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Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale

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Recent advances in biocatalysis have strongly boosted its recognition as a valuable addition to traditional chemical synthesis routes. As for any catalytic process, catalyst's costs and stabilities are of highest relevance for the economic application in chemical manufacturing. Employing biocatalysts as whole cells circumvents the need of cell lysis and enzyme purification and hence strongly cuts on cost. At the same time, residual cell wall components can shield the entrapped enzyme from potentially harmful surroundings and aid to enable applications far from natural enzymatic environments. Further advantages are the close proximity of reactants and catalysts as well as the inherent presence of expensive cofactors. Here, we review and comment on benefits and recent advances in whole cell biocatalysis.

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Introduction

In search of novel techniques towards a sustainable and biobased economy, biocatalysis contributes to various areas from the production of bulk chemicals from renewable resources to highly selective syntheses of fine chemicals or pharma products [1–4]. In all these applications, whole cell biocatalysts supply some unique advantages over the use of crude, purified, or immobilized enzyme preparations. In addition to being the cheapest form of catalyst formulation, whole cells already supply the cofactors needed for biotransformations and simplify their regeneration, usually making an external and expensive cofactor addition obsolete (see Section 'Cofactor regeneration'). Furthermore, residual cell wall compounds act in a protective manner also for resting or dead cells and thus enable enzyme applications under harsh reaction conditions or in unconventional (non-aqueous) reaction media [5]. Especially for hydrophobic substrates, applications in such media are strongly desired, as the originally proclaimed inherent greenness of biocatalytic reactions under aqueous conditions had to make way for a new perception: unconventional media can be ecologically advantageous, due to less generation of solvent waste during the reaction (less dilution) and during downstream processing (no extraction). Furthermore, such reactions become economically favorable due to higher product titers [6,7]. As a result, the combination of whole cell catalysts and unconventional reaction media is highly appealing for economically and ecologically beneficial process designs (see Section 'Applications in unconventional media'). These and other advantages and advances of whole cell biocatalysis are highlighted in this review. To keep a reasonable scope, we focus mostly on publications since 2012 and do not consider synthetic biology approaches utilizing entire natural or artificially introduced reaction pathways, which have been recently summarized [8–10]. The dynamic field of immobilization of whole cell catalysts will only be considered in context of particularly remarkable or novel techniques and reactor concepts, as immobilization of *Escherichia coli* whole cell has been nicely reviewed by Zajkoska *et al.* recently [11]. The second part focusses on approaches to overcome or alleviate common limitations in application of whole cell biocatalysts and presents novel reactor setups.

Advantages of whole cell catalysis

Catalyst costs

Whole cell catalysts, either in form of naturally occurring organisms or genetically modified expression hosts, represent the cheapest catalyst formulation possible. In a study on the cost contribution of biocatalyst production to biocatalytic processes, Tufvesson and co-workers found purified enzyme roughly 10-fold more expensive than whole cells [12]. Subsequent catalyst immobilization added even more costs to the analyzed process, with the carrier material being particularly impactful to the cost increase. Additionally, the scale in which whole cells are produced is decisive for the final catalyst price, meaning the bigger the scale the cheaper the catalyst (economy of scale). As soon as a sufficient market size for an aspired product is given, it is advisable to either invest into large scale production vessels or search for a contract manufacturer with appropriate facilities.

Cofactor regeneration

Especially in the case of cofactor-dependent reactions, whole cell biocatalyst is frequently used, as the inherent

presence of cofactors generated by the host itself and the ease of recycling grants significant advantages over the expensive external addition thereof. Consequently, especially reactions catalyzed by oxidoreductases, depending on nicotinamide cofactors, are often performed in whole cells, while the cofactor is traditionally regenerated by conversion of a sacrificial cosubstrate or by regeneration in an enzyme-coupled approach [13•]. Despite advances in the fields of electrochemical or photochemical regeneration methods [13•], the coupling of enzymatic reactions in redox-neutral multi-step reactions found great interest recently. Here, monooxygenase and alcohol dehydrogenase-catalyzed processes are frequently combined [14,15,16•]. Of course, such approaches rely on proper balancing of reaction rates of both enzymatic reactions and on the effectiveness and efficiency of such a route to access the product of interest. Another novel strategy is the use of 'smart' diol cosubstrates (Figure 1) [17]. After double oxidation of the diol, a stable lactone ring is formed, which drives the thermodynamic equilibrium to the product side. Because of this shift and the supply of two reduction equivalents per molecule of cosubstrate, 90 mol% of cosubstrate are saved in comparison to traditional alcohol cosubstrates, which usually require at least a five-fold molar excess. At the same time, many of the lactones produced in these approaches find potential applications as polymer building blocks and hence represent valuable co-products [18].

Many more examples of redox-neutral cascades [16•,19,20••,21,22] and other co-factor regeneration strategies can be found in the publications by Hummel and Gröger [14] and Kara *et al.* [13•,23•].

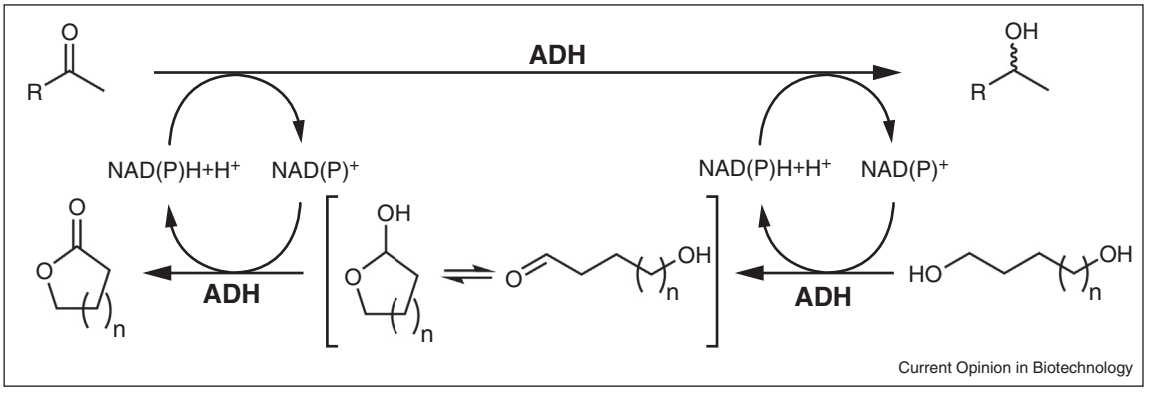
Applications in unconventional media

Productivity metrics of biocatalytic processes often suffer from low substrate and product loads, given the fact that biocatalytic reactions are either investigated in aqueous environments, which enable only poor solubility of many synthetic hydrophobic substrates, or because maximum concentrations are not tested. Nevertheless, increasing research effort has been spent on enzymatic conversions in unconventional media and the number of publications grows together with the increasing number and type of non-conventional solvents [5]. Because of numerous publications in these fields, we will neither consider lipase-catalyzed applications (which are frequently investigated in organic environment) [24] nor biphasic systems of water-immiscible organic solvent (which we consider as already widely known) [25].

Neat substrate systems

Neat substrate systems (NSSs; also termed solvent-free systems) represent the ideal case of a reaction setup, since they are built from the minimum components needed as the substrate(s) represent the solvent(s) at the same time. To add smallest amounts of water to restore activity of dried catalyst, the catalyst and substrates are pre-equilibrated over saturated salt hydrate solution resulting in a defined water activity [26]. Alternatively, water or buffer is added resulting in a micro-aqueous system, in which ideally no second phase is observed as the water is taken up by the solvent or the catalyst [27]. In 2011, Jakoblinnert *et al.* applied lyophilized *E. coli* whole cells expressing *Candida parapsilosis* carbonyl reductase (CPCR) in a mixture of only acetophenone as main substrate and n-propanol as a cosubstrate for cofactor regeneration. After optimization, product titers of 300–500 g L^{-1} were reached and downstream processing involved only catalyst removal and the evaporation of cosubstrate and coproduct to get hands on gained (*S*)-phenylethanol (*ee* > 99%) [26]. Hibino and Ohtake took a similar approach, but expressed two *Thermus thermophiles* HB27 alcohol dehydrogenases in the hydrophobic expression host *Rhodococcus rhodochrous* NBRC15564. Because of the high affinity of the catalyst to organic media/substrates, 97% of the model substrate 2,2,2-trifluoroacetophenon could be converted within 48 h to a remarkable product concentration of 634 g L^{-1} [28]. The concept of neat substrate systems was further adapted to the stereoselective production of (*R*)-and (*S*)-2-butanol

Figure 1



'Smart' diol cosubstrates are oxidized twice thus generating two equivalents of regenerated cofactor before lactone formation [17].

[29••]. This synthesis is particularly challenging, as the reversibility of the reaction leads to racemization of the product, which decreases the optical purity. To allow thorough control of the reaction progress, a continuous process was developed, retaining lyophilized *E. coli* cells, either harboring CPCR or *Lactobacillus brevis* alcohol dehydrogenase (LbADH), inside a membrane reactor. This reactor was constantly flushed with a mixture of 2-butanon, 2-propanol (as cosubstrate for cofactor regeneration) and 10% triethanolamine buffer. The reactions in this almost neat, monophasic substrate system could be optimized for maximum productivity yielding 2278 g L^{-1} d^{-1} (*R*)-2-butanol with a stereoselectivity of 96.6% and 180–461 g L^{-1} d^{-1} (*S*)-2-butanol (*ee* 98%), respectively [29••]. The simplicity of such processes together with the small waste volume generated make these approach particularly impactful, even though the success of these reaction systems is strongly depending on the catalyst's tolerance towards such high substrate concentrations, which might not be given in cases of substrate excess inhibition or catalyst inactivation due to excessive substrate loads.

Micro-aqueous solvent systems

Micro-aqueous solvents are an alternative when the high substrate loads of NSSs are prohibited, for example due to catalyst inactivation or safety concerns. Still, the advantages of high substrate loads, minimum water addition, and working in a monophasic system remain when diluting the substrates in organic solvent. As an example, the benefits of a micro-aqueous solvent system were demonstrated for the biocatalytic synthesis of cyanohydrins using recombinant *E. coli* cells containing *Arabidopsis thaliana* hydroxynitrile lyase (AtHNL). Since product formation also proceeds non-catalytically in the presence of water, only the application in micro-aqueous methyl *tert*-butyl ether (MTBE) enabled the hydrocyanation in a stereoselective manner (*ee* > 98% for (*R*)-mandelonitrile) [30]. For a similarly executed carboligation of aldehydes, pronounced aldehyde reactivity/toxicity towards the catalyst forbade using a NSS. Nevertheless, substrate concentrations of 500 mM could be achieved in micro-aqueous MTBE in a 2-step reaction employing recombinant whole cell catalyst for carboligation and oxidoreduction, respectively. Even with highly inactivating acetaldehyde and benzaldehyde as substrates, space-time-yields and product concentration of the batch reactions were outstandingly high, with up to 327 g L^{-1} d^{-1} and 49 g L^{-1} for (1*R*,2*R*)-phenylpropane-1,2-diol production [27].

Despite the benefits obtained from these organic systems, a current challenge remains in the desired switch to more benign solvents or those which can be produced from renewable resources such as 2-methyltetrahydrofuran [31].

Ionic liquids and deep eutectic solvents

In the last decades ionic liquids (ILs) evolved as a potentially greener alternative to the use of organic solvents, granting the advantages of lower toxicity, flammability, and vapor pressure [32,33]. As multiple recent and comprehensive reviews on ionic liquids [32,33] and their application in whole cell catalysis [34,35] can be found, we would like to only highlight some particularly impressive publications.

A systematic study on the influence of 90 different ionic liquids on cell viability was performed by Wood *et al.* [36]. Even though the compatibility was screened for living cells, and not with resting/dead cells in biotransformations, the results serve as a useful hint for IL selection. Good compatibility was generally found for imidazolium-based salts of short alkyl chain length, especially when they were mixed with alkyl sulfate anions [36], which might be another reason why these cations represent the most frequently used ILs in biocatalytic contexts in addition to their good commercial availability and wide application [35]. A trend of increasing catalyst activity with decreasing alkyl chain length described by Wood *et al.* was confirmed in the stereoselective reduction of ethyl acetoacetate using immobilized *Acetobacter* cells in biphasic systems [37]. In that study, immobilized whole cell catalyst retained activity in ten repetitive batches better in an IL-buffer-mixture than in pure aqueous buffer and the best performing organic-solvent-buffer mixture [37]. As an example of outstanding product concentration achieved with ILs, Wang *et al.* recently managed to yield remarkable 987 mM of (*R*)-[3,5-bis(trifluoromethyl)phenyl] ethanol using recombinant *E. coli* expressing an engineered carbonyl reductase from *Leifsonia* [38].

When highlighting ILs, it needs to be mentioned that the often proposed greenness of ILs has undergone some re-evaluation upon consideration of their ecotoxicity and the waste generation during production [39]. As a result, deep eutectic solvents (DESs) currently evolve as a more sustainable alternative granting similar advantages as ILs in comparison to classical organic solvents [39,40]. Compatibility of DESs with purified enzyme preparations has been demonstrated already for enzymes of different classes, but also recently for whole cell catalysts, such as wildtype *Arthrobacter* [41] or *Acetobacter* [42] species, baker's yeast [43,44], and recombinant *E. coli* [45].

Multi-step biocatalysis

Combining enzymes into multi-step cascade reactions can grant access to complex and valuable products from inexpensive starting substrates. Coexpression of multiple enzymes in one cell allows for an efficient reaction cascade due to the close proximity of catalysts for consecutive steps within a confined space. On the other hand, coexpression of multiple proteins may lead to a high metabolic burden during cell growth, which can result in poor overexpression and thus impaired catalytic performance [46]. Therefore, in some cases it can be beneficial to express each enzyme in single and later combine

the cells into cascade reactions flexibly [27]. Anyhow, cascade biocatalysis becomes particularly sophisticated yet appealing when redox-neutral (see Section 'Cofactor regeneration') or recycling cascades [47] are designed. A couple of excellent and comprehensive reviews on synthetic cascade reactions can be suggested to the reader [48–50].

Overcoming limitations of whole cell biocatalysis

Despite the advantages outlined so far, some drawbacks remain for whole cell catalysts. In the following we like to comment on frequently named downsides and show up novel strategies to overcome limitations.

Optimizing stability, recovery, and recyclability by immobilization

To facilitate recovery, recyclability and ideally stability of the catalyst, immobilization of whole cell catalysts is often desired and researchers strive for novel materials and techniques. After the first publication in 1998 employing the 'Lentikat' technology, numerous studied have been published on these lense-shaped polyvinyl alcohol immobilizates. A recent in depth characterization of Lentikat particles unveiled a pronounced physical strength of the lenses [51], making them well suited for stirred tank reactor setups. Of further importance, Lentikats including *E. coli* whole cell catalyst were proven storable for up to 15 month [52], enable almost identical reaction rates as free cells, and maintain their catalytic activity much better than freely suspended catalyst [53]. Another stabilizing technology is the silicone coating of recombinant *E. coli* adhesively grown on ion exchange resin. In doing so, hydrophobic as well as hydrophilized silicone coatings maintain the initial rate activity in comparison to uncoated immobilizate, whereas the hydrophobic coating even enables the immobilizate application in NSSs [54•].

Applications in which immobilization was used to enable highly productive reactor concepts were published recently with glutaraldehyde-crosslinked whole cell catalyst [55,56]. In the example given by Stojkovič and Žnidaršič-Plazl a 25 μL microreactor could be loaded with such a high catalyst load that space-time-yields 3.5 times higher than in a comparable 1 L membrane reactor were achieved [56].

As a carrier-free and particularly easy-to-prepare option for whole cell immobilization, we have recently transferred the handy and modularly applicable concept of 'teabag catalysis' to whole cell applications, by entrapment of lyophilized cells into containers of polyvinylidene difluoride membrane (cut-off 0.2 μm). The catalytic 'teabags' were not only shown to properly retain active catalyst, but also showed excellent recyclability even under challenging micro-aqueous conditions and extraordinarily high substrate loads (400 mM) [57].

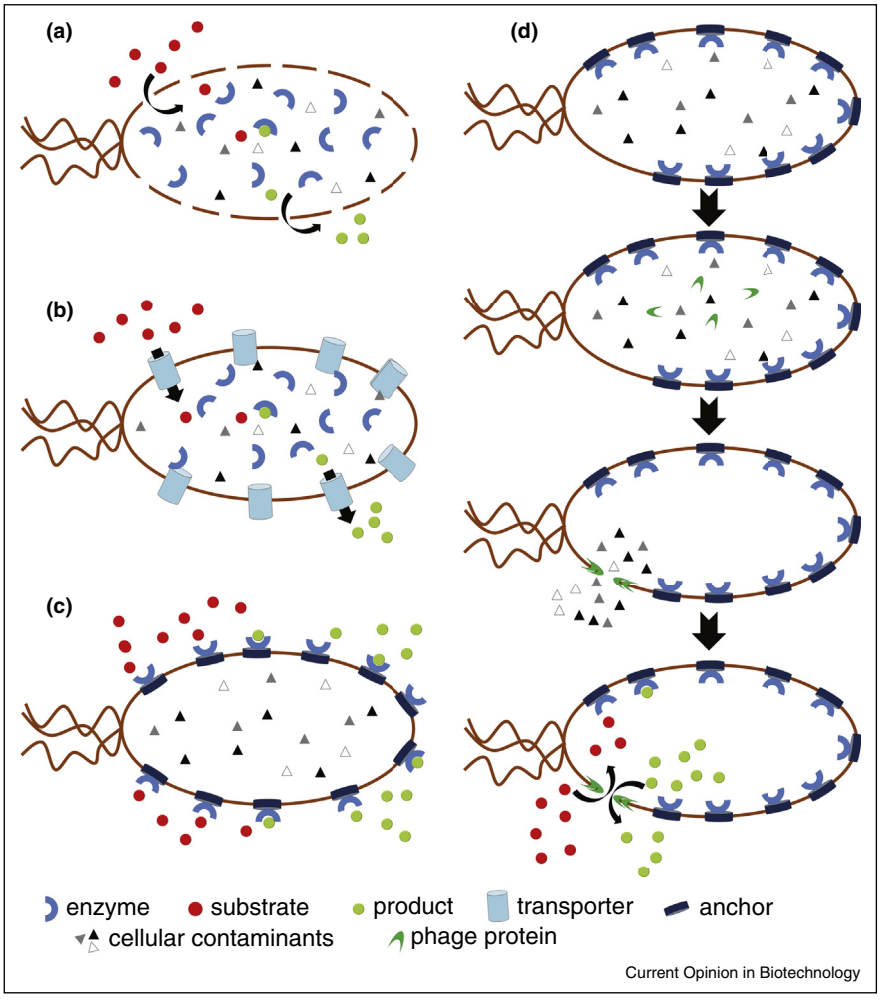
Regulatory issues

Some regulatory concerns might be raised when whole cell catalyst is used in the production of active pharmaceutical ingredients, which might prevent some researchers from using whole cells in such projects. Since catalyst formulation choice is a case to case decision usually based on economic considerations, we point out that numerous applications of whole cell reactions can be found in established pharmaceutical processes [58]. In some cases the use of dead whole cells can fullfill regulatory contraints. For almost any downstream processing challenge following biocatalytic reactions, technical solutions can be found [59]. Furthermore, the often proclaimed lowered selectivity of whole cell catalyst compared to purified enzyme, which may lead to unwanted sideproducts, can widely be overcome by a selective knockout of host cells enzymes if necessary, but is mostly obsolete when using powerful overexpression techniques [60,61].

Overcoming mass transfer limitations

Among the commonly named disadvantages of whole cell biocatalysts, the limitation in mass transfer due to the catalysts' cell membrane is the most prominent one. A classical way to decrease this negative effect is the deliberate permeabilization of the cell wall compounds by treatment with surfactants, chelating agents, or organic solvent (Figure 2a) [11,62]. An alternative to enhance mass transfer is the coexpression of membrane transporters as recently shown for the biocatalytic conversion of C7 to C16 alkanes (Figure 2b). Coexpression of the alkL gene from *Pseudomonas putida* enhanced the cell specific product yield up to 100-fold, due to an increased substrate uptake [63••]. Many more such examples of influx as well as efflux transporters potentially useful for biocatalysis have been summarized recently by Kell *et al.* [64]. Instead of enhancing the transfer of substrate towards the enzyme, others employed cell surface display techniques to rather bring the enzyme to the substrate (Figure 2c). As an example, *Candida antarctica* lipase B was displayed on *Pichia pastoris*, with the additional result of increased solvent and thermal stability [65]. Also for *E. coli* whole cell catalyst expressing a monooxygenase, the benefits of such systems have been demonstrated by Ströhle *et al.* [66]. Because of the facilitated recovery and reusability of the catalyst, a remarkable turnover number (TON) of >54,000 was reached, representing the highest TON ever reported for P450 monooxygenase according to the authors [66]. Sührer *et al.* proposed a different strategy enabling almost 28,000 fusion proteins per cell, by fusing galactosidase to an inner membrane anchor from rabbit liver cytochrome b_5. In addition, they introduced a lytic phage protein in order to induce temperature-controlled cell lysis by pore formation after biocatalyst production (Figure 2d). As a result, host cells interior components leach to the outside and can be washed away, leaving only the cellular envelope, carrying the recombinantly integrated enzyme, behind. This approach did not only

Figure 2



Modifications in cell morphology to increase mass transfer. **(a)** Cell permeabilization [11,62]. **(b)** Coexpression of membrane-integrated transport proteins for active influx and efflux [63••,64]. **(c)** Fusion protein of enzyme and membrane anchors to display enzymes on the cell surface [65,66]. **(d)** Fusion protein of enzyme and inner membrane anchors followed by belated induction of phage protein induction, leading to pore formation, efflux of cellular contaminants, and increased mass transfer of substrates and products [67•].

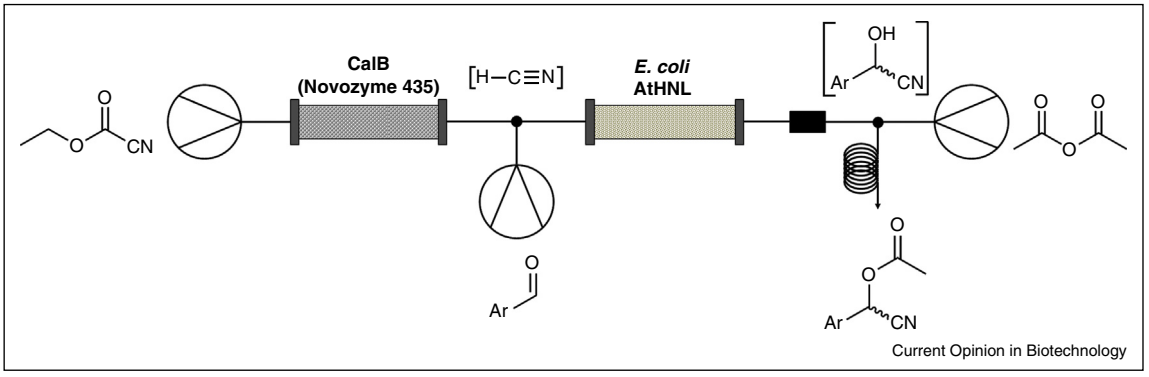
increase enzyme activity by 35% compared to free enzyme (presumably by stabilization of the tetrameric enzyme structure), but also increased activity of the catalytic cell envelope particles more than 3-fold when compared to intact whole cell catalyst [67•].

Advances in reactor concepts for whole cell biocatalysis

For preparative biocatalytic processes, reactors are needed which give the best compromise of maximum mass transfer at minimum mechanical abrasion of the catalyst. Additionally, the reliable scalability from investigatory scale to large scale manufacturing is desired, especially for challenging, unconventional biphasic or viscous media. Therefore, novel reactor concepts as well as screening platforms are constantly optimized to enable a fast and reliable process development. In a recent advance, a platform of 48 parallelized 10 mL reactors has been tested for biocatalytic reactions with whole cells in biphasic systems with 20% ionic liquids [68]. Each reactor was stirred with a small impeller, which was proven to allow identical local energy dissipation as a comparable 200 mL reactor equipped with a rushton turbine. When compared in the reduction of 2-octanone mediated by recombinant *E. coli* including recombinantly expressed LbADH, the reaction progress was the same on the 10 mL scale as on 200 mL scale for four different ionic liquids (content $\leq$ 40%) [68].

In another work, even the application of 24 well microtiter plates was shown to be adjustable to screen for scalable reaction conditions in biphasic systems [69]. Prerequisite is the utilization of small amounts of surfactant (0.1%

Figure 3

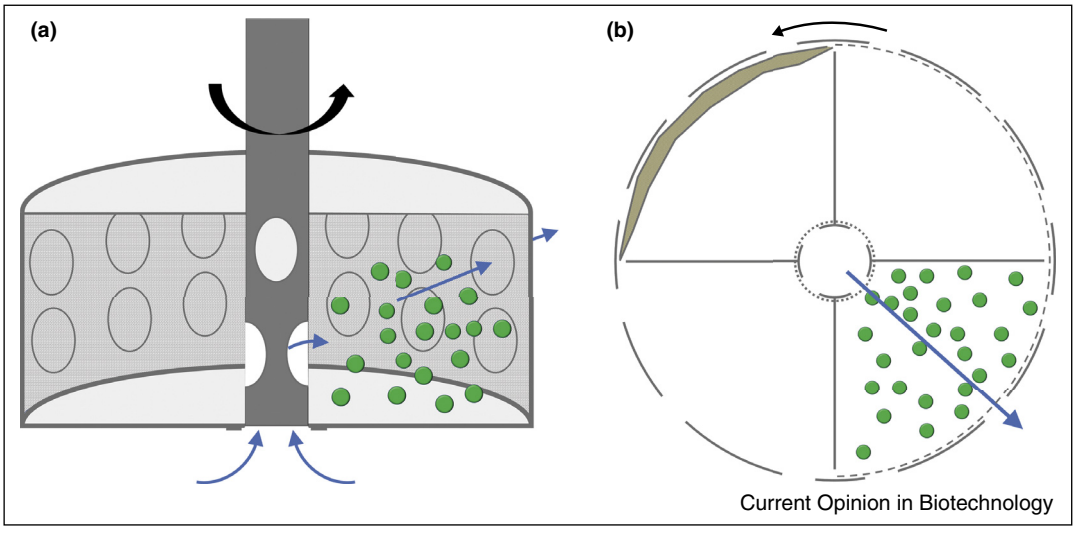


Biocatalytic 2-step synthesis of aromatic cyanohydrins. The entirely closed flow system allows a safe in situ generation of cyanide which is stereoselectively converted to various aromatic cyanohydrins. An in-line acetylation step further enables an in situ protection of the generated products [72•].

Triton X-100) and sealing with a special silicone cover. By these optimization, the conversion of n-dodecane into 1-dodecanol by recombinant *E. coli* in a 1.2 mL well proceeded with similar conversion rates than those obtained in a 120 mL shake flask and in a 1.2 L stirred tank reactor [69].

An emerging field in chemical catalysis is flow chemistry, as it offers the advantages of continuous operation, on-line-analytics and simple scale-up [70]. The first application of whole cell biocatalysis in flow has been published by Tamborini and co-workers in 2013 [71]. The deracemization of flurbiprofen was catalyzed by lipase within dried mycelium of *Aspergillus oryzae*. The esterification efficiency could greatly be increased by adding a module to capture generated water, thus shifting the thermodynamic equilibrium, and by an in-line capturing method to recover unreacted (*S*)-flurbiprofen for racemization and re-application into the reactor. In another example, the 2-step formation of cyanohydrins from cyclic aldehydes using recombinant *E. coli* whole cells was performed [72•]. Cyanide was released from ethyl cyanoformate by immobilized *C. antarctica* lipase B (Novozyme 435), mixed with different cyclic aldehydes, and converted into cyanohydrins by recombinantly expressed *A. thaliana* hydroxynitrile lyase in *E. coli* (Figure 3). This in situ

Figure 4



Schematic side view **(a)** and top view **(b)** cross sections of the SpinChem reactor concept. Rotation of the reactor leads to suction of reaction medium from the bottom of the hollow stirrer shaft inside the reactor. The flow streams through four immobilizate-filled compartments and is extruded to the outside. Alternatively to immobilizate particles polymeric membrane bags including lyophilized whole cell catalyst can be used (b, top left) [74,75].

generation and conversion of cyanide in the closed flow reactor greatly increased the process safety compared to classical batch processes.

Without the need for novel flow chemistry equipment, similar setups of continuous reactors can be set up by means of traditional plug flow or fluidized bed applications. Simply adding filter paper of appropriate cutoff to both ends of an empty HPLC column enables utilization of recombinant, lyophilized *E. coli* whole cell catalyst, as recently shown for the lipase-mediated conversion of glyceryl triacetate with methanol [73].

Another novel reactor technology enabling high mass transfer at a minimum mechanical force has been developed by Nordic ChemQuest AB. The so-called SpinChem has been proven feasible in biocatalytic transformation using immobilized enzyme, but also for whole cell catalyst entrapped in alginate beads (Figure 4). In comparison to stirred tank reactors, mechanical abrasion of catalyst is reduced while maintaining identical reaction rates [74]. As an alternative to the entrapment of whole cell catalyst into alginate beads, we recently demonstrated the application of catalytic 'teabags' inside the SpinChem reactor, exhibiting the same reaction rate as in a 140 mL model stirred tank reactor and 5 mL vial scale. Furthermore, the simple catalyst exchange when using the SpinChem reactor makes it particularly appealing for set-up and optimization of biocatalytic multi-step reactions [75].

Conclusion and future perspective

Whole cell biocatalysis offers many advantages over the use of purified or immobilized enzymes, among them (i) significantly reduced catalyst costs, (ii) an inherently higher stability due to the residual cell wall compounds, and (iii) no need for external cofactor addition. Disadvantages in terms of mass transfer limitations can be conquered at multiple stages, either by modifications of the cell wall or by engineering tools. In order to increase product concentrations, we see a huge potential of whole cell biocatalysts in the field of unconventional reaction media, which provides increasing numbers of biocompatible and sustainable solvents. In combination with novel, particularly atom-efficient cofactor regeneration tools, one can profit from two main advantages of whole cell catalysts: the high catalysts' stability in unconventional media and the cost savings for catalyst and cofactor production. Especially when enzymes are recombinantly expressed at high titers, when catalyst recyclability is facilitated by immobilization, and when a continuous reaction mode is applicable, whole cell catalysts can exhibit productivities far beyond industrial benchmarks. Such processes will become even more appealing when the production of fine chemicals or pharmaceutical building blocks is connected to precursors from renewable resources, thus not only boosting valorization, but also getting closer to the common goal of a more biobased economy. Here we see big potential, especially if synthetic biology techniques from the metabolic engineering field as well as recent advances in multi-step biocatalysis are combined.

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- •• of outstanding interest

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