Flow bioreactors as complementary tools for biocatalytic process intensification

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Abstract. Biocatalysis has widened its scope and relevance since new molecular tools, including improved expression systems for proteins, protein and metabolic engineering, and rational techniques for immobilization, have become available. However, applications are still sometimes hampered by low productivity and difficulties in scaling up. A practical and reasonable step to improve the performances of biocatalysts (including both enzymes and whole-cell systems) is to use them in flow reactors. This review describes the state of the art on the design and use of biocatalysis in flow reactors. The encouraging successes of this enabling technology are critically discussed, highlighting new opportunities, problems to be solved and technological advances.

Biocatalysis in flow reactors: why?

Bioprocesses (including biocatalysis) and continuous processing have been identified as the foremost key green research areas for sustainable manufacturing from pharmaceutical and fine chemicals industries [1]. Biocatalysis has widened its scope and application thanks to the massive advances in protein and metabolic engineering, together with biocatalyst immobilization [2]. The performances of a single enzyme can be improved in terms of substrate scope, operational stability and selectivity by advanced protein engineering, whereas biosynthetic pathways can be engineered in suitable microbial hosts leading to the preparation of molecules of different degree of complexity starting from cheap and largely available natural substrates (metabolic engineering). Biocatalysis is now a reliable tool to develop green and intensified processes, as long as proper reactor configurations are designed. Biocatalysis is customarily carried out in batch reactors, which are flexible and simple. However, biocatalysis in continuous flow reactors can be more productive, controlled and environmentally sustainable [3,4]. Specifically, **flow chemistry** (see Glossary) has rapidly developed both at industrial and academic level [5-8], encompassing the design of compact and reconfigurable manufacturing platform for the synthesis, and even formulation, of active pharmaceutical ingredients [9,10].

The transition from batch to continuous flow micro- and meso-reactors involves other two key concepts of modern industrial biotechnology: **green chemistry** and **process intensification** (see Glossary) [11]. Flow processing has the potential to accelerate biotransformations due to enhanced mass transfer, making large-scale production more economically feasible in significantly smaller equipment with a substantial decrease in reaction time, from hours to a few minutes, and improvement in space-time yield, with increases of up to 650-fold as compared to batch processes. The small dimensions of the reactors facilitated control of the reaction parameters, which can result in improved yields and productivities [12]. Better process control makes the reaction more efficient and waste generation is minimized. The modular nature of flow reactors also enables for the flexible increase of production volume by simply numbering-

up (putting flow reactors in series and/or parallel). Overall, these features result in reduced inventory, waste and energy requirements of the flow biocatalytic process, as compared to the conventional batch mode. Moreover, biocatalyst stability (Box 1) is enhanced by working in an environment where harsh mixing is avoided.

Biocatalytic processes in continuous flow reactors have attracted attention in recent years for carrying out continuous manufacturing systems with high level of intensification; here, we discuss the most significant innovations, new developments, trends, and future directions. Unfortunately, lack of clarity concerning basic lexicon and definitions of this multidisciplinary field is often encountered. This review aims also to clearly define the terminology and definitions, which apply to this rapidly expanding field and to provide recommendations for reporting of biocatalytic reactions in flow reactors.

Box 1. Biocatalyst stability

A general concern when using a biocatalyst is its lifetime. Enzyme stability is typically affected by several factors, such as temperature, pH, surfactants, etc., which can disrupt the catalyst structural interactions. Immobilization is often used to enhance enzyme stability: it limits heat and mass transfer, minimizing access of destabilizing agents to the enzyme; moreover, immobilization onto a surface introduces additional interactions that stabilize the tertiary structure of the enzyme; and loss of quaternary structure can be minimized by cross-linking unbound subunits to those already bound to the support.

In a batch operation, if immobilized enzyme is used, the biocatalyst can be recovered and reused. If needed, fresh biocatalyst can be added or/and the reaction time can be adjusted to maintain product quality. However, the amount of solid particles (biocatalysts) that batch reactor can handle is limited, and tuning of reaction time may impact downstream processes. In a flow reactor with immobilized enzymes in continuous operation, the temperature profile can be gradually increased to compensate for enzyme deactivation with increased reactivity, but a more practical approach consists of adjusting the

flow rate to the profile of enzyme deactivation, so that the quality of product concentration stays constant. A window of operation to evaluate the effective use of immobilized biocatalysts has been suggested based on the turnover number (tn), defined as the amount of product formed per amount of catalyst used [94,95]. Janssen and co-workers suggested that if the tn of a catalyst is too low, immobilization is not economically viable [94]. On the other hand, the tn may be very high, or the products may have high added value, resulting in catalyst costs under 0.05% of the added value. In this case, catalyst recycling is generally not justifiable. However, if a tn falls within these limits then enzyme immobilization is adequate. Additionally, the impact of catalyst costs on the product costs can be estimated based on the total turnover number (ttn), defined as the moles of product formed (or of substrate converted) per mole of catalyst employed. Accordingly, the ttn should exceed 1000 for high-value compounds produced at small scale, or 50000 for commodity goods [95]. The biocatalyst half-life (time required to reduce its activity to 50%) is a key parameter and can be determined under operational conditions [95]. Again, there is no gold standard for enzyme half-life, it is factored in the cost of the process.

Working principles in of biocatalysis in flow reactors

The market accessibility of automated and easy-to-control instrumentation for flow chemistry has increased in recent years, making controlled and reproducible work feasible. Different companies (*e.g.*, Vapourtec Ltd, Corning, Syrris, ThalesNano Nantechnology Inc., Accendo Corporation, AM Technology, Uniqsis Ltd, Future Chemistry Holding BV, Chemtrix BV, Advion Inc., Ehrfeld, and YMC Co. Ltd) offer now modules for micro- and meso-fluidic flow chemistry at lab scale.

The main components of a flow reactor for biocatalysis are schematically represented in Figure 1. The biocatalytic vessel can be a flow coil, a microfluidic device, a meso packed bed reactor (PBR, see Glossary), a membrane reactor or a wall-coated (surface immobilized) reactor. Mixing can be modulated

and placed at different points in the reactor, including at downstream of the reactor to aid product extraction. Heat and mass transfer can be accurately modulated by adjusting the material and size of the reactor. The outflowing stream can be analyzed in real-time [13]. Finally, in-line liquid-liquid extraction, solid adsorption, quenching, membrane separation, and solvent evaporation can be integrated with the biotransformation [14].

Figure 1 to be inserted here

Biocatalysis in micro- and meso-reactors: types and definition

Miniaturization is an important feature in flow chemistry and efficient micro- and meso-fluidic flow reactors are among the most important tools for the development of new and efficient continuous processes (Box 2). The opportunities and challenges for carrying out biocatalysis in microfluidic reactors have been previously reviewed [15]. Microreactors are often classified as either chip-type or microtube (microcapillary) devices [16,17]. Chip-type reactors usually have either a bankcard or microscope slide footprint with external dimensions not exceeding a few centimeters [18,19]. This allows for easy control of microfluidics and for the integration in a single device of more than one function or process, *e.g.,* sensors and control units, upstream and/or downstream processes. The integration of downstream processes enables facilitated in-situ product recovery (ISPR) [20,21,22]. A microcapillary reactor simply uses a microchannel as the reaction space. It can be made of glass, plastic or metal, and it is often prepared using gas or liquid chromatography parts. Besides its simplicity, this type of microreactor can be easily scaled up by assembling together a bundle of microcapillaries [18,21].

Box 2. Microreactors and mesoreactors

Miniaturization for continuous flow reactors has been typically associated with devices displaying channels or tubes with internal diameters between 10 μm to a few mm [20,39,41,96]. This scale range can be divided to comprise microreactors (also called microfluidic reactors) and mesoreactors at a threshold corresponding to an inner diameter sized 500 μm. Microreactors have diameters under this threshold, displaying a volume in the μL range, and specific areas (area/volume) between 5000–50,000 m^2/m^3, allowing: a) extremely effective heat and mass transfer; b) operation under low Reynolds number, corresponding to laminar flow, *e.g.,* smooth and orderly, like thin sheets gliding over each other where mixing is diffusion-limited, and c) fast and effective temperature control. Dean vortices are created in the bends of the channels, which enhance mixing along the length of the microreactor. The small dimensions have a penalty, since throughput is limited and high pressure drops and channel blockage may occur [15,39,97,98]. Channel blocking due to the presence of solids, either as reaction products or non-solubilized starting materials, can be minimized through the use of ultrasounds, that applied in proper level of energy and frequency can disperse aggregates; or by introducing in the channel a solvent where the solid is soluble, that may dissolve a part of it [99,100].

Mesoreactors have diameters sized between 500 μm and a few mm, corresponding to volumes in the mL range, and display specific areas between 100–10 000 m^2/m^3. Mesoreactors have higher flow capability and lower pressure drops but poorer heat transfer and diffusion-mixing features than microreactors. Moreover, for diameters exceeding 1 mm, high Reynolds numbers corresponding to turbulent flow, *e.g.,* highly disordered and chaotic, with frequent fluctuations, such as eddies and vortices superimposed on the main motion are likely to result except at extremely low flow rates [20,39,96]. For diameters within 500 μm and 1 mm, intermediate Reynolds numbers may occur, depending on the flow conditions. In this "grey" area of unpredictable flow, microfluidic behavior may be observed [20].

Mesoreactors are available with various designs to overcome the decreased mixing efficiency as compared to microreactors. Thus, mesoreactors may incorporate static mixing devices to cope with

mixing and back-mixing issues, such as Couette-Taylor devices to create vortices that induce a turbulent flow pattern or oscillatory baffled reactors, where a piston oscillates the flow to create eddies around each baffle, thus promoting turbulent mixing [41].

Either cell-free enzymes or whole cells can be used as biocatalysts. Immobilized enzyme reactors (IMERs) and free enzyme reactors (FERs) are the most common because cell-free systems offer advantages versus the whole-cell approach such as generally faster flow and lack drawbacks such as the additional barriers between the substrate(s) and the catalyst, the possibility of side reactions, and the need to maintain the cell wall integrity. Whole-cell biotransformations are particularly advantageous for co-factor depending enzymes, as the presence of native metabolic pathways, as well as endogenous cofactors, can make these processes self-sufficient. Whole cells can be used in a tubular reactor (or in a back-mix reactor), but to avoid washout during continuous operation and simplify cell recycle and downstream processing they can be immobilized (immobilized whole cells reactors, IWCRs). A recent review by Polakovič and colleagues describes the use of immobilized whole cells in packed bed meso-reactors and in micro-reactors [23]. Many applications regard immobilized whole cells with hydrolase activity, in particular in the field of biodiesel production [24]. In another recent application, the mycelium of *C. cladosporioides* MUT 5506, a strain endowed with transfructosylating activity, was used as alginate beads in an IWCR to produce a new mixture of fructooligosaccharides [25]. Whole cells of *Aspergillus oryzae* have also been used in a PWCR for the kinetic resolution (see Glossary) of flurbiprofen, in pure organic solvent [26].

Flow reactors are often used with free biocatalysts (Configuration 1, Figure 2, Key Figure), but they can be successfully combined with immobilized biocatalysts, allowing for their repeated use and easing downstream processing. Different arrangements for immobilized reactors are used: i) biocatalyst immobilized on beads that are packed in the reactor, allowing for high enzyme load but being prone to

excessive back-pressure (Configuration 2, Figure 2) [27]; ii) biocatalyst immobilized on the inner surface of the channels (coated wall reactor) (Configuration 3, Figure 2) [28]; iii) biocatalyst immobilized on a monolith contained in the microchannel (Configuration 4, Figure 2) [15], which minimizes the limitation of configurations 2 and 3; iv) biocatalyst immobilized on a membrane (Configuration 5, Figure 2), as reviewed recently [29,30]. A number of immobilization techniques are nowadays available for either using packed immobilized biocatalysts, also including the innovative use of magnetic nanoparticles, [31,32] or for directly attaching enzymes onto the reactor surface, also exploiting tagged enzymes [33-35]. Immobilization within the reactor allows to localize the enzyme in a microfluidic environment and to perform multienzymatic reactions where the sequential distribution of each enzyme across the structure of the reactor may be crucial to control the cascade reactions [36].

Figure 2 (Key figure) to be inserted here

An important issue of flow processes concerns their application on a large scale. For batch reactions, the scale has implications on the mass and heat transfer within the system, so the process conditions must often be re-optimized. Although scaling up microreactors for producing compounds at least at the gram scale seems simple on paper, the cost of individual microchip type reactors and the challenge of pumping liquid throughout the microreactors limit this approach. Larger mesoreactors can overcome these limitations, ultimately allowing for throughputs from g/h to tons/year. They may consist of scaled-up versions of the planar chip-type microreactor, single tubular reactors or parallel capillary reactors [37-40]. The amount of product generated is determined by the duration over which the entire flow regime is operated, once flow rates and reactor volumes are defined.

Flow in micro- and meso-reactors can occur in either monophasic flow or in slug flow (also known as segmented flow or Taylor flow), where two immiscible phases are present, producing discrete droplets

of solution (Figure 2, Configurations 6-9). Recirculation occurs within segments of the two-phase segmented flow, enabling a large surface area to be exposed to the second phase at any given time. By varying the relative flow rates of each stream, the size and periodicity of the slugs can be modulated and controlled. Slug-flow is often favored in mesoreactors to overcome back-mixing [41]. Bolivar and Nidetzky previously discussed key critical issues (*e.g.,* critical mixing, possible blockage, phase separation), re-dimensioning multiphase flow reaction performance and gave guidelines to design scalable multiphase biocatalytic microreactors [28].

Further knowledge about flow behavior in flow reactors can be obtained by using computational fluid dynamics (CFD), a tool that combines momentum, mass and heat transfer equations in complex geometries. CFD can theoretically evaluate the performance of flow reactors. Simulations can be compared with experimental data to provide relevant information on process conditions, such as the rate of substrate transport and how it is affected by channel geometry, the flow conditions and the physical properties of the fluid, to ultimately optimize the flow conditions and reactor configuration [29,42,43].

Analytical techniques (*e.g.*, liquid and gas chromatography and mass spectroscopy) have been interfaced with flow systems to provide real-time reaction monitoring [44]. Integrating sensors to monitor variable process conditions (*e.g.*, temperature, pH, dissolved oxygen, concentration of molecules) represents an important research topic; optical sensors, able to operate on-line or at-line, are particularly attractive, given their non-invasive, non-destructive nature, and footprint compatible with microfluidic devices [45]. In reactions where some parameters (such as oxygen or pH) can change, on-line monitoring is preferable, as it provides a direct measure of the progress of the reaction. This issue was recently successfully addressed by Gruber and colleagues, who used an optical pH sensor layer integrated in a microfluidic side-entry reactor to measure the pH at multiple points in narrow channels. This sensor could map the progression of a transketolase- and a penicillin G acylase-catalyzed reaction and constantly adjust the pH in the enzymatic reaction [46].

218 Finally, essential information about the bioreactor is required to reproduce and understand the results of
219 biocatalytic processes in flow reactors (see Box 3).
220
221 **Box 3. Reporting of biocatalytic reactions in flow reactors**
222 Key parameters need to be properly reported to reproduce and compare the performance of
223 biotransformation in flow reactors (Table I).
224 **Table I.** Key parameters of biocatalytic reactions in flow reactors.

Parameter	**Information required**
Reaction time, determined by the time the reagents take to flow through the reactor.	Residence time (τ); tracer experiments can be performed to determine the residence-time distribution function Specific reaction rates (see below)
Substrate concentration	Concentration of the substrates ingoing the reactors; stoichiometric ratios must be specified
Biocatalyst loading	Amount of biocatalyst used (mg or g of immobilized biocatalysts) and activity (U) at zero time
Reactor size	Available reactor volume; the dimension of the channels should be specified as well as the void volume (or total porosity, *i.e.*, void volume/geometric volume) for packed-bed and monolith reactors
Reactor productivity	Space-time yield normalized by the reactor volume
Reactor stability	Conversion at different times of operation (observed at optimal τ)
Biocatalyst productivity	Amount of product synthesized per amount of enzyme used. This information can be expressed as ttn, measured as the quotient of the k_{cat} (apparent turnover number) and the k_d (first-order deactivation rate constant), both measured at the same temperature [101].

225
226 Specific reaction rates in continuous-flow systems (r_{flow}) are generally calculated using the equation [90]
227 $$r_{flow} = [P] \times \frac{f}{m_{biocatalyst}}$$

where [P] is the product concentration flowing out of the reactor (commonly expressed as mmol mL^{-1}), *f* is the liquid flow rate (commonly expressed as mL min^{-1}), and $m_{biocatalyst}$ [g] is the amount of biocatalyst loaded in the column. Alternatively, the amount of biocatalyst can be replaced by its activity. Rigorous comparison between the rates of batch and continuous-flow biotransformations cannot be made, since the reaction rate is dependent on substrate/product concentrations. A major advantage of continuous processes is that they can be modulated through residence times so that the flow stream leaving the reactor contains a constant concentration of substrate/product; thus, different rates can be compared only at similar degree of conversions. Good examples are kinetic resolutions, where the desired degree of conversion of a racemic mixture is around 50%, therefore, a batch and flow reaction can be compared by evaluating the time (for batch biotransformations) and residence time (for flow biotransformations) necessary to reach this conversion.

Biocatalysis in flow reactors: selected examples

Hydrolases

Many examples of hydrolases, mainly lipases, can be found in the literature; reports from 1991 to 2013 have been previously reviewed [47,48]. Here, we review applications that bring significant innovation to the field.

Two-liquid phase systems consisting of water and a water-immiscible organic solvent are often used in biocatalysis to convert water-insoluble reactants, favoring the equilibrium of the reaction, and helping product recovery. Controlling liquid-liquid flow regimes in microchannels may provide large specific interfacial area: the formation of micro-droplets of hydrophobic organic solvents in water may allow for improved enzymatic activity in a continuous dispersed regime phase. Novak and colleagues described a microfluidic reactor integrated with a membrane separator for the preparation of isoamyl acetate, catalyzed by free *Candida antarctica* lipase B; optimizing the flow regime produced *n*-heptane droplets

252 in the aqueous phase containing the enzyme, furnishing a dramatic increase in the overall productivity
253 [49]. The in-line liquid-liquid separation permitted product recovery in the organic phase and the reuse
254 of biocatalyst for several consecutive biotransformations.
255 A new concept called flow Pickering emulsion involves compartmentalizing a biocatalyst in water
256 droplets (based on a water-in oil Pickering emulsion). The organic phase flows at the interface of the
257 droplet, promoting catalysis with a 10-fold improved efficiency when compared to a batch process [50].
258 Ionic liquids have also raised interest as enzyme stabilisers, and they have been used in continuous liquid-
259 liquid conditions where the substrates are dissolved in a non-polar phase immiscible with the enzyme-
260 containing ionic liquids. This method has been applied to lipases for the synthesis of chiral esters and
261 amides in flow with high turnover numbers and space-time yields with respect to batch [51,52]. Covalent
262 and non-covalent immobilization for a β-glucosidase has been reported for thin film continuous flow
263 processing; this technique requires a minimal amount of enzyme and relies on the large surface area
264 available for efficient biotransformation [53]. Britton and colleagues devised an interesting system where
265 different enzymes can be rapidly segregated through a metal coordinating poly-histidine tag in a
266 continuous-flow, vortex fluidic device (VFD). The approach allowed the formation of distinct thin-layer
267 enzymatic zones for multi-step biocatalysis, which the authors exemplified by combining an alkaline
268 phosphatase and phosphodiesterase in sequence for the two-step hydrolysis of bis(*p*-
269 nitrophenol)phosphate into *p*-nitrophenol phosphate [54]. Another important development is the
270 evolution of flow systems with increased complexity, such as the dynamic kinetic resolution of a
271 protected amino acid derivative (*N*-Boc-phenylalanine thioethyl ester) in continuous flow, mediated by
272 an alcalase, exploiting an alternating cascade of PBR and racemization reactors [55]. One advantage of
273 the flow mode was that the biocatalyst proved to be more stereoselective than in the batch mode by
274 minimizing the non-catalyzed reaction of benzylamine with the starting thioester, and the overall reaction
275 was strongly accelerated. A three-enzyme cascade capillary monolithic bioreactor consisting of an

immobilized deoxyribonuclease I, a snake venom phosphodiesterase, and an alkaline phosphatase, was recently reported to efficiently digest genomic DNA into single nucleosides [56]. Finally, a first application of a micro- reactor based platform to study enzymatic polymerization reactions in continuous flow mode was reported by Kundu and colleagues [57]; a versatile microreactor design enabled enzyme-catalyzed ring-opening polymerization of ε-caprolactone to polycaprolactone in continuous mode, in organic media, and at elevated temperatures.

Transferases

Using transaminases (TAs) in flow is very appealing because these enzymes are of great interest in pharmaceuticals. However, using cofactor-dependent enzymes under flow conditions is much more challenging. Interestingly, only a few examples of TAs in flow have been reported to date. Recombinant *E. coli* containing (*R*)-selective TAs were immobilized in a flow reactor; moderate residence times, clean production, and high biocatalyst stability were observed [58]. A cell-free TA from *H. elongata*, covalently immobilized on epoxy resin, proved to be very stable in a PBR at high flow rate for the synthesis of amines, purified in-line through a basification followed by extraction with EtOAc [59]. By tuning the reaction conditions, the same enzyme could be used for the mild oxidation of a large range of amines with excellent conversion yields [60]. Lentikats have also been shown to be valid alternatives for TAs in flow [61], as have silica monoliths, onto which the enzymes can be covalently attached [62]. Monoliths are particularly appropriate for designing continuous flow reactions, in particular because they exhibit high void fractions to minimize **pressure drop** (see Glossary)[28,63,64].

Transketolases (TKs) have also been reported in flow systems; one example describes the combination of a TK and a TA in sequential PBRs for the synthesis of 2-amino-1,3,4-butanetriol from hydroxypyruvate and glycolaldehyde. However, this setup used metal coordination, and the applicable flow was extremely low (between 2 and 30 µL/min). The enzymatic efficiency was lost within two weeks

for the TK, while the selected TA was almost completely inactive within 5 days [65]. A biocatalytic microfluidic multi-input reactor was developed using a soluble transketolase to address the limitations of single-point feeding [66]. With this strategy, an 8-fold improvement of productivity was obtained over fed-batch microplate reactions, an approach used to counter enzyme inhibition at high substrate concentrations.

The use of a cyclodextrin glycosyltransferase cross-linked to chitosan spheres in a PBR was reported for the production of β-cyclodextrin from renewable materials. The microfluidic enzymatic system resulted in high operational stability without loss of activity after 100 h of continuous use [67].

Lyases

Ammonia lyases have been of interest for their potential use in the synthesis of cinnamic acid derivatives and non-natural amino acids. Until recently, the immobilization of cell-free catalysts had been limited to methodologies that are poorly suitable for flow, such as cross-linked enzyme aggregates (CLEAs) and microcapsule entrapment. A phenylalanine ammonia lyase (PAL) was immobilized on carboxylated single-walled carbon nanotubes, but the performance of the catalyst in flow was stable only for 72 h [68]. The same enzyme was immobilized on magnetic nanoparticles and used in a Magne-Chip microfluidic reactor, where the enzyme was efficiently used for substrate screening [69]. Brahma and colleagues reported on the safe handling of HCN in flow for the synthesis of cyanohydrines mediated by hydroxynitrile lyase (HNL) from *Arabidopsis thaliana* in a telescoped two-step biotransformation combining CalB and HNL [70].

A flow enzymatic cascade with three different immobilized enzymes was developed to synthesize complex chiral carbohydrate analogues from aldehydes and dihydroxyacetone in the presence of pyrophosphate [71]. This configuration could shift the equilibrium of the aldolase-catalyzed transformation reaction towards the synthesis of the carbohydrates, eliminating retroaldol reactions and

affording the desired products with high yields and stereochemistry dependent on the aldolase employed. Flow reactors appear particularly suited for assisting the conditions in which enzymatic cascade reactions can efficiently take place; namely, favourable thermodynamics, controlled kinetics and high selectivity.

Oxidoreductases

Oxidoreductases are industrially relevant enzymes, however they rely on cofactors which are expensive and often not spontaneously regenerated in the catalytic cycle, a crucial issue to consider when employing these enzymes in flow reactors [72]. Šalić and Zelić assembled two microreactors where an alcohol dehydrogenase was used to oxidize *n*-hexanol in the first reactor with the concomitant reduction of NADH, whereas the second reactor was used for the recycling of the cofactor through reduction of acetaldehyde catalyzed by the same enzyme [73].

Co-immobilizing enzymes can assemble a coupled enzymatic system that specifically addresses the cofactor regeneration in close proximity and within the same bioreactor; Dall'Oglio and colleagues reported the efficient enantioselective reduction of bulky ketones in flow by combining a ketoreductase from *P. glucozyma* with a glucose dehydrogenase, which were compatible under the selected working conditions and catalytically active for several weeks despite the presence of 20% DMSO in the buffer [74]. An immobilized ketoreductase (P1B2 from Codexis) was used in a plug flow reactor (PFR, see Glossary) for the preparation of various chiral alcohols; the immobilized enzyme is highly active and stable in organic solvents, thus greatly simplifying product recovery [75-77]. An example of co-immobilization of both enzyme and cofactor was developed by Lopez-Gallego and co-workers where they successfully trapped NAD^+ (as well as FAD and PLP) in the same solid phase where the enzyme was immobilized. The cofactor remained available for catalysis and exogenous supplementation was no longer required [78].

Efficient O_2 -liquid transfer is required in bio-oxidation: this can be achieved in conventional batch reactors by high levels of aeration (also by using O_2-enriched air) and agitation. However, scaling up gas/liquid reactions in batch reactors is critical because when size of the reactor increases, maintaining a uniform gas/liquid dispersion, and efficient distribution of mixing energy is problematic. Biocatalyzed flow reactions can address the scale constraints of batch reactors. Among the developed multiphasic flow reactor configurations, tube-in-tube reactors and segmented-flow proved more efficient for gas-liquid transfer [79,80]. In a tube-in-tube reactor, a pressurized gas permeates through a Teflon AF-2400 membrane and reacts with the substrate in the presence of biocatalyst in liquid phase. Slug-flow reactors have been operated for the oxidation of (*R*)-limonene to (*R*)-perillic acid (using both wild-type *Pseudomonas putida* and recombinant *P. taiwanensis* VLB120) and for the stereoselective oxidation of achiral 1,3-diols with immobilized *Acetobacter aceti* [81,82]. In both the cases, poor oxidation was observed without gas-transferring devices. Recently, an automated tube-in-tube flow reactor system was developed for fast determination of the kinetics of oxygen-dependent enzymes [83].

A multiphase gas/liquid reaction also containing solids in the form of live cells and organic debris was successfully scaled up to 10 L using a dynamically mixed flow reactor, which avoided the accumulation of solids and blockage of the system [12,84]. This resulted in reduced cost of capital equipment, lower operating costs, and reduced catalyst consumption, due to faster throughput, for manufacturing processes. Finally, Table 1 reports selected examples of biocatalyzed flow reactions.

Table 1 to be inserted here

Concluding Remarks and Future Perspectives

Recently, Sheldon and Pereira introduced the concept of "biocatalysis engineering", which combines and entails the "engineering" of the different elements composing a biocatalytic process as a whole [91]. A

holistic approach, which integrates substrate engineering, medium engineering, protein engineering, metabolic engineering, immobilization engineering, biocatalytic cascade engineering, and reactor engineering should be considered for developing and optimizing biotransformations. In this context, this review shows that biocatalytic processes may be dramatically improved by continuous flow processing in both micro- and macro-structured reactors. Continuous manufacturing systems are particularly suited for making biocatalysis truly competitive with established preparative chemical methods. Many biocatalytic processes are carried out under heterogeneous conditions (immobilized biocatalyst, whole cells, liquid-liquid or liquid-gas systems): improved mixing efficiency achievable in flow reactors translates into improved mass transfer, consequently accelerating the overall process. The possibility to work in an environment where the biocatalyst suffers limited damages (controlled substrate concentration, continuous product removal, no mechanical stirring, highly overseen pressure and temperature) may lead to important increases in turnover number and frequency. Integrated processing and analytical control are easily realizable even on the microscale, facilitating progressive scale-up.

Moreover, the possibility to perform in-line work-ups or purification procedures aimed at isolating the sole pure product represents great potential for continuous flow processes. As most of the cost of manufacturing of a biotech process is attributed to the downstream processing, such advancement answers the need to circumvent this constraint typically associated to in-batch biotransformations. In-line purifications have been reported relying on extractions, catch and release strategies, use of scavengers or semi-preparative HPLC [26,89,92]. Integrated reaction and purification in microfluidic environment has been implemented for the complex cell free synthesis of protein, paving the way for the production of therapeutic proteins on location where required [93].

However, efficient membrane separators or suitable solid adsorbents still need to be developed to simplify recovery and purification of the products or reutilization of the biocatalyst, avoiding tedious and material-consuming downstream operations. On-line monitoring still remains one of the key

development needs for flow bioreactors, alongside strategies to handle slurries, which often lead to clogging. Moreover, the complexity of multi-enzymatic systems, and how to individually optimize reaction conditions for maximum throughput and process efficiency in flow, remain challenging (see Outstanding Questions). New approaches such as printing of customized 3D reactor device could facilitate these developments.

Therefore, further research on these issues is not only of interest for analytical and synthetic enzymatic reactions, enzyme stability studies or bioprocess development, but for a variety of biotechnological applications where continuous flow reactors can be envisioned. We feel there is a bright future for biocatalysis in flow reactors, with a number of biotransformations that may benefit from this approach towards more sustainable, greener production processes, possibly including modular and compact platforms up to production scale.

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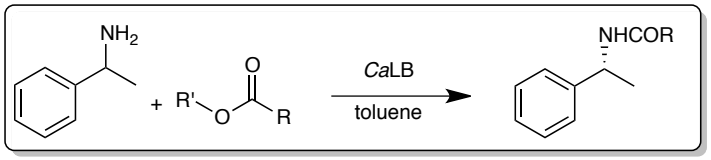
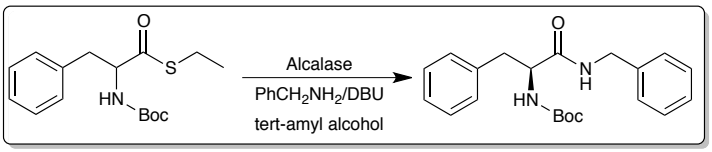
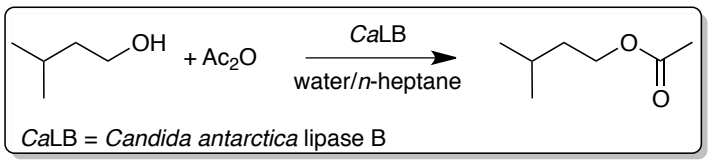
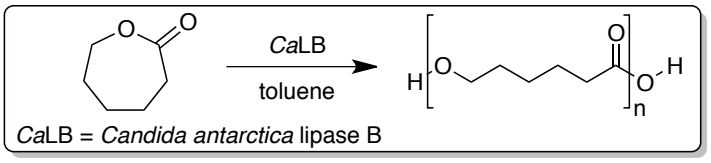
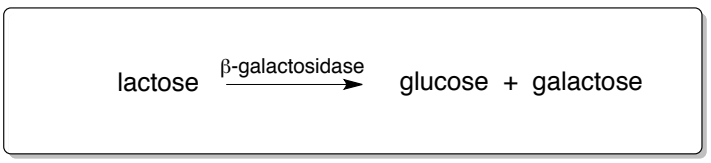
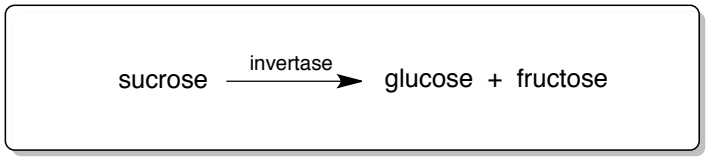
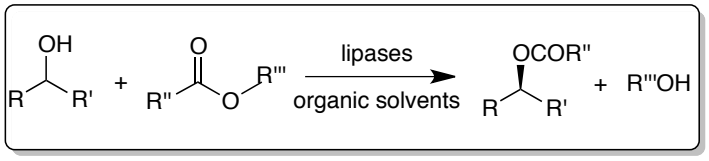
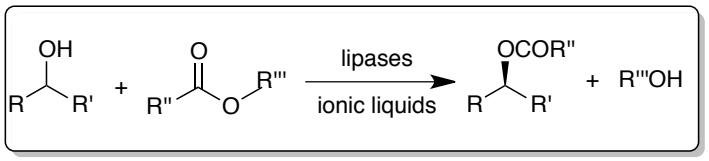
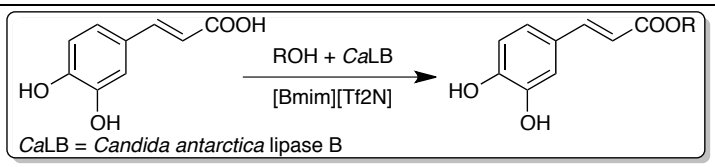
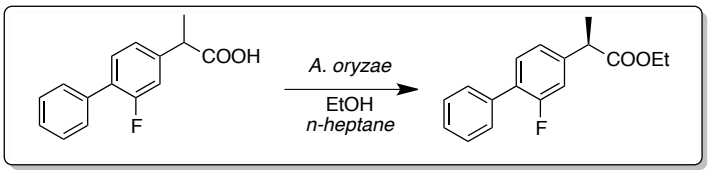
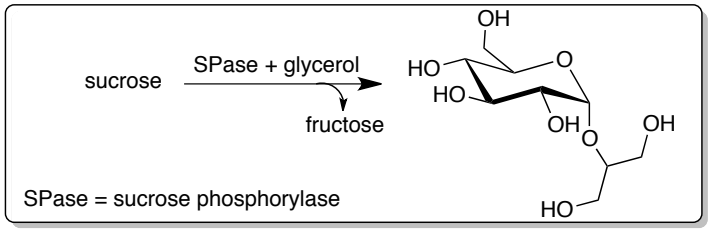
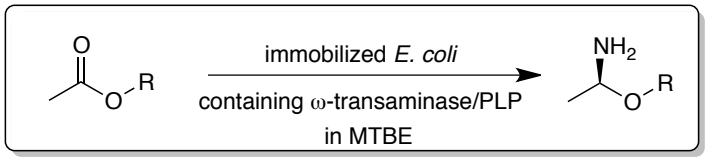
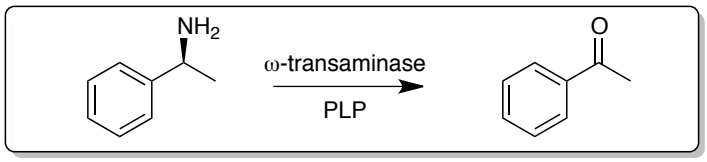
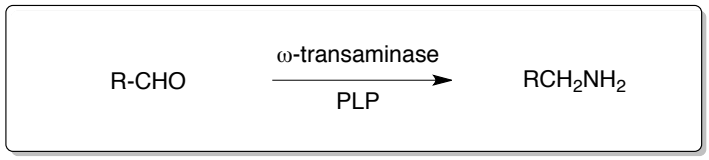
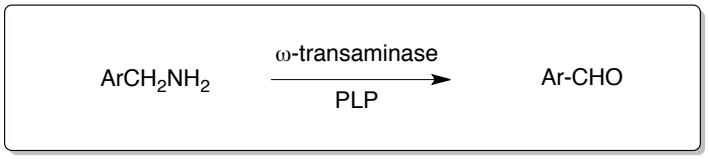
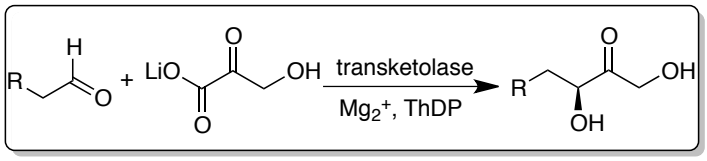
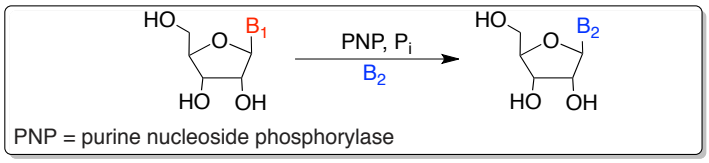
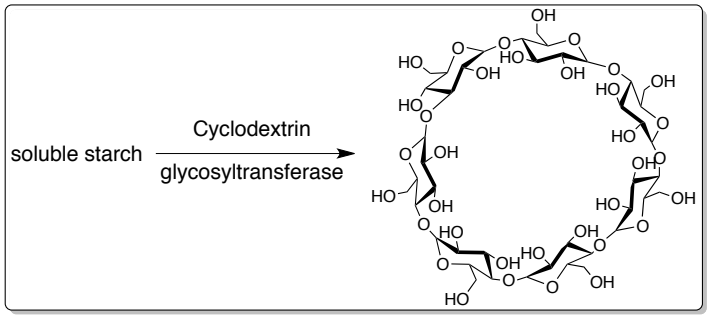
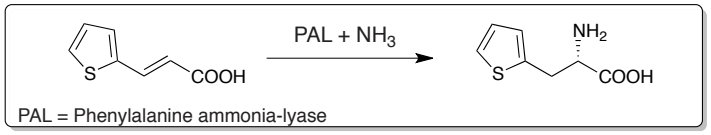
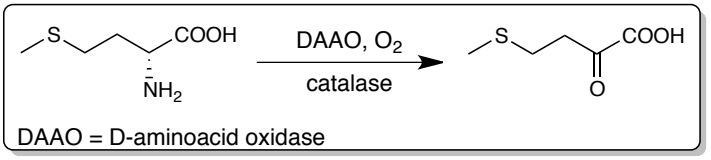
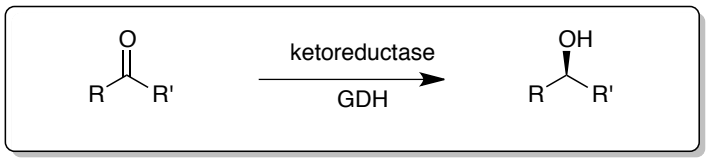
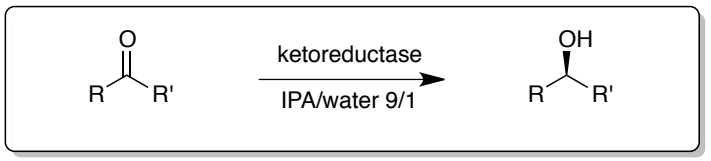
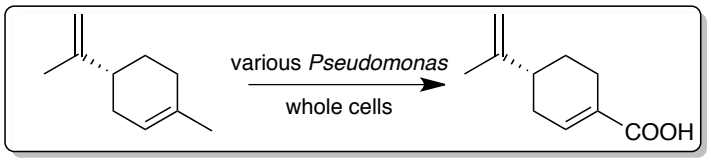
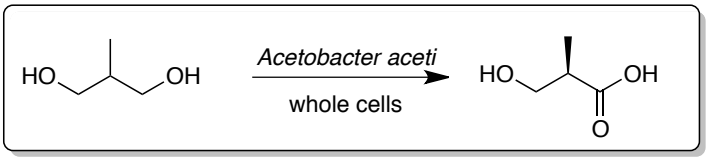
625

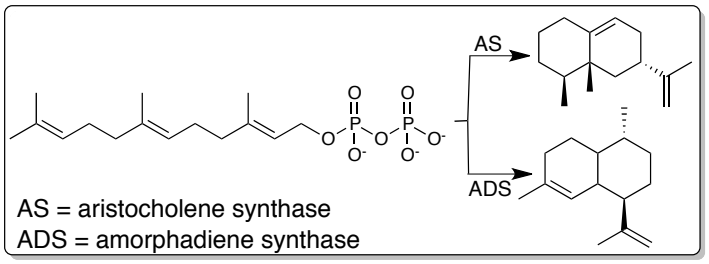
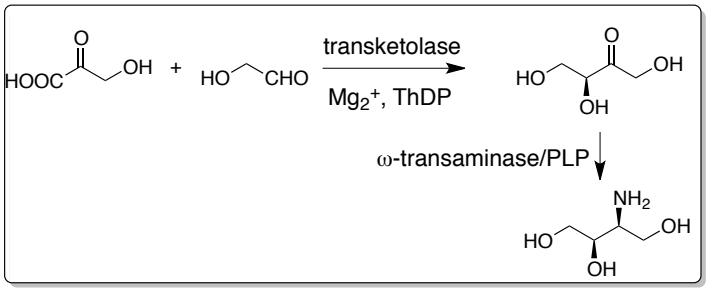
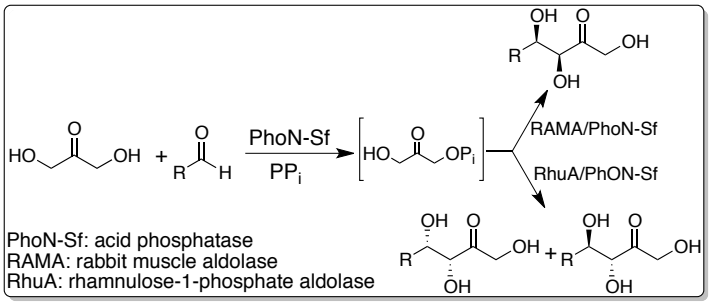
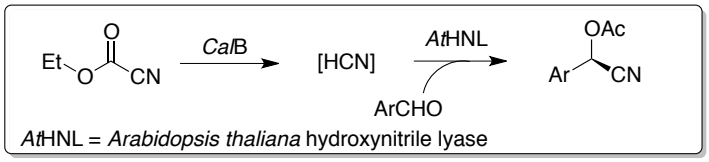
626 **Table 1.** Selected examples of biocatayzed flow reactions

Biotransformation	Reactor configuration and volume	Comments	Ref
Hydrolases			
	IMER, packed bed 0.82 mL	Productivities and space-time yields exceeding values for batch reactions by a factor of 3100 and 40	**41**
	IMER, packed bed, alternated with racemization columns 0.82 mL	Dynamic kinetic resolution; side reactions suppressed; enhanced productivity and overall acceleration	**45**
	FER, microfluidic connected with a l/l separator 0.50 mL	High productivity, flow regime of dispersed organic phase/water droplets with *in situ* extraction of product and enzyme recycling	**39**
	IMER, packed bed 0.52 mL	Enzyme-catalyzed polymerization in continuous mode; faster product formation compared to batch reactors	**46**
	IMER, micro- and multi-channel, wall coated 24.5 μL	Space-time yield of 500 mg glucose mL^{-1} h^{-1} (conversion 70%); Half-life of 15 days under the operational conditions.	**86**

	IMER, capillary wall-coated 0.45 mL	Residence time of 48.8 min, full conversion for 20 days	**87**
	IMER, packed bed 0.82 mL	High productivity, overall acceleration	**90**
	IMER, hybrid monolithic 2.49 mL	Improved biocatalyst productivity and space time yield	**41**
	IMER, packed bed, sandwich-like microchannel structure 0.40 mL	Almost complete conversion (99.5%), with overall enhanced acceleration	**42**
	IWCR, packed bed 3.50 mL	Improved enantioselectivity, overall acceleration, integrated with in-line product recovery; inline racemization of the unreacted substrate	**24**
Transferases			
	IMER, micro-channel, wall coated 24.5 μL	Space-time yields of 500 mmol L^{-1} h^{-1} at product titers of ~200 mM. Operational half-life of about 10 days.	**85**
	WCIR, packed bed with inline recovery 0.50 mL	Reaction in MTBE: no leaching of PLP from the cells; high	**47**

		enzyme stability (several days)	
	IMER, micro-channel packed bed 18.4 µL-3.2 mL	>80% activity retained after 21 days	**50**
	IMER, packed bed, inline recovery 0.9 mL	Low residence times, high conversions; in-line product recovery (ion exchange column)	**48**
	IMER, packed bed, inline recovery 0.9 mL	Low residence times/high conversions; in-line product recovery (liquid/liquid extraction)	**49**
	FER, microfluidic T-junction reactor 60 µL	Inline filtration device; complete conversion; 8-fold improvement of productivity over fed-batch bioconversion	**55**
	IMER, packed bed 0.830 mL	Bioconversion coupled with product purification; high yields = 52–89% within low τ, high biocatalyst stability	**89**
	IMER, packed bed 13.5 mL	Biocatalyst maintained 100% operational stability after 100 h of continuous use; productivity of 310 g/L h with flow rate of 5mL/min.	**56**
Lyases			

	IMER; carboxylated single-walled carbon nanotubes 0.2 mL	No loss of activity over 72 h up to 60 °C; enhanced overall productivity and acceleration	**57**
Oxidoreductases			
	IMER, micro-channel, wall coated 13 µL	operational half-life of the immobilized oxidase was 40 h.	**66**
	IMER, packed bed 0.90 mL	High biocatalyst productivity, high stability in the presence of 20% DMSO	**63**
	IMER, plug flow reactor 5 mL	High biocatalyst productivity, high stability and activity using IPA/water 9/1 as medium	**64**
	WCIR, catalytic biofilms with segmented air-liquid flow 0.31 mL	Max. volumetric productivity 33.8 g L^{-1} d^{-}Operation time 12 h	**68**
	WCIR, packed bed with segmented air-liquid flow 5.1 mL	Low τ (10 min) for reaching total conversion; recovery inline by catch-and-release strategy using a ion-exchange resin	**69**
Synthases			

	FER, water/pentane segmented flow in capillary tubes 2 mL	Water/organic segmented flow allows high mass- transfer rate without enzyme deactivation, leading to high yields	**88**
Cascade reactions			
	Sequential two packed bed IMER 1.5 mL each	Transaminase loading was much higher than transketolase due to low amination rate: matching enzyme loading allowed to optimize the dual- step enzyme reaction.	**54**
	Sequential two packed bed IMER 0.52 mL (PhON); 2.50 mL (PhON+aldolase)	The two sequential reactors allowed for favorable thermodynamics, controlled kinetics and high selectivity.	**60**
	Sequential two packed bed IMER 0.70 mL (*Cal*B); 0.35 mL (*At*HNL)	Acceleration of the reaction time over the batch protocols (40 min *vs* 345 min); safe generation in situ of HCN.	**59**

627

628

Glossary

Biocatalysis: the use of biological systems (mostly enzymes) as catalysts. Enzymes can be used as isolated proteins, crude cell-extract, or in whole cells; enzymatic preparations are often utilized as free or immobilized. Biocatalysis has widened its scopes and relevance owing to the development of different biotechnological techniques which allow for the production of satisfactory amounts of robust and selective enzymes.

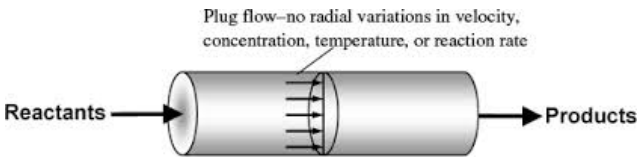
Flow chemistry: chemical reactions performed in a continuous flow stream. Reactants are pumped at known flow rates from reservoirs into a reactor where the chemical reaction takes place as flow runs through the reactor.

Green chemistry: is directed towards the development of chemical processes that reduce waste, conserve energy, and replace hazardous reagents and solvents with renewable substances. Paul Anastas and John Warner outlined 12 rules that would make green(er) a chemical process, concerning prevention, atom economy, less hazardous chemical syntheses, designing safer chemicals, safer solvents and auxiliaries, design for energy efficiency, use of renewable feedstocks, reduce derivatives, catalytic reagents *vs* stoichiometric reagents, design for degradation, real-time analysis for pollution prevention, inherently safer chemistry.

Packed bed reactors (PBRs): also known as fixed bed reactors, consist of a tube, filled with particulate biocatalyst (immobilized enzyme/cells) that remains in fixed positions relative to one another, as reaction medium is fed at either the top or the bottom of the column used, to form a continuous liquid phase between the particles. Screens placed on the liquid outlet to prevent the biocatalyst from leaving the column. The reaction medium must be free of solids to prevent clogging the bed. In conventional PBRs high flow rates may be required to ease mass transfer from the liquid medium to the solid catalyst, but this may lead to high pressure drop. If a PBR is operated in upflow mode at high liquid flow rates, the particles become suspended in the liquid and move about constantly. The outcome is a fluidized bed

reactor where, due to the movement of the particles, clogging of the bed and channeling (maldistribution of the flow) that may occur in PBR are avoided. Axial dispersion is significant in fluidized beds unlike PBR, hence the latter is closer to the PFR model.

Plug flow reactor (PFR): a cylindrical, fully segregated flow reactor, in which the whole content is radially mixed at any given location along the reactor length (flow direction), and no mixing occurs along the direction of flow. Concentration and temperature gradients are therefore absent in the radial direction and considered in one spatial dimension, *i.e.,* the distance along the reactor length. In this ideal steady-state reactor, the residence time (τ) in the reactor is the same for all elements of fluid, as the fluid velocity is alike from the wall to the centerline of the reactor and it is determined by the flow rate (f) applied in a reactor of fixed volume. In practice, as a result of velocity variations (parabolic profile at low Reynolds number), molecular diffusion and turbulent diffusion (at high Reynolds number) some elements of fluid will reside longer in the reactor than others, promoting axial dispersion and interaction between reacted and unreacted elements of the feed (backmixing). These reactors can operate with downflow, upflow and horizontal feed of the fluid. Fluid dynamics in microreactors closely resemble the PFR model. The continuous stirred tank reactor (CSTR, also called back-mix reactor) also features continuous input and output of material and steady state. However, the contents in a CSTR are perfectly mixed, hence, the concentrations and temperature are identical anywhere inside the reactor and in the exit stream. Therefore, the reactor size for similar conversion will be smaller for PFR as compared to CSTR. On the other hand, CSTR is preferred for reactions involving substrate inhibition. In real CSTR, pockets of stagnant zones may occur, ultimately resulting in an overall conversion in the outlet lower than ideally predicted.



Pressure drop: the pressure decrease observed between the two ends (length) of the channel. It results from the Navier-Stokes equation when the pressure forces balance the viscous forces. For steady-state laminar flow of a non-compressible fluid with viscosity μ, the pressure drop (ΔP), can be determined from the Hagen-Poiseuille equation, from the volumetric flow rate (Q) or the linear velocity (v). In a channel with circular cross section of radius r (or diameter D) and length L, this is given by:

$$\Delta P = \frac{8 Q \mu L}{\pi r^4} = \frac{32 \mu L v}{D^2}$$

Process intensification: rational use of manufacturing and development tools that allows for an increased efficiency, yield and sustainability of manufacturing processes, enhanced applicability of hazardous reactions, and a significant reduction in the time frame of process development and time-to-market. Concomitantly process intensification encompasses a marked decrease in reactor size, at least of 100-fold, while complying with a given production goal**.**

Enzymatic kinetic resolution: a process leading to the separation of enantiomers from a racemic mixture by means of an enzymatic reaction operating at different rates on the two enantiomers. Kinetic resolutions result in an enantioenriched sample of the less reactive enantiomer.

Figure 1 captions

A) Pumps: used to deliver reproducible quantities of solvents and reagents at flow rates; the usual types are piston, peristaltic, syringe or gear centrifugal pumps

B) Reaction loops: used to introduce small volumes of reagents

C) T-piece: primary mixing point, where reagents streams are combined

D) Coil reactor: provides homogeneous mixing for the reaction

E) Column reactor: packed with immobilized biocatalyst (immobilized enzymes or whole cells)

F) Back pressure regulator: controls the pressure of the system

G) Downstream unit: in-line analytics, work-up operations, etc.

H) Syringe pumps

I) Microfluidic reactors

Figure 2 captions

1. Free biocatalyst

2. Immobilized biocatalyst in a packed bed reactor

3. Biocatalyst immobilized on the inner surface of the channel

4. Biocatalyst immobilized on a monolith

5. Biocatalyst immobilized on a membrane

6. Free biocatalyst in a l/l biphasic parallel flow stream

7. Immobilized biocatalyst in a l/l biphasic flow stream

8. Immobilized biocatalyst in a g/l biphasic flow stream

9. Free biocatalyst in a tube-in-tube reactor