Advances on whole cell biocatalysis in flow

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Abstract

The combination of enabling technologies, such as biocatalysis and flow chemistry, represents an important opportunity to expand the chemical toolbox for the preparation of fine chemicals and pharmaceuticals under ambient and environmentally benign conditions. Whole cells are considered as the cheapest form of catalyst for bioconversion for many reasons among which the ready and cheap preparation, no need of expensive cofactor and the increased enzyme stability due to protective barriers offered by cell compartments. This review highlights some of the most recent advances in the field of biotransformations under flow conditions using whole cells. Different examples are provided where the use of continuous flow techniques enables the development of very efficient processes and multiple reaction steps to be combined into a single continuous operation.

Introduction

Continuous flow biocatalysis is fast becoming a key area of focus for researchers with applications in the production of fine chemicals, small-molecule active pharmaceutical ingredients (APIs), biotherapeutics, and biofuels, to name a few [1-9]. Either cell-free enzymes or whole cells can be used as biocatalysts in flow reactors. Although the great potential of whole cells and their wide use for industrial processes, their application in continuous flow environments is still not frequent. Whole-cell biocatalysts offer some unique advantages over the use of crude, purified, or immobilized enzyme preparations (Table 1) [10-14]. In addition to being the cheapest form of catalyst formulation, whole cells are particularly advantageous for cofactor-dependent enzymes, as the presence of native metabolic pathways, as well as endogenous cofactors, can make these processes self-sufficient. Moreover, residual cell wall represents a protective barrier also for resting or dead cells and thus enables biocatalyst applications under harsh reaction conditions or in unconventional (non-aqueous) reaction media [15]. In addition, importantly, the use of recombinant cells with non-natural cascades of enzymes as biocatalysts for complex reaction sequences is very attractive [16-21].

Table 1. Comparison between whole cells and isolated enzymes as biocatalysts

	Isolated enzymes	**Whole cells**
Advantages	No side reactions Higher permeability Tolerance to high substrate concentrations	No exogenous addition of cofactors Possibility to perform cascade reactions with the same biocatalyst No purification processes
Disadvantages	Addition of exogenous cofactors Increased time and costs associated with their purification processes Lower stability outside the cell environment	Side reactions due to metabolic pathways Difficult product recovery Low permeability due to cellular membrane

Soluble whole cells can be used in reactor channels that can integrate membranes enabling biocatalyst separation and recycling [22]. However, to simplify cell recycle and downstream processing, cells can be immobilized (immobilized whole cells reactors; IWCRs). Since these reactions can only be performed by viable cells, their immobilization should keep the cells in a viable state allowing stabilization of their catalytic efficiency and enable their repeated use. Several methods for whole cells immobilization are available and have been used extensively for production of useful chemicals via biotransformation (Figure 1) [12, 23, 24]. Among them, one of the most used technique of whole-cell immobilization is based on the formation of stable porous gels based on ionotropic gelation of water-soluble polyelectrolytes, usually polysaccharides containing charged functional groups (e.g., alginate, carrageenan, chitosan) with oppositely charged ions [25,26].

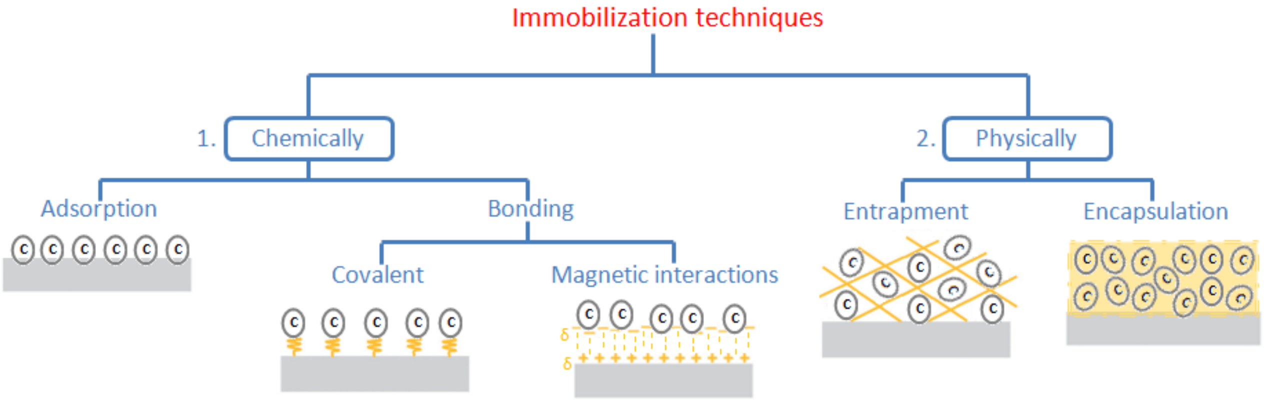

Figure 1. Whole cell immobilization methods

Whole cell biocatalysis in microreactors

Microbioreactors are a subject of intensive biochemical engineering research since they remarkably accelerate biocatalyst screening and engineering, as well as evaluation of process parameters, and intensify biocatalytic processes in multiphase systems. A microcapillary reactor is an engineered fluidic device that uses a microchannel as the reaction space; it is usually made of glass, silicon or polymeric materials that create a favorable microenvironment for the biomolecules. Whole cells can be immobilized on the channel surfaces by biofilm formation, by adherence to the liquid/liquid surface, by entrapment in porous matrices, or by individual confinement in aqueous droplets [27]. The high surface-to-volume ratio of microbioreactors offers the possibility to obtain high biocatalyst loading, which, together with short diffusional paths, leads to excellent accessibility of a biocatalyst for the substrate and thereby high biocatalyst productivity [28].

Polona Žnidaršič-Plazl's research group has been very active in this field of research for many years [29]. Recently, they described a microreactor with *S. cerevisiae* whole-cell biocatalyst immobilized in a copolymer hydrogel matrix (Fig. 1, Panel 2, entrapment mode). They prepared and fully characterized in terms of physico-chemical properties hydrogels of various compositions. Thin hydrogel films with yeast cells were integrated in a two-plate microreactor and efficiently used for L-malic acid production starting from fumaric acid with high volumetric productivities (2.86 g L^{-1} h^{-1}) and excellent stability [30]. Another very interesting work from the same group concerns the development of microscale reactors with surface-immobilized amine-transaminase (ATA). In this study, *E. coli* cells overexpressing ATA-wt have been immobilized on the surface of cycloolefin polymeric (COP, Zeonor®) meander channel chips previously treated with APTES and glutaraldehyde (Fig. 1, Panel 1, covalent bonding). The performances of the whole cell bioreactor were compared with other isolated enzyme microreactor systems. High volumetric productivities were observed with *E. coli* cells, which, considering the simplicity of biocatalyst preparation compared to isolated enzymes, might also present a very cost-effective approach for the synthesis of high value chemicals containing a chiral amine moiety [31].

The use of magnetic-field assisted microreactors using magnetic particles (microparticles, nanoparticles, or beads) for retainment of cells was also found as very promising approach for easily controlled biocatalyst immobilization (Fig. 1, Panel 1, magnetic interactions). Application of an external magnetic field to control the behavior of the biocatalyst inside the reactor was found to improve the reactor stability in absence of strong shear forces and at constant pressure, making the system an ideal host for whole cells [32, 33].

A unique and highly efficient whole cell microreactor with integrated dispersion membrane was recently developed for the biocatalyzed hydration of acrylonitrile to acrylamide, which is an important bulk chemical used for producing polyacrylamide, widely applied in diverse fields, such as enhanced oil recovery and water treatment. Acrylamide is currently produced using a tons-scale industrial biotransformation. The microreactor design ensured the dispersion of acrylonitrile into very small droplets, providing high specific surface area for adsorption of *Rhodococcus ruber* free cells containing nitrile hydratase (Fig. 1, Panel 1, cell adsorption). The faster substrate dissolution results in a significant acceleration of the apparent reaction rate due to the high catalytic activity of the free cells [34, 35].

Whole cell biocatalysis in packed bed reactors

Immobilized whole cells can be packed into the reactor channels, which is regarded as a packed bed-type channel. This ensures high surface area to volume ratios and relatively short diffusion distances between substrates and biocatalysts [36]. Some very interesting examples have been reported by Poppe's research group. In the paper by Nagy-Győr et al. [37], the authors have demonstrated an interesting strategy for the preparation of pure (*S*)-alcohols starting from racemic amines. The methodology involved the co-immobilization of whole cells from different microbial origins: *E. coli* expressing a mutant of *Chromabacterium violaceum* *S*-selective ω-transaminase (CvTA) and the unconventional yeast *Lodderomyces elongisporus* endowed with ketoreductase activity (LeKRED). The use of hollow silica microspheres with good mechanical properties and large surface area led to an entrapment-mode immobilization of the whole-cell suspension and its integration to flow reactors (Fig 1, Panel 2, entrapment mode). After optimization of the batch reaction conditions (e.g., ratio of bacterial/yeast whole cells, amount of silica particles mixed to the suspension), 4-phenylbutan-2-amine and heptan-2-amine were selected as substrates since the corresponding enantiopure products are important synthetic intermediates. At the end, a comparison between serially connected columns with separately immobilized CvTA and LeKRED whole cells and co-immobilized system was carried out in flow mode. The mixed cell TA-KRED reactor demonstrated better catalytic performances thanks to the beneficial effect of KRED, which enhances the TA reaction by removing the formed ketone from the equilibrium. The targeted biotransformations were subsequently performed in one-step on a preparative scale under flow conditions. The kinetic resolution of amines through immobilized transaminase-expressing *E. coli* whole-cells was further expanded by the same research group [38]. The use of transaminases with opposite stereopreference provided access to both enantiomers of the corresponding amines in continuous-flow mode. Very recently, they also published an excellent study in which different activities of wild-type yeast microbial cells were preserved by sol-gel entrapment immobilization resulting in "switchable" biocatalysts suitable for various biotransformations [39]. The enantiotope selective reduction of prochiral phenylacetone in the presence of fresh or recovered NADH cofactor and the following acyloin condensation using benzaldehyde were deeply studied and the corresponding products were obtained with good-to-moderate yields (47-91%) and high enantiomeric excess (96-99%). A good operational stability of yeast-cell-based immobilized biocatalysts was observed under flow conditions. The switchable biocatalytic activity of the immobilized yeast cells was demonstrated by consecutive biotransformations under continuous-flow conditions. Firstly the bio-reduction of phenylacetone was carried out using *L. elongispurus* whole cells and obtaining the (*S*)-alcohol in excellent yield and enantiomeric excess. Then, the reactor-column was washed with citrate buffer and the condensation reaction was performed giving the desired product with acceptable conversion (50%, e.e. 97%). In the third run, after rebuffering with phosphate buffer, the bio-reduction of phenylacetone was carried out again, providing comparable results to the first run, thus demonstrating the usefulness of yeast immobilized whole cells as multipurpose biocatalyst.

As mentioned above, the use of whole cells is particularly advantageous in the case of cofactor dependent enzymes because no additional cofactor is required for the reaction. Recently, De Vitis et al. exploited immobilized whole cells of *Acetobacter aceti* MIM 2000/28 for the continuous flow oxidation of prochiral diols to chiral 2-hydroxymethyl alkanoic acids, useful building blocks in the synthesis of high value chemicals [40]. The aerobic oxidation was performed using a segmented air-buffer flow, necessary for the bio-oxidation through acetic acid bacteria, as molecular oxygen takes part in the regeneration of cofactors during microbial oxidation. The reactor consisted of a packed bed column filled with wild-type whole cells of *A. aceti* MIM 2000/28 trapped into alginate beads (Fig. 1, Panel 2, entrapment mode). All the tested substrates gave the desired chiral 2-hydroxymethyl alkanoic acid as the major product, as only small amounts of the intermediate aldehydes were observed. No side-reactions (e.g., dehydration) previously detected with the free catalyst were observed, making the immobilized cells much more selective. Finally, an in-line purification step was applied, consisting of an ion exchange resin able to catch the acids in the out-flowing stream that were recovered with high chemical purity.

Finally, among challenges that need to be addressed for a more widespread use of biocatalyzed systems is the engineering of the reaction media that would be compatible to subsequent reaction steps where enzymatic parts typically prefer aqueous solutions, while many substrates are usually more soluble in organic solvents. Examples in which whole cells are used in pure organic medium or biphasic systems are rare. In this context, Hinzamann et al. described a biocatalytic transformation running in pure organic reaction environment. The process is based on the encapsulation of whole-cell catalyst in a superabsorber (polyacrylic acid with high affinity in binding water) as a “solid phase” in combination with an organic solvent with a high log P as flow stream (Fig. 1, Panel 2, encapsulation mode) [41]. This approach has been successfully applied for the preparation of *n*-octanenitrile from *n*-octanalaoxime using whole cells of *Escherichia coli* with over-expressed aldoxime dehydratase from *Bacillus sp.* Oxb-1 (OXdB). An efficient flow-setup with a packed-bed reactor containing the superabsorber-immobilized whole cells was fine-tuned. Almost complete conversion with high substrate loading was observed using cyclohexane as the solvent. Its high log P value ensures a low solvent concentration in the aqueous super-absorber phase as well as a negligible “dry-out effect” of removing water from the superabsorber to the organic medium.

Conclusion

The transfer of the exquisite catalytic efficiency shown by biocatalysts in Nature to chemical processes is a key element to implement the sustainability of a reaction. Moreover, a crucial issue for sustainable manufacturing is process intensification and the development of reactors for realizing productivity enhancements, in terms of increased throughput, better yield, conversion, and selectivity, smaller environmental footprint and intrinsically safer operations. In this context, biocatalytic processes in continuous flow reactors have attracted attention in recent years for carrying out continuous manufacturing systems with high level of intensification. This synergistic combination is attractive for developing greener synthetic protocols, in which biocatalyst stabilization is increased and the reuse is simplified. Whole cell biotransformations in continuous flow appear to be a very promising approach in terms of productivity, versatility and costs. Although its great potentiality, the application of this approach is quite limited. The main disadvantage could be that the cell membrane limits the penetration of the substrate and product, making the reaction slower compared to purified protein. Currently, technological developments in molecular biology, whole cell immobilization and reactor engineering are contributing towards enhancing the use of whole cells in flow reactors. Moreover, for a successful implementation of these methodologies for chemical manufacture, a critical issue is the integration of several catalytic steps in multistep organic syntheses, mimicking the metabolic pathways found in Nature, with the downstream processes.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

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