification and

thus phase separation problems, this obstacle is reduced in microflow reactors.[39,78]

Applications of slug flow include various reactions and enzymes, from reactions catalyzed by alcohol-dehydrogenase (ADH),[79–82] to hydroxynitrile lyase-catalyzed C-C bond formation,[83] a reduction with pentaerythritol tetranitrate reductase,[84] terpene production catalyzed by aristolochene synthase,[77] penicillin acylase-catalyzed antibiotic synthesis,[75,85,86] and lipase-assisted biodiesel production,[87,88] among others.

An interesting reactor design was reported by Karande *et al.* who combined different sized capillaries from 2.5 mm i.d. to 0.5 mm i.d. to comply with lower substrate concentration along the tubular reactor, where the ADH-catalyzed reaction takes place. This allowed optimization of the conversion of selected aldehyde to corresponding alcohol dissolved in an organic phase and contacted in a slug flow regime with an aqueous phase containing enzyme and cofactor, as well as a cofactor dehydrogenase-based regeneration system.[79] To circumvent the interfacial deactivation of ADH in segmented flow, the addition of surfactant and immobilization of the enzyme in porous beads carried along the tubular reactor within the aqueous segments were tested. Both approaches resulted in very efficient stabilization of the enzyme, with surfactant addition being preferred due to better enzyme activity, less complexity, and ease of implementation in slug flow microreactors.[80]

Significant mechanical energy savings have been reported for lipase-catalyzed soybean oil hydrolysis using a slug flow microreactor. The hydrodynamically well-controlled slug flow generated in a T-shaped microfluidic channel and continued in submillimeter reaction capillaries, ensured uniform residence time of all slugs and enabled the recovery of well-defined products. Further integration with two microfluidic separators resulted in phase separation and the possibility to reuse the dissolved enzyme.[89]

Recently, lipase-catalyzed biodiesel production in a slug-flow microreactor has received considerable attention. Very pure biodiesel with glycerol content below the detection limit was produced in an integrated system with two microchips connected in series. The first Y-shaped microchannel was used for biodiesel production with methanol in one feed and an emulsion of oil, lipase and surfactant in the second. In the Y-shaped microchannels connected in series, simultaneous purification, i.e., glycerol removal, was achieved with DES based on choline chloride and ethylene glycol.[87] In another study by the same group, DES based on choline chloride and glycerol was used for biodiesel production based on lipase-catalyzed transesterification of edible and waste sunflower oil with methanol. The reaction, which was carried out in a Y-Y-shaped microchannel as well as in a mm-scale tube, resulted in a 3-4-fold higher productivity than in the stirred tank reactor operated in batch mode.[88]

Environmentally friendly ATPS prepared from PEG and phosphate buffer was used for an enzyme-catalyzed synthesis of the β-lactam antibiotic cephalexin, which is produced industrially on a multi-tones annual scale. The microfluidic setup shown in Figure 6 b included a slug-flow microreactor that supported efficient mass transfer between penicillin acylase dissolved in the bottom ATPS phase and the substrates in the ATPS's top phase, as well as *in situ* product separation in the latter. Integration of the settler resulted in phase separation and enabled further recycling of the reaction phase containing the dissolved enzyme. The recycling circuit also included a microdyalizer operated in counter-current regime to remove phenylglycine, which tended to clog the system. The system could be operated continuously for 5 h, and the operating time appeared to be limited only by the washout of the enzyme.[75] The same group has also applied various ATPSs in the production of 6-aminopenicillanic acid (6-APA) from penicillin G using dissolved penicillin acylase. Criteria for the selection of ATPS were optimal separation of 6-APA from the enzyme, high buffering capacity to reduce undesirable pH decrease due to dissociation of phenylacetic acid – a byproduct of the reaction, relatively low cost of ATPS components, and the possibility of electrophoretic transport of fine droplets and reaction products to both accelerate phase separation and increase the 6-APA concentration in the product stream. The possibility of electrophoretic transport of the salt-rich droplets in the system was verified in a simple microfluidic device.[85] A continuation of this study led to the development of electric-field-enhanced selective separation of the reaction byproduct in a membrane microcontactor. Application of DC electric field resulted in enhanced mass transfer through a semi-permeable membrane for rapid, continuous, and selective separation of electrically charged low-molecular-weight phenylacetic acid from the original reaction mixture containing free penicillin acylase. Furthermore, the electroosmotic flow through the membrane, which counter-directs the transport of phenylacetic acid, was advantageously used to concentrate the separated product in the acceptor phase.[86]

The importance of minimizing stable emulsion formation, typical of the stirred tank batch processing, was highlighted for the enzymatic reduction of hydrophobic ketone in a biphasic methyl tert-butyl ether (MTBE) - buffer carried out in a segmented flow formed in a Y-shaped mixer and guided in a poly(fluorenylene ethynylene) (PFE) coil of 0.8 mm diameter, and compared with the batch process. While the conversions in both process operations were similar under comparable conditions, emulsification and precipitation were strongly suppressed when the biocatalytic reactions were carried out in flow mode, significantly simplifying and minimizing the effort required for biphasic biocatalytic reaction systems.[90]

The pioneering work of Karande *et al.*[80] inspired the study of segmented flow, in which segments containing a

heterogeneous biocatalyst surrounded by another liquid phase flowed in the microreactor. A segmented hydrogel-organic solvent system was developed based on superabsorbent polymer consisting of partially neutralized cross-linked polyacrylic acid, in whose matrix enzymes and whole cells could be embedded. Such a "fluid heterogeneous phase" was investigated with the ADH-catalyzed reduction of acetophenone and the aldoxime dehydratase-catalyzed dehydration of octanal oxime. Especially for solvent-labile catalytic systems, this approach offers an alternative for the application of immobilized biocatalysts in a continuously running process beyond conventional packed bed and wall-coated reactors.[90]

4. Microreactors With Biocatalysts Containing Gaseous and Liquid Phase

Enzymes can be used as highly selective catalysts for the oxyfunctionalization of unactivated carbons in organic synthesis. Insufficient oxygen supply is often a bottleneck in O_2-dependent reactions, which is why a high influx of the gas phase and intensive mixing are required in conventional stirred tank reactors. In biocatalytic processes, this can lead to enzyme deactivation and gas stripping of substrate and product.[32,91] To circumvent this, a tube-in-tube reactor (TiTR), in which the gaseous substrate enters the reaction chamber along the entire length of the tube, is a promising alternative. A flow-through chemistry apparatus developed a decade ago allows contact between gasses and liquids *via* a semipermeable Teflon AF-2400 membrane of a submillimeter i.d.[92]

The application of such a high-pressure reactor setup providing oxygen supply across the membrane surface from the outside of the reactor system was demonstrated for the synthesis of 3-phenylcatechol using a continuous segmented flow of the aqueous phase with the enzyme and decanol with the substrate as shown in Figure 7. 2-Hydroxybiphenyl- 3-monooxygenase was applied as a biocatalyst for the hydroxylation reaction and also required cofactor regeneration, which was provided by formate dehydrogenase dissolved in an aqueous phase. Very high

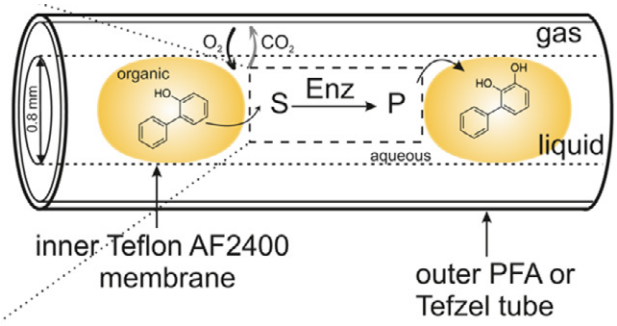

Figure 7. Scheme of a tube-in-tube reactor used for enzymatic hydroxylation using gaseous oxygen as a substrate. Reproduced with permission from Tomaszewski *et al.*, *Org. Process Res. Dev*. 2014, 18, 1516–1526.[91]

volumetric productivities were obtained when the reactor was of sufficient length providing the required residence times, emphasizing the potential of the TiTR as a promising technology for the realization of gas-dependent enzymatic reactions.

The same reactor configuration was also used to study the kinetics of oxygen-dependent reactions catalyzed by glucose oxidase, where the challenges of conventional systems can be avoided by creating a bubble-free aeration system. The TiTR setup was fully automated and computer controlled, allowing characterization of an oxygen-dependent enzyme within 24 hours with minimal manual labor, outperforming the conventional batch setup approach. By pressurizing the system, the dissolved oxygen concentration can reach 25-times the values achievable by air supply under atmospheric conditions. Operation in the low dispersed flow regime allowed the generation of time-series data with an enzymatic catalyst, despite its low diffusivity, and the resulting data were in good agreement with experiments conducted in a batch system.[93]

Direct introduction of the gas phase into enzymatic microreactors, allowing efficient supply of gaseous substrate, has been reported for many biocatalytic processes involving immobilized enzymes that were reviewed elsewhere.[14,16,20-28] A report on the generation of a three-phase slug flow in a microchannel used for dissolved enzyme-catalyzed reaction revealed the benefit of introducing an inert gas phase (nitrogen) into a liquid-liquid slug flow to stabilize the liquid-liquid interface, and improve uniformity and reproducibility of the flow. In this way, uniform reaction-transport properties were created in a heterogeneous reaction system with an unstable interface in a long microchannel, as demonstrated in the lipase-catalyzed hydrolysis of soybean oil.[94]

Among the commercial meso-scale flow reactors enabling efficient direct gas-liquid contact, Corning® Advanced Flow Reactors, such as presented in Figure 6a, are commonly used in chemical industry, but to the author's knowledge, no report of enzymatic reaction with the gas phase in these reactors has been reported. On the other hand, the Coflore™ agitated cell reactor (ACR) and the agitated flow reactor (ATR) have been used for the chiral resolution of DL-alanine using non-immobilized whole *Pichia pastoris* cells with D-amino acid oxidase, where reaction is oxygen limited due to the gas–liquid mass transfer constraints of the conventional vessels. Comparison of a batch process performed in a 250 mL stirred tank reactor at various stirring speeds with a 100 mL Coflore ACR, a dynamically mixed plug flow reactor that uses to promote mixing, revealed a slightly increased reaction rate in the flow reactor. Further comparison of 1 L stirred tank batch reactor with 1 L Coflore ATR tubular reactor using lateral movement showed much greater improvement in volumetric productivity for the flow reactor due to improved gas-liquid mass transfer. In addition, virtually the same results were obtained with AFR when scaling up from 1 to 10 L

without seeing the losses already evident when moving from 1 to 4 L in conventional batch reactor.[95]

5. Process Analytics in Microreactors With Biocatalysts

As described in the chapter on droplet microfluidic platforms used for fast biocatalyst screening, evolution and characterization, highly automated and controlled devices using numerous analytical techniques are under development. Miniaturization and integration of several analytical techniques such as chromatography, electrophoresis, or flow injection analysis in devices referred to as micro Total Analysis Systems (μTAS) were the first applications of microfluidics starting in the 1990s.[96] In contrast, most studies presented in this review use off-line analytical techniques such as HPLC or spectrophotometry. Optical sensors for non-invasive and non-destructive monitoring of e.g. oxygen, pH, carbon dioxide, glucose, and temperature reviewed by Gruber *et al.* (2017) have great potential for on-line and at-line monitoring, both of which have some advantages and disadvantages as listed in Table 1.[97]

An example of in-line analysis of dissolved oxygen and substrate or product concentration in the microchannel outlet stream is shown in Figure 8. Dissolved oxygen concentration was measured using a fiber microsensor within the needle inserted into the stream, while substrate or product concentration was evaluated using the flow-through miniaturized optical detector that measures absorbance at the specific wavelength of interest.

Oxygen can also be monitored on-line by introducing sensory nanoparticles into the fluid and monitoring them using a fluorescence microscope, or by creating measurement points in the channel.[98] As mentioned in Chapter 2, on-line pH measurement has been established based on a similar approach.[97]

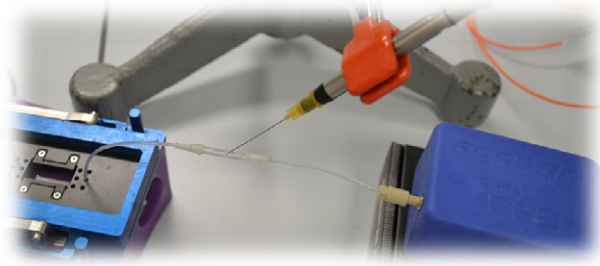

Figure 8. Monitoring of the microchannel outlet stream regarding the dissolved oxygen concentration using fiber microsensor within the needle inserted in the flow, and the substrate or product concentration based on flow-through miniaturized spectrophotometer.

The use of novel manufacturing capabilities offered by 3D printing technology and the integration of novel materials will pave the way for better process monitoring that will also enable process control, which is crucial for the efficient application of biocatalysts.

6. Conclusions and Future Perspectives

If four technological advances evolved in the last decades of the previous century have been recognized as crucial for the acceptance of enzymes as "alternative catalysts" in industry, viz the development of i) techniques for large-scale isolation and purification of enzymes, ii) techniques for large-scale immobilization of biocatalysts, iii) biocatalytic processes in organic solvents, and iv) recombinant DNA technology enabling biocatalyst engineering,[9] the fifth technological advance that can now be added is the development of continuous processing in miniaturized devices designed to efficiently harness these unique cata-

Table 1: Summary of the advantages of on-line and at-line monitoring in microfluidic systems as proposed by Gruber *et al.*, *Lab Chip*, 2017, 17, 2693, published by The Royal Society of Chemistry.[97]

	Advantages	**Disadvantages**
on-line	Real time analysis possible	Possible interaction of sensors with the flow or reactants
	Rapid feedback allows real time process control	Sensors need to be recalibrated and replaced over time
	No manual sampling required	Increase of system complexity (fabrication, design, operation, maintenance)
	Measurement at real temperature	Cross sensitivity with other analytes or interferences can be difficult to quantify
	No sampling required	Limitation to a specific analytical problem and a certain concentration range
	Less risk of contamination	
	Production flow undisrupted by sampling or redirecting	
at-line	Significant number of assays/analytical methods available	Changes in sample before analysis possible
	Can be cost-efficient	Analysis limited to on-site equipment
	Flow cells available	Certain sample volume necessary
	Feedback available quickly	Risk of contamination through sampling

lysts.

The application of microflow systems for biotransformations with free biocatalysts offers several advantages, from reduced shear stress on fragile molecules and cells, to reduced mechanical energy requirements for efficient mixing, to very efficient contacting of multiple phases that allows compartmentalization of biocatalyst and often inhibitory substrates and/or products. Microfluidics-based droplets manipulated in highly automated microfluidic devices provide a revolutionary tool for ultrahigh-throughput biocatalyst evolution and efficient biocatalytic process development. Furthermore, continuous process operation in microflow reactors also allows for easy downstream process integration, enabling enzyme or cell recycling and thus very high total catalyst turnover number, defined as the total moles of product produced per mole of enzyme over the lifetime of the enzyme. The use of environmentally friendly solvents in such production systems can ensure that the goals of green chemistry as well as the bioeconomy are achieved. The requirements for fully controlled microflow systems are driving the intensive development of integrated analytics, with new manufacturing technologies such as 3D printing together with novel materials offering endless possibilities. The use of model-based approaches that allow quantification of mass transfer in various reaction systems and microreactor configurations, as well as apparent reaction rates at different process conditions, will help to exploit the potential of microreactor technology. The use of engineering tools, such as characteristic times analysis and dimensionless numbers evaluation, could be of great value in this endeavor.[22,79,80]

Industrial implementation of flow biocatalysis requires the knowledge transfer between the various disciplines involved in process development. Understanding the fundamental phenomena underlying the structure and function of biocatalysts, biocatalytic reaction mechanisms and kinetics, and the performance of microreactors is therefore a basic requirement and should be implemented in the curricula of chemical, biochemical, and engineering study programs.

Acknowledgements

Financial support from the Slovenian Research Agency (Grants P2-0191, N2-0067 and J4-1775) and from the EC H2020 project COMPETE (Grant 811040) is gratefully acknowledged. The author would like to thank M. Seručnik, L. Ostanek Jurina, B. Perić, T. Pilpah and M. Klemenčič from University of Ljubljana for providing graphical material.

Abbreviations

ACR	Agitated cell reactor
ADH	Alcohol-dehydrogenase
ATR	Agitated flow reactor
6-APA	6-Aminopenicillanic acid
AMTPS	Aqueous micellar two-phase system
ATPS	Aqueous two-phase system
CaLB	Candida antarctica lipase B
DC	Direct current
DES	Deep eutectic solvent
Dex	Dextran
E-factor	Environmental factor
ESI-MS	Electrospray ionization – mass spectroscopy
FADS	Fluorescence-activated droplet sorting
FCDS	Flow cytometric droplet sorting
GSB	Gel-shell beads
HPLC	High-performance liquid chromatography
IL	Ionic liquid
MTBE	Methyl tert-butyl ether
PEG	Polyethylene glycol
PFA	Perfluoroalkoxy
PFE	Poly(fluorenylene ethynylene)
$scCO_2$	Supercritical CO_2
SpliMLiB	Split-and-Mix Library on Beads
TiTR	Tube-in-tube reactor

7. References

1. D. T. McQuade, P. H. Seeberger. Applying flow chemistry: methods, materials, and multistep synthesis. *J. Org. Chem.* **2013**, 78, 6384–6389. **DOI:** 10.1021/jo400583m. **DOI:**10.1021/jo400583m
2. K. F. Jensen. Flow chemistry—Microreaction technology comes of age. *AIChE J.* **2017**, 63, 858–869. **DOI:**10.1002/aic.15642
3. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, Center for Veterinary Medicine: Guidance for Industry: Process Validation: General Principles and Practices. **2011.**
4. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research: Advancement of Emerging Technology Applications to Modernize the Pharmaceutical Manufacturing Base Guidance for Industry, **2015**.
5. P. Kleinebudde, J. Khinast, J. Rantanen (Eds.) Continuous Manufacturing of Pharmaceuticals; Wiley, Hoboken, NJ. **2017**, ISBN 9781119001324.
6. R. A. Sheldon. The E factor 25 years on: the rise of green chemistry and sustainability. *Green Chem.* **2017**, 19, 18–43. **DOI:**10.1039/C6GC02157C
7. C. Jiménez-González, P. Poechlauer, Q. B. Broxterman, B.-S. Yang, D. am Ende, J. Baird, C. Bertsch, R. E. Hannah, P. Dell'Orco, H. Noorman, S. Yee, R. Reintjens, A. Wells, V. Massonneau, J. Manley. Key Green Engineering Research Areas for Sustainable Manufacturing: A Perspective from Pharmaceutical and Fine Chemicals Manufacturers. *J. Org. Proc.*

Res. Dev. **2011**, *15*, 900–911. **DOI:**10.1021/op100327d
8. R. Wohlgemuth. Biocatalysis – Key enabling tools from biocatalytic one-step and multi-step reactions to biocatalytic total synthesis. *New Biotechnol.* **2021**, *60*, 113–123. **DOI:**10.1016/j.nbt.2020.08.006
9. Đ. Vasić-Rački. History of Industrial Biotransformations – Dreams and Realities. In: A. Liese, K. Seelbach, C. Wandrey (Eds.): Industrial Biotransformations, 2nd Ed., Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, **2006**, pp. 1–36. **DOI:**10.1002/9783527608188.ch1
10. R. A. Sheldon P. C. Pereira. Biocatalysis engineering: the big picture. *Chem. Soc. Rev.* **2017**, *46*, 2678—2691. **DOI:**10.1039/C6CS00854B
11. R. A. Sheldon, J. M. Woodley. Role of Biocatalysis in Sustainable Chemistry. *Chem. Rev.* **2018**, *118*, 801–838. **DOI:**10.1021/acs.chemrev.7b00203
12. K. Faber, W.-D. Fessner, N. J. Turner. Biocatalysis: Ready to Master Increasing Complexity. *Adv. Synth. Catal.* **2019**, *361*, 2373–2376. **DOI:**10.1002/adsc.201900610
13. U. Kragl. The Role of Reaction Engineering in Bioprocess Development. *Chimia.* **2020**, *74*, 378–381. **DOI:**10.2533/chimia.2020.378
14. R. Wohlgemuth, I. Plazl, P. Žnidaršič-Plazl, K. V. Gernaey, J. M. Woodley. Microscale technology and biocatalytic processes: opportunities and challenges for synthesis. *Trends Biotechnol.* **2015**, *33*, 302–314, **DOI:**10.1016/j.tibtech.2015.02.010
15. A. Liese, K. Seelbach, A. Buchholz, J. Haberland. Processes. In: A. Liese, K. Seelbach, C. Wandrey (Eds.): Industrial Biotransformations, 2nd Ed., Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, **2006**, pp. 147–514. **DOI:**10.1002/9783527608188.ch6a
16. P. Žnidaršič-Plazl. Biotransformations in microflow systems: Bridging the gap between academia and industry. *J Flow Chem*. **2017**, *7* (3-4), 111–117. **DOI:**10.1556/1846.2017.00021
17. J. M. Bolivar, J. Wiesbauer, B. Nidetzky. Biotransformations in microstructured reactors: more more than flowing with the stream? *Trends Biotechnol.* **2011**, *29*, 333–342. **DOI:**10.1016/j.tibtech.2011.03.005
18. J. M. Bolivar, B. Nidetzky. Multiphase biotransformations in microstructured reactors: opportunities for biocatalytic process intensification and smart flow processing. *Green Process. Synth*. **2013**, *2*, 541–559. **DOI:**10.1515/gps-2013-0091
19. P. Žnidaršič-Plazl. Enzymatic microreactors utilizing non-aqueous media. *Chim. Oggi – Chem. Today* **2014**, *32*, 54–61. https://www.teknoscienze.com/tks_article/enzymatic-microreactors-utilizing-non-aqueous-media/.
20. L. Tamborini, P. Fernandes, F. Paradisi, F. Molinari. Flow Bioreactors as Complementary Tools for Biocatalytic Process Intensification. *Trends Biotechnol.* **2018**, *36*, 73–88. **DOI:**10.1016/j.tibtech.2017.09.005
21. J. Britton, S. Majumdar, G. A. Weiss. Continuous flow biocatalysis. *Chem. Soc. Rev*. **2018**, *47*, 5891–5918. **DOI:**10.1039/C7CS00906B
22. P. Žnidaršič-Plazl. The promises and the challenges of biotransformations in micro-flow. *Biotechnol. J.* **2019**, *14*, 1800580, **DOI:**10.1002/biot.201800580
23. J. M. Bolivar, F. López-Gallego. Characterization and evaluation of immobilized enzymes for applications in flow reactors. *Curr. Opin. Green Sustain. Chem.* **2020**, *25*, 100349. **DOI:**10.1016/j.cogsc.2020.04.010
24. M. Romero-Fernandez, F. Paradisi. Protein immobilization technology for flow biocatalysis. *Curr. Opin. Chem. Biol.* **2020**, *55*, 1–8. **DOI:**10.1016/j.cbpa.2019.11.008
25. P. De Santis, L. Meyer, S. Kara. The Rise of Continuous Flow Biocatalysis – Fundamentals, Very Recent Developments and Future Perspectives. *React. Chem. Eng.* **2020**, *5*(12), 2155–2184. **DOI:**10.1039/D0RE00335B
26. M. S. Thomsen, B. Nidetzky. Continuous-flow Microchannel Reactors with Surface-immobilized Biocatalysts. In: W.-D. Fessner, T, Anthonsen (Eds.): Modern Biocatalysis. Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, **2009**, pp. 43–54. **DOI:**10.1002/9783527623839.ch3
27. M. Bajić, P. Žnidaršič-Plazl, M. Kingston, V. Hessel. General aspects of immobilized biocatalysts and their applications in flow. In: M. B. Nielsen (Ed.): Science of Synthesis: Knowledge Updates 2018/1. Georg Thieme, Stuttgart; New York. **2018**, pp. 397–443.
28. D. Valikhani, J. M. Bolivar, B. Nidetzky. Enzyme Immobilization in Wall-Coated Flow Microreactors. In: J. Guisan, J. Bolivar, F. López-Gallego, J. Rocha-Martín (Eds.): Immobilization of Enzymes and Cells. Methods in Molecular Biology, Humana, New York, NY, **2020**, vol. 2100. pp. 243–258. **DOI:**10.1007/978-1-0716-0215-7_16
29. G. Stojkovič, P. Žnidaršič-Plazl. Covalent Immobilization of Microbial Cells on Microchannel Surfaces. In: J. Guisan, J. Bolivar, F. López-Gallego, J. Rocha-Martín (Eds.): Immobilization of Enzymes and Cells. Methods in Molecular Biology, Humana, New York, NY, **2020**, vol. 2100. pp. 417–426. **DOI:**10.1007/978-1-0716-0215-7_28
30. R. DiCosimo, J. McAuliffe, A. J. Poulose, G. Bohlmann. Industrial use of immobilized enzymes. *Chem. Soc. Rev*. **2013**, *42*, 6437–6474. **DOI:**10.1039/c3cs35506c
31. J. J. Straathof. Quantitative Analysis of Industrial Biotransformations. In: A. Liese, K. Seelbach, C. Wandrey (Eds.): Industrial Biotransformations, 2nd Ed., Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, **2006**, pp. 515–520. **DOI:**10.1002/9783527608188.ch7
32. C. R. Thomas, D. Geer. Effects of shear on proteins in solution. *Biotechnol Lett.* **2011**, *33*, 443–456. **DOI:**10.1007/s10529-010-0469-4
33. C.-H. Ho, J. Yi, X. Wang. Biocatalytic Continuous Manufacturing of Diabetes Drug: Plantwide Process Modeling, Optimization, and Environmental and Economic Analysis. *ACS Sustain. Chem. Eng.* **2019**, *7*, 1038−1051. **DOI:**10.1021/acssuschemeng.8b04673
34. N. Miložič, M. Lubej, U. Novak, P. Žnidaršič-Plazl, I. Plazl. Evaluation of Diffusion Coefficient Determination using a microfluidic device. *Chem. Biochem. Eng. Q.* **2014**, *28*, 215–223. **DOI:**10.15255/CABEQ.2014.1938
35. M. Tišma, B. Zelić, Đ. Vasić-Rački, P. Žnidaršič-Plazl, I. Plazl.

Modelling of laccase-catalyzed L-DOPA oxidation in a microreactor. *Chem. Eng. J*. **2009**, *149*, 383–388. **DOI:**10.1016/j.cej.2009.01.025

36. C. K. Savile, J. M Janey, E. C. Mundorff, J. C Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman, G. J. Hughes. Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture. *Science* **2010**, *329*, 305–309. **DOI:**10.1126/science.1188934
37. J. Lawrence, B. O'Sullivan, G. J. Lye, R. Wohlgemuth, N. Szita. Microfluidic multi-input reactor for biocatalytic synthesis using transketolase. *J. Mol. Catal. B: Enzym*. **2013**, *95*, 111–117. **DOI:**10.1016/j.molcatb.2013.05.016
38. P. Gruber, M. P. C. Marques, P. Sulzer, R. Wohlgemuth, T. Mayr, F. Baganz, N. Szita. Real-time pH monitoring of industrially relevant enzymatic reactions in a microfluidic side-entry reactor (μSER) shows potential for pH control. *Biotechnol. J.* **2017**, *12*, 1600475. **DOI:**10.1002/biot.201600475
39. P. Žnidaršič-Plazl, I. Plazl. Steroid extraction in a microchannel system: mathematical modelling and experiments. *Lab Chip* **2007**, *7*, 883–889. **DOI:**10.1039/B704432A
40. M. Panić, M. Cvjetko Bubalo, I. Radojčić Redovniković. Designing a biocatalytic process involving deep eutectic solvents. *J. Chem. Technol. Biotechnol.* **2021,** *96*, 14–30. **DOI:**10.1002/jctb.6545
41. M. Cvjetko Bubalo, I. Sabotin, I. Radoš, J. Valentičič, T. Bosiljkov, M. Brnčić, P. Žnidaršič-Plazl. A comparative study of ultrasound-, microwave-, and microreactor-assisted imidazolium-based ionic liquid synthesis. *Green Proc. Synth*. **2013**, *2*, 579–590. **DOI:**10.1515/gps-2013-0086
42. R. A. Sheldon, R. Madeira Lau, M. J. Sorgedrager, F. van Rantwijk, K. R. Seddon. Biocatalysis in ionic liquids. *Green Chem*. **2002**, 4, 147–151. **DOI:**10.1039/b110008b
43. U. Novak, A. Pohar, I. Plazl, P. Žnidaršič-Plazl. Ionic liquid-based aqueous two-phase extraction within a microchannel system. *Sep. Purif. Technol.* **2012**, *97*:172–178. **DOI:**10.1016/j.seppur.2012.01.033
44. S.-X. Meng, L.-H. Xue, C.-Y. Xie, R.-X. Bai, X. Yang, Z.-P. Qiu, T. Guo, Y.-L. Wang, T. Meng. Enhanced enzymatic reaction by aqueous two-phase systems using parallel-laminar flow in a double Y-branched microfluidic device. *Chem. Eng. J.* **2018**, *335*, 392–400. **DOI:**10.1016/j.cej.2017.10.085
45. F. A. Vicente, I. Plazl, S .P. M. Ventura, P. Žnidaršič-Plazl. Separation and purification of biomacromolecules based on microfluidics. *Green Chem.* **2020**, *22*, 4391–4410. **DOI:**10.1039/C9GC04362D
46. M. Seručnik, F. A. Vicente, Ž. Brečko, J. A. P. Coutinho, S. P. M. Ventura, P. Žnidaršič-Plazl. Development of a microfluidic platform for R-phycoerythrin purification using an aqueous micellar two-phase system**.** *ACS Sustain. Chem. Eng.* **2020**, *8* (46), 17097–17105. **DOI:**10.1021/acssuschemeng.0c05042
47. T. Wang, C. Xu. Liquid–liquid–liquid three-phase microsystem: hybrid slug flow–laminar flow. *Lab Chip* **2020**, *20*, 1891–1897. **DOI:**10.1039/D0LC00292E
48. T. Maruyama, J. Uchida, T. Ohkawa, T. Futami, K. Katayama, K. Nishizawa, K. Sotowa, F. Kubota, No. Kamiya, M. Goto. Enzymatic degradation of *p*-chlorophenol in a two-phase flow microchannel system. *Lab Chip* **2003**, *3*, 308–12. **DOI:**10.1039/b309982b
49. P. Žnidaršič-Plazl, I. Plazl. Modelling and experimental studies on lipase-catalyzed isoamyl acetate synthesis in a microreactor. *Process Biochem*. **2009**, *44*, 1115–1121. **DOI:**10.1016/j.procbio.2009.06.003
50. Y. S. Huh, Y. Jun, Y. K. Hong, W. H. Hong, D. H. Kim. Microfluidic separation of (*S*)-ibuprofen using enzymatic reaction. *J. Mol. Catal. B: Enzym*. **2006**, 43, 96–101. **DOI:**10.1016/j.molcatb.2006.06.017
51. K. Marik, L. Ticha, L. Vobecka, M. Pribyl. Theoretical study on enzyme synthesis of cephalexin in a parallel-flow microreactor combined with electrically driven ATPS microextraction. *React. Chem. Eng*. **2020**, *5*, 570–583. **DOI:**10.1039/C9RE00482C
52. Y. Ding, P. D. Howes, A. J. DeMello. Recent Advances in Droplet Microfluidics. *Anal. Chem.* **2020**, *92*, 132–149. **DOI:**10.1021/acs.analchem.9b05047
53. T. S. Kaminski, P. Garstecki. Controlled droplet microfluidic systems for multistep chemical and biological assays. *Chem. Soc. Rev.* **2017**, *46*, 6210–6226. **DOI:**10.1039/C5CS00717H
54. S. L. Sjostrom, Y. Bai, M. Huang, Z. Liu, J. Nielsen, H. N. Joensson, H. Svahn. A. High-throughput screening for industrial enzyme production hosts by droplet microfluidics. *Lab Chip* **2014**, *14*, 806–813. **DOI:**10.1039/c3lc41398e
55. P. Mair, F. Gielen, F. Hollfelder. Exploring sequence space in search of functional enzymes using microfluidic droplets. *Curr. Opin. Chem. Biol.* **2017**, *37*,137–144. **DOI:**10.1016/j.cbpa.2017.02.018
56. B. Kintses, C. Hein, M. F. Mohamed, M. Fischlechner, F. Courtois, C. Laine, F. Hollfelder. Picoliter cell lysate assays in microfluidic droplet compartments for directed enzyme evolution. *Chem. Biol.* **2012**, *19*, 1001–1009. **DOI:**10.1016/j.chembiol.2012.06.009
57. D. Hess, T. Yang, S, Stavrakis. Droplet-based optofluidic systems for measuring enzyme kinetics. *Anal. Bioanal. Chem.* **2020**, *412*, 3265–3283. **DOI:**10.1007/s00216-019-02294-z
58. F. Ma, T. Guo, Y. Zhang, X. Bai, C. Li, Z. Lu, X. Deng, D. Li, K. Kurabayashi, G. Yang. An ultrahigh-throughput screening platform based on flow cytometric droplet sorting for mining novel enzymes from metagenomic libraries. *Environ. Microbiol.* **2020. DOI:**10.1111/1462-2920.15257
59. M. S. Packer, D. R. Liu. Methods for the directed evolution of proteins. *Nat. Rev. Genet*. **2015**, *16*, 379. **DOI:** 10.1038/nrg3927.
60. R. Obexer, A. Godina, X. Garrabou, P. R. E. Mittl, D. Baker, A. D. Griffiths, D. Hilvert. Emergence of a catalytic tetrad during evolution of a highly active artificial aldolase. *Nat. Chem.* **2017**, *9*, 50–56. **DOI:**10.1038/nchem.2596
61. F. W. Y. Chiu, S. Stavrakis. High-throughput droplet-based microfluidics for directed evolution of enzymes. *Electrophoresis* **2019**, *40*, 2860–2872. **DOI:**10.1002/elps.201900222
62. M. Fischlechner, Y. Schaerli, M. F. Mohamed, S. Patil, C. Abell, F. Hollfelder. Evolution of enzyme catalysts caged in

biomimetic gel-shell beads. *Nat. Chem.* **2014**, 6, 791–796. **DOI:**10.1038/nchem.1996

63. L. Lindenburg, T. Huovinen, K. van de Wiel, M. Herger, M. R. Snaith, F. Hollfelder. Split & mix assembly of DNA libraries for ultrahigh throughput on-bead screening of functional proteins. *Nucleic Acids Res.* **2020**, 48, e63,. **DOI:**10.1093/nar/gkaa270
64. F. Gielen, R. Hours, S. Emond, M. Fischlechner, U. Schell, F. Hollfelder. Ultrahigh-throughput–directed enzyme evolution by absorbance-activated droplet sorting (AADS). *Proc. Natl. Acad. Sci. U. S. A*. **2016**, 113, E7383. **DOI:**10.1073/pnas.1606927113
65. X. Wang, Y. Xin, L. Ren, Z. Sun, P. Zhu, Y. Ji, C. Li, J. Xu, B. Ma. Positive dielectrophoresis–based Raman-activated droplet sorting for culture-free and label-free screening of enzyme function in vivo. *Sci. Adv.* **2020**, 6, eabb3521. **DOI:**10.1126/sciadv.abb3521
66. D. G. Horvath, S. Braza, T. Moore, C. W. Pan, L. Zhu, O. S. Pak, P. Abbyad. Sorting by interfacial tension (SIFT): Label-free enzyme sorting using droplet microfluidics. *Anal. Chim. Acta* **2019**, 1089, 108e114. **DOI:**10.1016/j.aca.2019.08.025
67. X. W. Diefenbach, I. Farasat, E. D. Guetschow, C. J. Welch, R. T. Kennedy, S. Sun, J. C. Moore. Enabling Biocatalysis by High-Throughput Protein Engineering Using Droplet Microfluidics Coupled to Mass Spectrometry. *ACS Omega* **2018**, *3*, 1498–1508. **DOI:**10.1021/acsomega.7b01973
68. C. Holtze, S. A. Weisse, M. Vranceanu. Commercial Value and Challenges of Drop-Based Microfluidic Screening Platforms–An Opinion. *Micromachines* **2017**, *8*, 193. **DOI:**10.3390/mi8060193
69. J. H. Li, J. Chen, Y. J. Wang, G. S. Luo, H. M. Yu. Hydration of acrylonitrile to produce acrylamide using biocatalyst in a membrane dispersion microreactor. *Bioresour. Technol.* **2014**, *169*, 416–420. **DOI:**10.1016/j.biortech.2014.07.034
70. J. H. Li, M. Guo, S. Jiao, Y .J. Wang, G.S. Luo, H. M. Yu. A kinetic study of the biological catalytic hydration of acrylonitrile to acrylamide, *Chem. Eng. J.* **2017**, *317*, 699–706. **DOI:**10.1016/j.cej.2017.02.100
71. U. Novak, P. Žnidaršič-Plazl. Integrated lipase-catalyzed isoamyl acetate synthesis in a miniaturized system with enzyme and ionic liquid recycle. *Green Proc Synth*. **2013**, *2*, 561–568. **DOI:**10.1515/gps-2013-0082
72. https://www.corning.com/worldwide/en/innovation/corning-emerging-innovations/advanced-flow-reactors.html
73. F. Zhang, C. Cerato Noyerie, P. Woehl. E. D. Lavric. Intensified liquid/liquid mass transfer in Corning® Advanced-Flow™ reactors. *Chem. Eng. Trans.* **2011**, *24*, 1369–1374. **DOI:** 10.3303/CET1124229.
74. U. Novak, D. Lavric, P. Žnidaršič-Plazl. Continuous lipase B-catalyzed isoamyl acetate synthesis in a two-liquid phase system using Corning® AFR™ module coupled with a membrane separator enabling biocatalyst recycle. *J. Flow Chem.* **2016**, *6*, 33–38. **DOI:**10.1556/1846.2015.00038
75. L. Vobecká, L. Tichá, A. Atanasova, Z. Slouka, P. Hasal, M. Přibyl. Enzyme synthesis of cephalexin in continuous-flow microfluidic device in ATPS environment. *Chem. Eng. J.* **2020**, *396*, 125236. **DOI:**10.1016/j.cej.2020.125236
76. T. A. Hakala, F. Bialas, Z. Toprakcioglu, B. Bräuer, K. N. Baumann, A. Levin, G. J. L. Bernardes, C. F. W. Becker, T. P. J. Knowles. Continuous Flow Reactors from Microfluidic Compartmentalization of Enzymes within Inorganic Microparticles. *ACS Appl. Mater. Interfaces* **2020**, 12, 32951–32960. **DOI:**10.1021/acsami.0c09226
77. X. Tang, R. K. Allemann, T. Wirth. Optimising Terpene Synthesis with Flow Biocatalysis, *Eur. J. Org. Chem.* **2017**, 414–418. **DOI:**10.1002/ejoc.201601388
78. N. Adebar, J. E. Choi, L. Schober, R. Miyake, T. Iura, H. Kawabata, H. Gröger. Overcoming Work-Up Limitations of Biphasic Biocatalytic Reaction Mixtures Through Liquid-Liquid Segmented Flow Processes. *ChemCatChem* **2019**, *11*, 5788–5793. **DOI:**10.1002/cctc.201901107
79. R. Karande, A. Schmid, K. Buehler. Miniaturizing Biocatalysis: Enzyme-Catalyzed Reactions in an Aqueous/Organic Segmented Flow Capillary Microreactor. *Adv. Synth. Catal.* **2011**, *353*, 2511–2521. **DOI:**10.1002/adsc.201100394
80. R. Karande, A. Schmid, K. Buehler. Enzyme Catalysis in an Aqueous/Organic Segment Flow Microreactor: Ways to Stabilize Enzyme Activity. *Langmuir* **2010**, *26*(11), 9152–9159. **DOI:**10.1021/la9048727
81. R. Karande, A. Schmid, K. Buehler. Applications of Multiphasic Microreactors for Biocatalytic Reactions. *Org. Process Res. Dev*. **2016**, *20*, 361–370. **DOI:**10.1021/acs.oprd.5b00352
82. A. Šalić, B. Zelić. ADH catalysed hexanol oxidation with fully integrated NADH regeneration performed in microreactors connected in series. *RSC Adv*. **2014**, *4*, 41714–21. **DOI:**10.1039/C4RA05421K
83. K. Koch, R. J. F. van den Berg, P. J. Nieuwland, R. Wijtmans, H.E. Schoemaker, J.C.M. van Hest, F.P.J.T. Rutjes. Enzymatic enantioselective C–C-bond formation in microreactors. *Biotechnol. Bioeng.* **2008**, *99*, 1028–1033. **DOI:**10.1002/bit.21649
84. S. Mohr, K. Fisher, N. S. Scrutton, N. J. Goddard, P.R. Fielden. Continuous two-phase flow miniaturized bioreactor for monitoring anaerobic biocatalysis by pentaerythritol tetranitrate reductase. *Lab Chip* **2010**, *10*, 1929–1936. **DOI:**10.1039/c003561k
85. L. Vobecká, A. Romanov, Z. Slouka, P. Hasal, M. Přibyl. Optimization of aqueous two-phase systems for the production of 6-aminopenicillanic acid in integrated microfluidic reactors-separators. *New Biotechnol.* **2018**, *47*, 73–79. **DOI:**10.1016/j.nbt.2018.03.005
86. A. Romanov, Z. Slouka, M. Přibyl. Electric-field-enhanced selective separation of products of an enzymatic reaction in a membrane micro-contactor. *Biotechnol. Bioeng.* **2020**, 1–10. **DOI:**10.1002/bit.27597
87. A. Šalić, A. Jurinjak Tušek, A. Sander, B Zelić. Lipase catalysed biodiesel synthesis with integrated glycerol separation in continuously operated microchips connected in series. *New Biotechnol*. **2018**, *47*, 80–88. **DOI:**10.1016/j.nbt.2018.01.007

88. M. Gojun, M. Bačić, A. Ljubić, A. Šalić, B. Zelić. Transesterification in microreactors—overstepping obstacles and shifting towards biodiesel production on a microscale. *Micromachines* **2020**, *11*, 457. **DOI:**10.3390/mi11050457
89. J. Čech, W. Schrott, Z. Slouka, M. Přibyl, M. Brož, G. Kuncová, D. Šnita. Enzyme hydrolysis of soybean oil in a slug flow microsystem. *Biochem Eng J.* **2012**, *67*, 194–202. **DOI:**10.1016/j.bej.2012.06.015
90. N. Adebar, H. Gröger. Heterogeneous Catalysts "on the Move": Flow Chemistry with Fluid Immobilised (Bio)Catalysts. *Eur. J. Org. Chem.* **2020**, 6062–6067. **DOI:**10.1002/ejoc.202000705
91. B. Tomaszewski, A. Schmid, K. Buehler. Biocatalytic Production of Catechols Using a High Pressure Tube-in-Tube Segmented Flow Microreactor. *Org. Process Res. Dev*. **2014**, *18*, 1516–1526. **DOI:**10.1021/op5002116
92. M. O'Brien, I. R. Baxendale, S. V. Ley. Flow Ozonolysis Using a Semipermeable Teflon AF-2400 Membrane To Effect Gas-Liquid Contact. *Org. Lett.* **2010**, *12*,1596. **DOI:**10.1021/ol100322t
93. R. H. Ringborg, A. Toftgaard Pedersen, J. M. Woodley. Automated Determination of Oxygen-Dependent Enzyme Kinetics in a Tube-in-Tube Flow Reactor. *ChemCatChem* **2017**, *9*, 3285–3288. **DOI:**10.1002/cctc.201700811
94. J. Čech, M. Přibyl, D. Šnita. Three-phase slug flow in microchips can provide beneficial reaction conditions for enzyme liquid-liquid reactions. *Biomicrofluidics* **2013**, *7*, 054103. **DOI:**10.1063/1.4821168
95. E. Jones, K. McClean, S. Housden, G. Gasparini, I. Archer. Biocatalytic oxidase: Batch to continuous. *Chem. Eng. Res. Design* **2012**, *90*, 726–731. **DOI:**10.1016/j.cherd.2012.01.018
96. A. Manz, N. Graber, H. M. Widmer. Miniaturized Total Chemical Analysis Systems: a Novel Concept for Chemical Sensing. *Sens. Actuators B* **1990**, *1*, 244–248. **DOI:**10.1016/0925-4005(90)80209-I
97. P. Gruber, M. P. C. Marques, N. Szita, T. Mayr. Integration and application of optical chemical sensors in microbioreactors. *Lab Chip* **2017**, *17*, 2693–2712. **DOI:**10.1039/C7LC00538E
98. B. Ungerböck, A. Pohar, T. Mayr, I. Plazl. Online oxygen measurements inside a microreactor with modeling of transport phenomena. *Microfluid Nanofluid.* **2013**, *14*, 565–574. **DOI:**10.1007/s10404-012-1074-8

Povzetek

Industrijska biokataliza je ena ključnih spodbujevalnih tehnologij, ki skupaj s prehodom na kontinuirno vodenje procesov nudi možnosti za razvoj ekonomsko učinkovitih proizvodenj visoko kvalitetnih produktov in tvorbo majhne količine odpadkov. Izpostavljeni članek osvetljuje vlogo miniaturiziranih pretočnih reaktorjev s prostimi encimi in celicami v teh prizadevanjih na osnovi nedavnih primerov njihove uporabe v eno ali večfaznih reakcijah. Kapljična mikrofluidika omogoča izvedbo ultra visokozmogljivostnih presejalnih testov in hiter razvoj biokatalitskih procesov. Uporaba mikroreaktorjev edinstvenih konfiguracij zagotavlja zelo učinkovito kontaktiranje večfaznih sistemov, kar se odraža v intenzifikaciji procesov in izognitvi problemom, prisotnih pri konvencionalnem šaržnem procesiranju. Nadaljnja integracija z zaključnimi procesi nudi možnosti recikliranja biokatalizatorjev, kar prispeva k ekonomski učinkovitosti procesov. Uporaba okolju prijaznih topil podpira učinkovito reakcijsko inženirstvo in tlakuje pot tem visoko selektivnim katalizatorjem k postavitvi trajnostne proizvodnje.