Cell-enzyme tandem systems for sustainable chemistry

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Abstract

The combination of isolated enzymes and whole cells for chemical biomanufacturing has recently arose as an alternative with multiple industrial advantages. Both isolated enzymes and whole-cell biocatalysis have benefits of their own that can be synergistically used in more efficient and sustainable bioprocesses. Those benefits range from decreasing the production times to generating products that are otherwise unobtainable. In this review we have studied the reports of cell-enzyme tandem systems applied as biocatalysts focusing on the different architectures used for their coupling. Combination of extracellular enzymes and microorganisms, enzyme display on whole cell walls and integration of enzymes and microorganisms into different materials are presented as the available alternatives for tandem enzyme-cell systems biotransfromations.

Keywords

Biocatalysis, biotransformations, enzymes, biocatalytic cascades, green chemistry, immobilization.

Introduction

It is now long known that migration towards greener industrial processes has boosted the application and studies of biotranformations as an alternative to harsher and more contaminant chemical conversions[1]. Scientists have more recently extended this concept towards the use of natural or unnatural cascades, mimicking Nature[2–6]. These cascades may be coupled systems of enzymes, microorganisms[7–9] or arrangements of both, aiming towards synthesis of more exquisite molecules, use of difficult substrates or extended half-life times of the overall biocatalytic systems.

The sustainability of bioprocesses based in cell-enzyme tandem systems lies not only in their greener approach but also usually in the use of renewable raw materials that are converted into commodities, minimizing the carbon footprint for their production. When transforming raw materials into high added value chemicals, the combination of isolated enzymes and whole cells creates a synergy that turns out to be very useful in chemical biomanufacturing. These hybrid systems afford the valorization of poorly soluble or metabolically inactive feedstocks (i.e cellulose) through the sequential action of extracellular enzymes that degrade the starting materials into metabolically active intermediates then utilized by the cell metabolism that ultimately yields the valorized produc[10,11]. In an opposite scheme, the microorganisms can transform a low-value material (i.e glycerol) into an intermediate that is secreted to the media and further upgraded by the extracellular enzymes under non-physiological conditions[12].

Tandem working enzymes and cells may be used as enzymatic solutions with suspended cells in either sequential processes in several reactors (Figure 1A) or in a concurrent process in one single reactor (Figure 1B). Moreover, free enzymes can be attached and display to the surface of microorganisms (Figure 1C) or co-immobilized with microbes into solid carriers (Figure 1D). Throughout this work, we will revise the recent literature on bioconversions based on the concerted cell and enzyme action with special emphasis on the biocatalytic system architecture. Although the literature on this type of systems is not extensive, their impact and ability to expand the scope of applications of sustainable biocatalytic processes is paramount.

Coupled systems of soluble enzymes and whole cells

The combinatorial use of isolated enzymes and whole cells have been mainly exploited to valorize agroindustrial wastes. This is well illustrated by the production of second-generation bioethanol where a cocktail of cell-free robust cellulases performs the saccharification of biomass feedstock to produce the glucose that is then fermented by yeasts to produce bioethanol. The enzymatic hydrolysis is often performed simultaneously to the fermentation as it is more cost-effective than performing both processes separately. Hemicellulose and lignin are other fractions of the biomass that have been also exploited to manufacture industrially relevant building blocks. To this aim, lignolytic enzymes and xylanases are combined with engineered microbes to valorize lignin and xylans into high-added value products such as furfural, isobutanol, aromatic acids, etc [13–15]. Like biomass feedstock, agro-food wastes are also appealing starting materials for the combination of isolated enzymes and whole cells. Particularly, dairy wastes like whey are attractive starting materials to produce high-added value sugars with an excellent purity. This biotransformation requires the extracellular hydrolysis of the lactose contained in whey to release the galactose that is ultimately isomerized to D-tagatose; the target product[16]. The limitation of this enzymatic system is the low purity of D-tagatose as it is produced as an undesired mix of D-glucose, D-galactose and D-tagatose. To increase the purity of D-tagatose, isolated enzymes that catalyze the hydrolysis of the lactose and the isomerization of the D-galactose to D-tagatose have been combined with yeasts whole-cell biocatalysts that metabolize the remaining D-glucose. Cervantes et al [16]. developed three sequential steps where a β-galactosidase was primarily incubated with the whey powder to quantitatively hydrolyze the lactose content into D-glucose and D-galactose. In a second step, the hydrolysate was treated with *Pichia pastoris* whole cells that fully consumed the D-glucose. Last, the cells were removed, the reaction crude was pH-adjusted to be finally incubated with free L-arabinose isomerase that aims transforming the left D-galactose into the targeted D-tagatose. Through individual optimization of each step, it was reported a D-tagatose titer of 30 g x L^{-1} starting from a whey permeate containing 180 g x L^{-1} galactose (Figure 2A). A similar titer but higher purity of D-tagatose was achieved using cell-free extracts of *E.coli*

constitutively expressing the endogenous β-galactosidase and recombinantly overexpressing L-arabinose isomerase. The resulting mixed sugar syrup was fermented by *Sacharomyces cerevisie* whole cells that metabolize the excesses of both D-glucose and D-galactose to concomitantly produce ethanol as a by-product[16] (Figure 2B). The co-immobilization of β-galactosidase and L-arabinose isomerase as bioorganic metal phosphates allowed recycling both enzymes up to five cycles maintaining up to 50% of the initial conversion of D-tagatose from the lactose of whey permeates[17]. A further advance of this biotransformation was recently published by Zheng and col [18] where a total of 23.5 g/L D-tagatose and 26.9 g/L bioethanol was achieved from cheese whey powder containing 100 g/L lactose.

Innovative combination of extracellular enzymes and engineered microbes are gaining momentum to upcycling plastics. The sequential use of a thermostable polyester hydrolase and evolved whole cells of *Pseudomonas umsongensis* GO16 were successfully combined to convert post-consumer polyethylene terephthalate (PET) into hydoxyalkanoyloxy-alkanoates, that are further used in the chemical synthesis of medium chain-length polyhydroxyalkanoates (PHAs) and poly(amideurethane) (bio-PU) [19]. This is a two-pot system where the polyester hydrolase degraded PET into terepthalic acid and ethyleneglycol in one pot, and the resulting hydrolysate, without intermediate purification steps, is directly transferred to a second pot where both degradation products are metabolized by the evolved microbe. These examples illustrate the potential of the synergies between free enzymes and engineered microbes to tackle two major challenges for our future society; the plastic pollution removal and the circular sustainable chemical manufacturing.

Although the combination of extracellular enzymes and microbes succeeds degrading and valorizing polymer-based substrates into industrially relevant small molecules, examples where both biocatalysts work concurrently in one-pot are scarce. As far as we know, the cost-efficient bioethanol production through the simultaneous saccharification/fermentation remains as the only industrial case where extracellular enzymes cooperate with microbes within the same vessel. Yet more examples of these synergistic combination will be demanded in the transition from a fossil fuel economy to a bio-based economy. Efforts should be orientated to match the fermentation conditions with the optimal ones for the free enzymes to work.

Nature inspired enzyme display at the surface of microbial factories

In nature, a great variety of enzymes are anchored to the cell walls to carry out diverse extracellular processes. The natural model to self-assemble multi-enzyme systems at the cell wall is the bacterial cellulosome[10]. Cellulosomes use protein scaffolds anchored to the outer cell wall to recruit and display cellulases. In addition, the natural cellulosomes are fused to cellulose binding domains (CBMs) that drive the cell adsorption to the lignoceullosic substrates[20]. This architecture is assembled through calcium mediated interactions between cohesin and dockerin domains fused to the scaffoldin and cellulases, respectively[21]. Cell surface display of biotic scaffold is certainly a key enabling technology to transform large insoluble raw materials unable to be either internalized into or metabolized by the cells. The displayed and scaffolded multi-enzyme systems at the cell surface is able to degrade those hardly metabolizable substrate and convert them into metabolizable ones. As cellulose biomass is one of the most abundant renewable feedstock that cannot be directly processed by the intracellular metabolisms, cell-surface enzyme display has been majorly applied in this [22–25].

Cellulosomes are proven to enhance the cellulose degradation by approaching the multi-enzyme cascades and the extracellular substrate – the cellulose –, which increases the concentration of the degradation intermediates and optimizes the stoichiometry of the enzymes involved in the degradation cascade. Inspired by nature, researchers have explored the boundaries of these biotic architectures to create synthetic cellulosomes. This idea was proposed for the first time by Bayer back in the 90´s[26] Later, several synthetic-(mini)-cellulosomes have been constructed for facilitating enzymatic hydrolysis rates on solid polysaccharides [27,28]. These artificial cellulosome have been created by combining enzyme chimeras fused to dockerin domains to dock a scaffoldin made of cognate specific cohesins from different species to be applied to different cascade enzymatic reaction[29–31].

This artificial cellulosomes have attracted the attention of synthetic biologists to display multi-enzyme systems at the surface of engineered microbes. To that aim, microorganisms like bacteria and yeast have been engineered to express outer membrane proteins that anchors extracellularly secreted or *ex-vivo* added protein scaffolds to spatially organize non endogenous multi-enzyme systems[25]. One of the most recent and illustrative examples of cell surface display in biotechnology is the use of engineered

Sacharomyces cerevisie to display a scaffold based on type II dockerin domains from *Clostridum thermocellum* fused to the yeast surface anchoring protein α-agglutinin subunit (Aga2). Such scaffoldin serves as anchoring point to assemble an extracellular consortium of celullases to enhance the bioethanol production in one-pot, starting from acid treated cellulose. This technology has proven successful to form cellulose-enzyme-microbe complexes that more efficiently convert the cellulose into metabolizing sugars[32]. However, the precise stoichiometry and nanometric organization of such complexes requires an extremely fine tuning of gene regulation to properly overexpress, display and secrete the whole system. For this reason, *in vitro* assembly of enzymes on the surface of microbes genetically programmed to display the scaffoldin is preferred to achieve more efficient multi-functional systems. One of the major limitations of such approach relies on the steric hindrances found by the enzyme to bind their corresponding protein cognates in the displayed scaffoldin. Interestingly, Smith et al.[32] found an optimal density of the scaffoldin units displayed at the yeast surface to maximize the density of the anchored cellulases, improving the packing efficiency of the assemblies. The optimal density of cellulases on the surface of yeasts achieved one of the highest ethanol titers ever reported for the one-pot cellulose degradation and ethanol fermentation. These insights are fundamental to boost the design of displayed multi-enzyme systems.

Beyond cellulases and cellulosomes, other enzyme families and anchorin proteins have been also displayed at the surface of different microorganisms as *Escherichia coli*[33]. For example, the widely used Spy-tag/Spy-catcher system was exploited by Gallus et al[34] to immobilize Spy-tagged oxidoreductases on the surface of engineered *E. coli* that expressed Lpp-OmpA fused to the spy-catcher. Recent examples of multi-enzyme systems anchored to the surface of microbes to cooperatively work with the intracellular metabolism are gaining scientific attention[35] but rather unexplored yet. Currently, there is a toolbox of anchoring/binding domains to display almost any protein at the surface of a variety of microbes. This could be exploited for the design of whole-cell biotransformations involving engineered microorganisms that degrade insoluble substrates into compounds that can be further transformed by the same microorganisms into high-added value molecules.

Hybrid biocatalysts: isolated enzymes and wholes cells confined within the same microenvironment.

The integration of whole cells and isolated enzymes into solid materials is even more rare in the fabrication of robust biocatalytic systems. Challenging as it may be, it is worth noting that the rational and tailored-made merge of whole-cells, isolated enzymes and solid materials may harness the best from the metabolic engineering, the cell-free biochemistry and the heterogenous biocatalysis to access more efficient biosynthetic pathways that involve intra and extracellular steps. Our group has applied this concept entrapping *Gluconobacter oxydans* and immobilized amine transaminases (iTA) into alginate-based hydrogels for the one-pot transformation of glycerol into serinol. In this system, *G.oxydans* resting cells oxidized glycerol to dihydroxyacetone without requiring the addition of redox cofactor, and the ketonic intermediate was further aminated by a ATA immobilized on agarose microbeads. The whole cascade took place within the alginate porous environment[12] (Figure 2C).

In a similar approach but aiming to produce and purify galacto-oligosaccharides (GOS) from lactose in one pot, Aburto et al [36] have co-immobilized β-galactosidase and *Saccharomyces cerevisiae* cells. Their biocatalysts design included crosslinked enzyme aggregates (CLEAS) of β-galactosidase entrapped in calcium alginate along with the whole cells. The strategy offered competitive advantages over conventional processes of sequential synthesis and purification without compromising the yield of the process. While β-galactosidase transferred or released galactose from lactose producing GOS enriched solutions, *S. cerevisiae* simultaneously consumed the glucose co-product that otherwise would have to be removed to use the syrup as probiotics in food industry. The combined immobilized biocatalyst proved efficiency in its reuse improving the amount of purified product per unit mass of biocatalyst. Hence, the co-immobilization of resting cells and isolated enzymes within the same solid carrier is an interesting approach to concurrently valorize wastes or low-value raw materials. Creating artificial microenvironments where the microbes synergistically work with isolated enzymes may improve the exchange of intermediates between biocatalysts.

Conclusion

Biocatalysis is increasingly being regarded as a powerful and green strategy in the industrial production of many chemicals. Although advancements in line with more

sustainable processes have been significant in the last years, many challenges still remain in the design and engineering of biotransformations for effective translational biotechnology sustainable solutions. This review has intended to show the benefits of combining both whole-cells and isolated enzymes as a recent enabling technology for step-wise biotransformations. Different configurations that merge the advantages of both catalysts have been reviewed. We have shown that a good number of these combined catalysts are applied to the valorization of industrial wastes or proposed to aid processes by decreasing production times, reducing or eliminating by-product generation, easing downstream processing and always increasing the half-life times of the biocatalysts. In the light of the evidence that we have herein analyzed, we envisage a bright future for cell-enzyme tandem systems in the improvement of established or novel processes.

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Figure Captions

Figure 1. Organization of cell-enzyme tandem systems. (A) Multi-pot system where whole-cell and cell-free biotransformation are physically segregated and performed in discontinuous pots (B) One-pot stepwise biotransformation where whole cells and isolated enzymes are mixed in the same reactor. (C) Enzyme display at the cell surface for step-wise biotransformation in one-pot. (D) Co-entrapped of whole cells and enzymes into microporous particles for step-wise biotransformation in one-pot.

Figure 2. Examples of alternate organizations of tandem systems for the synthesis of added value products. (A) Transformation of lactose into into D-tagatose through a cell enzyme tandem reaction separated in three sequential pots. β-gal: β-galactosidase. AI: L-arabinose isomerase. *P. pastoris*: *Pichia pastoris* [16] (B) A cell-enzyme tandem systems where several celullases are anchored to the surface of *Saccharomyces cerevisie* through an artificial cellulosome for the one-pot transformation of phosphoric acid pre-treated celullose (PASC) into bioethanol [32]. (C) One-pot conversion of glycerol into serinol

catalyzed by a hybrid composite that entraps *Gluconobacter oxydans* (*G. oxydans*) resting cells and an immobilized ω-transaminase (TA) immobilized on agarose microbeads[12].