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Modularized Biocatalysis: Immobilization of Whole Cells for Preparative Applications in Microaqueous Organic Solvents

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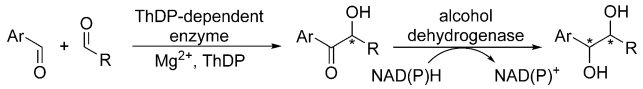
The use of whole-cell biocatalysts enables catalyst application in microaqueous reaction systems, in which the liquid phase consists of high substrate loadings in organic solvents, to enable access to high concentrations of easy-to-purify product. One current research focus is the modularization of single reaction steps to (i) enable flexible combinations into multi-step enzyme reactions, (ii) investigate ideal reaction conditions, and (iii) facilitate catalyst handling and recycling. Therefore, we published the easy-to-apply encapsulation of a lyophilized whole-cell catalyst in a polymeric membrane recently. These catalytic "teabags" were demonstrated to enable flexible catalyst combinations for multi-step reactions and excellent recyclability during repeated batch experiments. We now describe the applicability of these "teabags" on a larger scale by using the new SpinChem reactor and a classical stirred-tank reactor model. As an alternative, we investigate the described alginate entrapment approach and compare the results. The carboligation reaction towards (*R*)-benzoin, using lyophilized *E. coli* that enclose *Pseudomonas fluorescens* benzaldehyde lyase (EC 4.1.2.38), served as a model reaction. It was demonstrated that the catalytic "teabags" are scalable and perform equally on the investigatory 5 mL scale and the preparative 140 mL reactor scale. Tested in a more advanced application, the "teabags" were proven to be useful in a one-pot two-step reaction for the gram-scale production of 1-phenylpropane-1,2-diol by using the SpinChem reactor, which allowed to reach an industrially relevant product concentration (32.9 g L^{-1}) and space–time yield (8.2 g L^{-1} d^{-1}).

Introduction

Biocatalysis is becoming increasingly appealing to industrial applications if sustainability is a target because it is mostly a less toxic and more environmentally friendly technology in comparison to established chemical processes. In recent years, increasing attention in biocatalysis research has been paid to the combination of single biocatalytic steps into synthetic multi-step reactions to turn cheap starting materials into chiral building blocks.[1–4] To gain access to a broad product platform, we focused on the establishment of enzyme toolboxes. These toolboxes comprise numerous enzymes that catalyze one kind of reaction, but the catalysts differ in their substrate spectrum and stereoselectivity.[5,6] As an example, by an enzymatic two-step reaction, vicinal 1,2-diols can be generated. These compounds are versatile building blocks for active pharmaceutical ingredients[7] but their chemical synthesis often suffers from low stereoselectivity, toxic reactants, or low yields.[8–16] Biocatalytically, we are able to access a platform of differently substituted diols selectively by the reduction of the corresponding 2-hydroxy ketones using a diverse toolbox of alcohol dehydrogenases.[6] The 2-hydroxy ketones can also be produced biocatalytically by the carboligation of aldehydes using another toolbox of enzymes that depend on thiamine diphosphate (ThDP) (Scheme 1).[5]



Scheme 1. Multi-step reaction that employs the carboligation of two aldehyde substrates and subsequent oxidoreduction of the resulting 2-hydroxy ketone towards a 1,2-diol (Ar: aromatic residue, R: aliphatic residue, asterisk: chiral center).

Despite the advantages of biocatalysis, many enzymatic reactions are limited by poor substrate solubility, limited product concentrations, low catalyst stability, or expensive catalyst preparation, which decrease the potential of implementation into industrial-scale processes.[17,18] To overcome these restrictions, our approach combines a cheap lyophilized (freeze-dried) whole-cell catalyst[19–22] with microaqueous organic solvents. On one hand, whole cells might lead to the generation of by-products or less specific biotransformations from the interference of host-cell enzymes.[18] On the other hand, the application of a lyophilized whole-cell catalyst has the advantages of (i) no need for expensive enzyme purification, (ii) facilitated handling (compared to wet cells), (iii) excellent storability, (iv) an often increased catalyst stability (compared to free enzyme), and (v) no need to add any cofactor externally.[18,23–25] In combination with microaqueous solvents, high concentrations of poorly water-soluble compounds can be applied as

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the reaction medium is mainly composed of a suitable organic solvent. The organic solvents are supplemented with small amounts of buffer, just enough to restore the enzyme activity within the lyophilized cells.[24,25] Besides the high substrate loads, these media enable a facilitated downstream processing simply by solvent evaporation, which renders extraction steps needless.

To enable an easier investigation of catalyst combinations or reaction conditions and to further facilitate the recycling and handling of catalyst, we are striving for the modularization of single enzymatic steps. Well-established methods are, for example, immobilization by entrapment in alginate, agar, or polyvinyl alcohol.[26] As an alternative, we recently published the encapsulation of lyophilized catalyst into a polyvinylidene fluoride (PVDF) western blotting membrane (0.2 µm cut-off) to result in catalytic "teabags".[25] The "teabags" are (i) easily applicable, (ii) recyclable in at least five consecutive batches, (iii) applicable in microaqueous organic solvents at elevated substrate concentrations, and (iv) allow final product concentrations to be reached similar to those obtained with freely suspended cells.[24–25] However, the membrane seems to limit the diffusion of substrates and products, which manifests in low reaction rates. Furthermore, this "teabag" encapsulation has so far not been used in a properly scalable reactor setup. To investigate and possibly overcome a diffusion limitation as well as to shed light on the unproven scalability of "teabags" is the target of the presented work. We used the newly developed SpinChem reactor (Nordic ChemQuest AB, Umeå, Sweden), which was designed initially to be used in heterogeneous chemocatalysis and is available for small-lab scale up to industrial production dimensions. It was described to decrease mass transfer limitations and at the same time prevent catalyst degradation.[27] In a SpinChem reactor, immobilizate particles are loaded into a quartered cylinder (called the SpinChem). Upon the rotation of the SpinChem in the reaction vessel, the reaction medium is sucked through a hollow shaft at the bottom of the cylinder and pushed through the reactor compartments by centrifugal forces (Figure 1).

Here, we investigate whether the advantages of the "teabag" encapsulation can be combined with those of the SpinChem reactor, even though the manufacturer made us aware that the SpinChem was not intended for immobilizate preparations such as the catalytic "teabag". Additionally, to test a more ubiquitously usable alternative to scale up our reaction system, we investigated the applicability of the catalytic "teabags" in a stirred-tank model reactor on a 140 mL scale. As an established alternative to the use of "teabags" in microaqueous systems, we further investigated the alginate immobilization of a whole-cell catalyst[28] for comparison.

As a model reaction, we chose the carboligation of benzaldehyde towards (*R*)-benzoin (Scheme 2a) catalyzed by lyophilized *E. coli* SG13009 cells that enclose overexpressed *Pseudomonas fluorescens* benzaldehyde lyase[29–31] (BAL; EC 4.1.2.38; UniProt code Q9F4L3). To prove the applicability in more advanced applications, such as one-pot two-step reactions, the preparative synthesis of (1*R*,2*R*)-1-phenylpropane-1,2-diol (PPD) using BAL and *Ralstonia* sp. alcohol dehydrogenase[32,33] (RasADH; EC 1.1.1.1; UniProt Code C0IR58) in microaqueous solvent was performed (Scheme 2b).[6,24,25,34] We show that our "teabags" are applicable for single-step biotransformations as well as enzyme cascades not only on a small scale but also on a preparative scale.

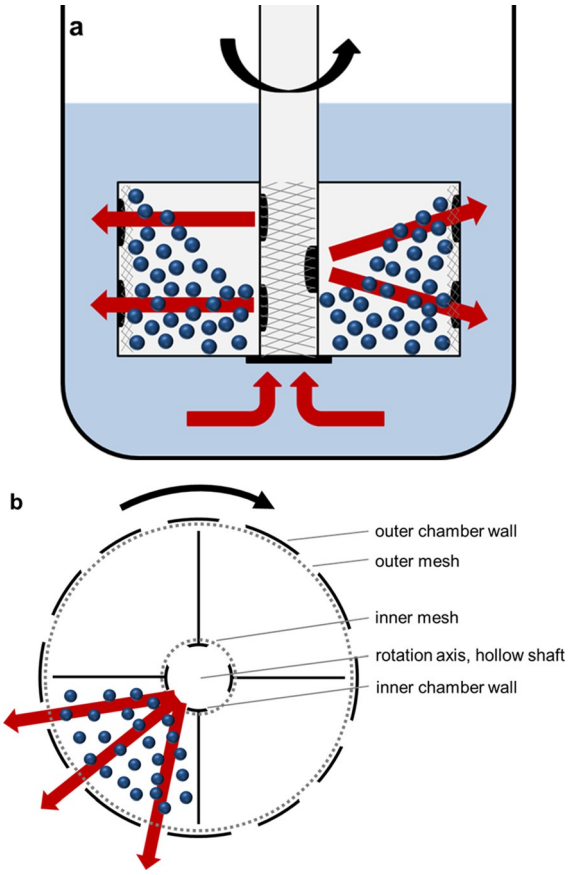
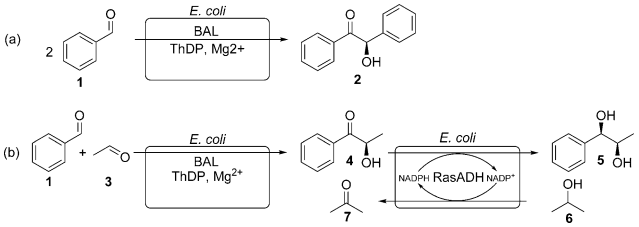


Figure 1. a) Schematic side view of the SpinChem, b) schematic top view of the SpinChem (blue balls: immobilizate particles, red arrows: liquid flux, black arrows: direction of rotation; meshes are used to retain catalyst were omitted in reactions in which the "teabag" ring was used; Scheme adapted from Byström, E. (November 20, 2012), Video: SpinChem-Intro, Retrieved February 2, 2015, from https://youtu.be/yF6cQblCgQQ.



Scheme 2. a) Carboligation of benzaldehyde towards (*R*)-benzoin that employs whole-cell catalyst that encloses overexpressed *P. fluorescens* benzaldehyde lyase, b) one-pot two-step reaction of carboligation and oxidoreduction using a whole-cell catalyst for the production of a chiral 1,2-diol (BAL: *P. fluorescens* benzaldehyde lyase overexpressed in *E. coli*, RasADH: *Ralstonia sp.* alcohol dehydrogenase overexpressed in *E. coli*, ThDP: thiamine diphosphate, NADPH: nicotinamide adenine dinucleotide phosphate, (**1**): benzaldehyde, (**2**): (*R*)-benzoin, (**3**): acetaldehyde, (**4**): (*R*)-2-hydroxy-1-phenylpropan-1-one, (**5**): (1*R*,2*R*)-1-phenylpropane-1,2-diol, (**6**): 2-propanol, (**7**): acetone).

Results and Discussion

Adaption of the "teabag" concept to the SpinChem reactor

As neither "teabags" nor microaqueous solvent systems for biocatalysis have been used in the SpinChem reactor so far, first an optimal setup of the reactor had to be developed. To force a constant flux of reaction medium through the catalytic "teabag", we had to ensure that the bag covers the entire inner reaction chamber wall properly. To do so, instead of using a single "teabag" in each compartment of the SpinChem, we decided to form a closed "teabag" ring of four adjacent segments each filled with lyophilized whole-cell catalyst (for "teabag" preparation see the Supporting Information SI-1). As previous experience revealed, the density of the catalyst loaded in the "teabag" has a strong influence on initial reaction rates. On one hand, a higher catalyst load enables higher initial reaction rates, but on the other hand, diffusion limitations within the "teabag" itself are increased with a denser filling.[25] Consequently, the catalyst load was varied to reach the highest reaction rates allowed by the limited "teabag" ring dimensions. A total of 10.3 g L^{-1} lyophilized whole-cell catalyst seemed to be an optimal catalyst load for the highest reactivity during benzoin formation (Figure 2; Scheme 2a). Higher loadings led to decreased reaction rates because of the dense packing, whereas lower cell amounts resulted in diminished reaction rates because of the limited availability of the catalyst.

At the same time, the catalyst specific production rate (mass of product per mass of catalyst per hour) increased steadily with a decrease of the cell loading. Nevertheless, as whole cells represent an inexpensive catalyst source,[17] we decided to proceed with a catalyst loading of 10.3 g L^{-1} for the highest absolute production rate.

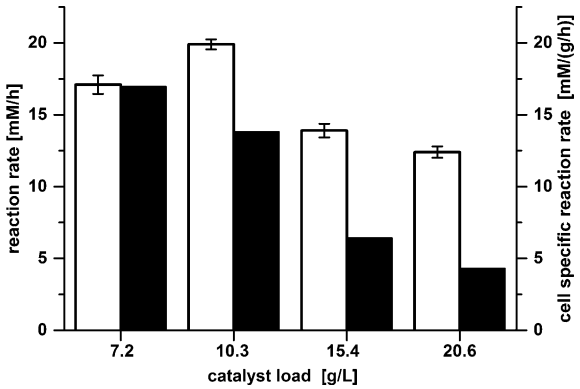


Figure 2. Impact of different catalyst loadings on the initial reaction rate (white bars) and cell-specific reaction rate (black bars) during benzoin condensation (Scheme 2a) using a "teabag" ring filled with lyophilized cells in the SpinChem reactor (measured in duplicate).

Impact of SpinChem stirring speed on the reaction rate

To stir the "teabag" ring in the SpinChem reactor at the optimal initial reaction rate, the stirring speed was varied during benzoin-forming reactions (Scheme 2a).

A maximum in reaction rate was found between 500 rpm (99.8 ± 1.9%) and 630 rpm, whereas 60 rpm was not sufficient to allow a proper flux of the reactants around the catalyst, as judged from only 59% relative initial rate activity (Figure 3). Speeds above 630 rpm appeared to be unsuitable as this led to the suction of air into the liquid and thus an irregular contact between reactants and catalyst (Supporting Information SI-2). Therefore, we performed all further investigations and reactions at 500 rpm as a compromise of reaction speed and material wear (of fittings in direct contact with the stirring shaft).

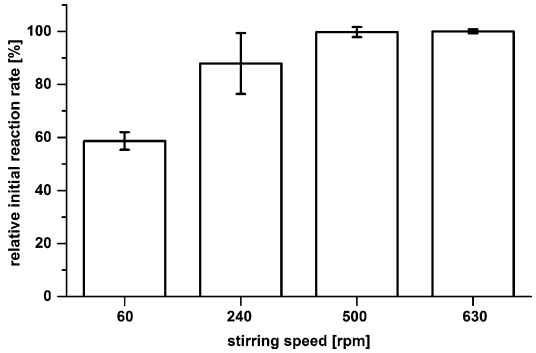


Figure 3. Impact of the stirring speed on initial reaction rate using a "teabag" ring filled with lyophilized cells in the SpinChem reactor (measured in duplicate).

Application of the "teabag" concept using different scales and reactor types

To test different combinations of catalysts and optimize reaction conditions of one-step or multi-step reactions, we generally operated on a vial scale (4–5 mL scale in overhead-shaken glass vials). The vials were agitated rather slowly by overhead shaking at 30 rpm. We obtained 18.7 mM h^{-1} of benzoin production optimally, which served as a reference value for the scaled-up reactions (Scheme 2a, Figure 4). When we performed the assay under the same conditions on a 140 mL scale in the SpinChem reactor, the reaction rate was almost identical with an overlapping standard deviation (16.3 mM h^{-1}). Therefore, the "teabag" system is scalable to the SpinChem reactor, although the reactor does not enable to overcome the diffusion limitations exhibited by the "teabag" membrane material.

To also test ubiquitously available lab equipment for the application of "teabag" catalysis on a larger scale, we used a simple 250 mL round-bottomed glass flask equipped with a stirrer bar as a model stirred-tank reactor. To avoid intense mechanical forces on the membrane material, we adjusted the stirring speed in such a way that the four freely suspended "teabags" were sucked under the liquid surface but not hit by the magnetic stir bar at the bottom of the vessel. We performed the benzoin condensation assay under the same reaction conditions as applied before and we reached a similar reaction rate (18.5 mM h^{-1}). Therefore, the model stirred tank is an appropriate reactor alternative to the SpinChem (for this particular application), although it does not lead to an increased mass transfer.

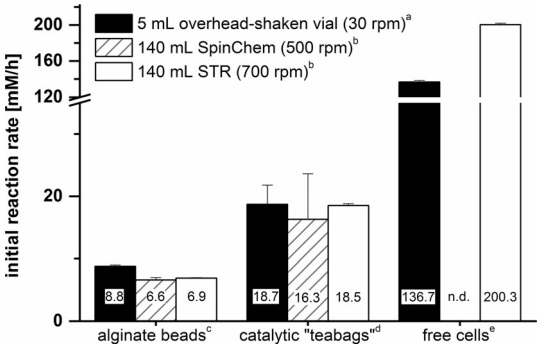


Figure 4. Initial reaction rates during benzoin formation using alginate immobilizates, catalytic "teabags", or freely suspended cells in different reactor types and volumes (n.d.: not determined, [a] measured in triplicate, [b] measured in duplicate, [c] benzaldehyde in MTBE (50 g L^{-1}, 200 mM), immobilizate (10.3 g L^{-1} dry cell weight), 90 min reaction time, [d] benzaldehyde in MTBE (200 mM), 10.3 g L^{-1} dry cell weight, 10.3 mL L^{-1}, 1 M TEA buffer (pH 10.0), 44.8 cm^2 membrane per gram cell dry weight, 90 min reaction time, [e] benzaldehyde in MTBE (10.3 g L^{-1}, 200 mM), dry cell weight 10.3 mL L^{-1}, 1 M TEA buffer (pH 10.0), 10.5 min reaction time).

After we operated the "teabags" in the SpinChem, we noticed a rather high standard deviation within repeated trials (±45%) together with an aqueous phase (at the bottom of the reactor vessel) that was present initially but disappeared over the course of the reaction. Therefore, we assumed that the standard deviation originated from the different hydration states of the catalyst during measurements as the SpinChem did not distribute the aqueous phase to an extent that it crossed the membrane reproducibly. To solve this problem, we incubated the "teabag" ring in buffer before we started the assay. In doing so, we not only reduced the standard deviation to only ±16%, but simultaneously increased the reaction rate to 29 mM h^{-1}. This result demonstrates the necessity of proper catalyst re-hydration if a lyophilized whole-cell catalyst is used. We had fixed the buffer/catalyst ratio after initial trials to (i) circumvent clogging of the catalyst in excess water and (ii) avoid the presence of a distinct second phase at the end of the reaction, which thus bypassed the necessity of extraction steps for product recovery (the latter of which is one of the main advantages of the microaqueous reaction system). Disadvantageously, when the ring had been pre-incubated, most of the buffer added to the reaction (to ensure the buffer saturation of the organic solvent) remained at the bottom of the reaction vessel and was not sucked into the "teabag" ring.

The impact of the "teabag" membrane on mass transfer becomes clear when the data of all three reactor setups that employ the catalytic "teabag" are compared with freely suspended cells, which reached an astonishing initial rate activity of 136.7 and 200.3 mM h^{-1} in the overhead-shaken vial and the model stirred-tank reactor, respectively (Figure 4).

Nonetheless, although the mass transfer limitation caused by the membrane material cannot be overcome by any of the mixing modes, space–time yields of more than 3 g L^{-1} h^{-1} are achieved during initial rate measurements with the catalytic "teabags", which is more than 30 times higher than the threshold of industrial feasibility as stated by Wenda et al.[35] Furthermore, new SpinChem designs, which have undergone further optimization with regard to improved mass transfer in heterogeneous catalysis, are now available on the market and might lead to higher reaction rates than those obtained here (Supporting Information SI-3). Both reactor concepts used should be easily scalable into the liter scale by using equipment of a bigger volume. On all scales, the "teabag" retains its advantages as a catalytic module that can be easily handled, stored (Supporting Information SI-4), removed, and recycled as described previously.[25] Thus it represents a valuable tool for modularized biocatalysis especially in the search for optimized new catalysts, catalyst combinations, and/or reaction- and process parameters.

Application of alginate immobilizates using different scales and reactor types

Despite the investigation of possibilities to scale up the "teabag" approach, we tested alginate entrapment as an alternative method of whole-cell immobilization to be used in the microaqueous systems. The method has already been applied in the SpinChem reactor for the biocatalytic whole-cell oxygenation of cyclohexanone towards ε-caprolactone in buffer.[27] Upon the first trials, it became clear that calcium alginate beads are not compatible with the highly concentrated triethanolamine (TEA) buffer (Supporting Information SI-2), which is needed to boost the reaction by BAL cells to its maximum.[24] Therefore, we decided to test the beads in a solely organic system as we expected the immobilizate (prepared from frozen wet cells in alginate solution) to already contain enough water to allow proper biocatalytic activity.

Similar to the "teabag" approach, we first optimized the preparation of alginate beads to find the optimal operation point (with respect to the maximum initial reaction rate). We varied the amount of alginate and the concentration of the $CaCl_2$ solution used for preparation and found a clear optimum with the use of 20 g L^{-1} alginate and 50 mM of $CaCl_2$ (Supporting Information SI-2). In addition, the impact of the stirring speed on the reaction rate in the SpinChem was investigated, which resulted again in an optimum at 500 rpm (Supporting Information SI-2). With these optimized parameters, we tested the immobilizates with the same volumetric catalyst load as that one used with the catalytic "teabags" in all three reactor types (Figure 4). Among all three scales and mixing modes tested, the alginate beads performed in a narrow range from 6.6 to 8.8 mM h^{-1}, which corresponds to less than half of the initial rate activity of biotransformations with catalytic "teabags". As for the "teabags", neither the vigorous mixing by the stirrer bar nor the application of the SpinChem allowed to overcome the mass transfer limitation of the alginate polymer. Nevertheless, the use of the SpinChem is preferred over the use of a stirred-tank reactor when alginate beads are used, as the minimization of mechanical forces in contrast to a stirred-tank reactor enables a better recyclability of the immobilizates.[27] Furthermore, the beads are easy to manage once prepared and storable for at least 30 days (Supporting Information SI-4).

The decreased initial reaction rate (at the same catalyst loading) in comparison to the utilization of catalytic "teabags" is most likely attributed to the impossibility of using the TEA buffer. Jakoblinnert and Rother have proven a tremendous reaction rate increase if this particular buffer is used.[24] As we did not use any other buffer during the preparation of the alginate beads, the pH inside the cells and in their surrounding remains unknown (although indicator paper gave a pH of 8–9 for the suspension of alginate and resuspended cells). As a result of the higher reaction rate and the faster preparation we favored the "teabag" approach over the alginate entrapment to be used in the preparative-scale synthesis of (1*R*,2*R*)-1-phenylpropane-1,2-diol.

Application of the SpinChem reactor for a one-pot two-step diol synthesis on a preparative scale

As a proof of concept for the application of multi-step biocatalysis and to take advantage of the flexible catalyst exchange using the "teabag" concept, catalytic "teabags" were used to perform a sequential one-pot two-step reaction (Scheme 2b) on a preparative scale of 140 mL by using the SpinChem reactor. The first carboligation step of the cascade was performed in a semi-automated fed-batch, in which acetaldehyde was fed in multiple pulses over time to avoid substrate toxicity and thus the inactivation of the catalyst.[24,25,36] To underline the big advantage of the "teabag" modules, the SpinChem was simply removed from the reaction chamber and the first "teabag" was replaced by one that contained lyophilized *E. coli* with RasADH to switch to the oxidoreduction step. At the same time, 2-propanol was added in excess to allow NADPH regeneration and push the reaction to the diol side.[24,25,33]

The concentration of the product of the first step, (*R*)-2-hydroxy-1-phenylpropan-1-one ((*R*)-HPP), increases together with that of the by-product (*R*)-benzoin during the first 4.5 h (Figure 5). Upon the addition of more acetaldehyde, the cleavage of (*R*)-benzoin is favored, which leads to a decrease in the

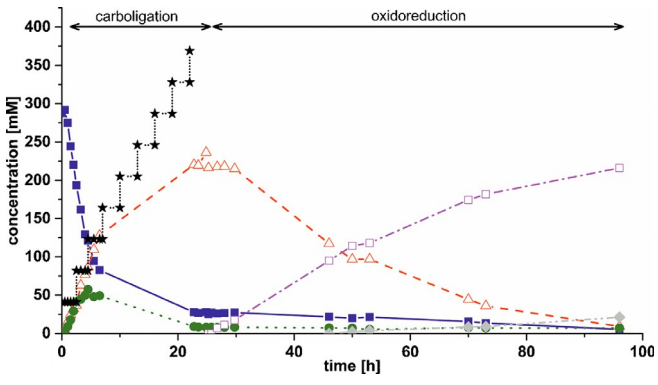


Figure 5. Concentration curves during the one-pot two-step 1,2-diol synthesis reaction of carboligation and oxidoreduction on a preparative scale using a "teabag"-encapsulated whole-cell catalyst in the SpinChem reactor (blue: benzaldehyde, green: (*R*)-benzoin, black: applied acetaldehyde (not measurable), red: (*R*)-2-hydroxy-1-phenylpropan-1-one, purple: (1*R*,2*R*)-1-phenylpropane-1,2-diol, grey: benzyl alcohol).

(*R*)-benzoin concentration. Within 24.8 h, a carboligation yield of 79% (*R*)-HPP up to a notably high product concentration of 236 mM was reached. At this point, 8 mM of benzoin remained uncleaved and 28 mM of the initial 300 mM benzaldehyde was unconverted (91% conversion). Negligible amounts of acetoin can be detected (< 5 mM), which results from the carboligation of two acetaldehyde molecules catalyzed by BAL.[37] Upon the addition of cosubstrate and buffer after 25.25 h (after the BAL-containing "teabags" were exchanged with RasADH-containing bags), the analyte concentrations decreased because of dilution. The following oxidoreduction yielded a 96% conversion of (*R*)-HPP. The resulting final diol concentration of 32.9 g L^{-1} (216 mM) can be considered to be in the lower industrially relevant order of magnitude.[35] The space–time yield of 8.2 g L^{-1} d^{-1} is not optimal[35] but it is likely to be improved by a more elaborate substrate feeding strategy during the carboligation step or the implementation of the better accepted, though more toxic, cyclohexanol as a cosubstrate for oxidoreduction.[24,33] Benzyl alcohol, which represents an unwanted by-product that results from the reduction of residual benzaldehyde by RasADH, is present in a concentration of 21 mM. Purification by column chromatography yielded 3.915 g (62% isolated yield) 1-phenylpropane-1,2-diol with a purity of > 99% according to GC and a target (1*R*,2*R*)- isomer content of 98.6% of all PPD isomers (for the precise distribution of isomers see Supporting Information SI-5). The product identity was confirmed by ^{1}H- and ^{13}C NMR spectroscopy (Supporting Information SI-5). In summary, the "teabag" approach has been demonstrated to be useful for the preparative scale application of modular multi-step reactions.

Conclusions

In the search for a suitable reactor to scale up the application of an immobilized whole-cell catalyst in microaqueous systems, we have tested two different cell retention techniques and compared their performance in two 140 mL reactors. Alginate beads and "teabag" encapsulation have been optimized and compared for maximum reaction rates. Alginate beads were not applicable in the chosen microaqueous systems as they decomposed in the presence of triethanolamine buffer and thus were used in the presence of only the organic solvent as the liquid phase. Even though the recyclability of the alginate preparations was not studied in depth, the recycling during initial rate measurements suggested good stability as reproducible initial rates were measured in three consecutive batches (Supporting Information SI-2). For each of both respective immobilization techniques we found the same initial reaction rates at all scales tested, whereas the application of "teabags" in microaqueous solvents enabled more than doubled initial reaction rates. On one hand, the scale-up works reliably whatever reactor was used, on the other hand, diffusion limitations induced by the "teabag" membrane and the alginate matrix could not be overcome. This was visible from the more than 10-fold higher initial reaction rates observed with freely suspended lyophilized whole-cell catalysts compared to both immobilizates. Still, because of its recyclability and the extraordi-

nary ease of addition and removal, the "teabag" approach is a very user friendly mode to screen optimal reaction conditions, process parameters, and/or catalyst combinations. Whether the SpinChem reactor or the stirred-tank reactor is recommended for a scaled-up application of "teabag"-encapsulated catalyst depends on the equipment available. Although the SpinChem reactor is the most elegant way to mix with the least mechanical force, it is limited by availability and maximum catalyst loading. As an alternative, our model stirred-tank reactor of a round-bottomed flask and a stirrer bar is easy to set up and ubiquitously available.

To prove "teabag" encapsulation as a new alternative for multi-step syntheses using whole-cell catalysts, the SpinChem reactor was chosen for a one-pot two-step reaction on a preparative scale. The reaction was performed under microaqueous conditions and afforded a concentration of 32.9 g L^{-1} (1*R*,2*R*)-1-phenylpropane-1,2-diol at a very good stereoselectivity (98.6% target isomer content) and with a reasonable space–time yield (8.2 g L^{-1} d^{-1}). This demonstrates that, despite the presence of a diffusion limitation by the applied "teabag" membrane, thresholds of industrial feasibility can still be exceeded.

In summary, an alternative way to implement whole-cell catalysis for use in the emerging SpinChem technology was presented and its applicability was confirmed by the production of a model fine chemical on a gram scale. Additionally, the combination of "teabag" encapsulation and whole-cell catalysis was also proven to be scalable to classical reactor concepts. Therefore, this technique has become even more appealing to users with and without a profound experience in biocatalysis and the handling of biological systems. It combines the advantage of (i) facile and flexible catalyst combination screening, (ii) investigation of ideal reaction parameters and operation modes, (iii) simple catalyst recovery, exchange, and recycling, and (iv) applicability to various scales and reactor types.

Experimental Section

Chemicals

All chemicals were purchased in high chemical grade. Aldehydes and benzoin were received from Fluka (Buchs, Switzerland), methyl *tert*-butyl ether (MTBE) from Carl Roth (Karlsruhe, Germany), benzyl alcohol from Sigma Aldrich (Munich, Germany), and alginic acid sodium salt from AppliChem (Darmstadt, Germany). (*R*)-HPP and PPD were synthesized in-house as described elsewhere.[6,24]

Preparation of lyophilized cells

E. coli SG13009 cells that contained overexpressed BAL were lyophilized from a frozen cell pellet that was produced by fermentation as described elsewhere.[38,39] *E. coli* BL21 (DE3) cells that contained RasADH were cultivated for 48 h in a shake flask at 20 °C in an autoinduction medium according to a recipe published elsewhere.[40] DNA and amino acid sequences of both enzymes are listed in Supporting Information SI-6. Cell pellets were lyophilized for at least two days, and the lyophilized cells were stored as crude powder at −20 °C.

Preparation of alginate immobilizates

Alginate was weighed into a small beaker and deionized water (10 mL) was added to achieve a concentration of 20–30 g L^{-1}. The heated mixture (45–55 °C) was stirred for 30 min. Frozen cell pellet (10 g) was resuspended in deionized water (10 mL) under gentle stirring (25 °C). The alginate solution was allowed to cool to 30–35 °C and mixed with the cell suspension. For bead formation, 20 μL of the mixture was dropped into stirred $CaCl_2$ (25–100 mM) solution using a dispenser pipette. The beads were allowed to further harden in the $CaCl_2$ solution overnight at 4 °C (protocol was modified from the method published by Hartmeier).[24] Before use, the beads were taken from the solution and dried for several seconds between two paper towels.

"Teabag" preparation

"Teabags" were prepared from PVDF western blotting membranes with a cut-off of 0.2 μm (Bio-Rad Laboratories GmbH, Munich, Germany). The ring structure consisted of four adjacent compartments, each filled with a quarter of the total catalyst loading, covering the inner wall of the SpinChem chamber. Precise manufacturing details for this ring can be found in Supporting Information SI-1. For 5 mL scale transformations, membrane material of 1.4× 2.7 cm was cut, folded, and sealed to give a small bag to be filled with 51.5 mg lyophilized whole-cell catalyst.

Benzoin condensation on 5 mL scale in an overhead-shaken vial

Benzaldehyde was weighed into a graduated flask that was filled with MTBE to give a 200 mM benzaldehyde solution. For "teabag"-catalyzed reactions, the solution was 202 mM to compensate for dilution by the addition of buffer as described below. For the use of alginate immobilizates in free suspension, alginate beads (250 mg, which correspond to 10.3 mg mL^{-1} cell dry weight within the reaction; see Supporting Information SI-7) were weighed into 8 mL glass vials. Benzaldehyde/MTBE solution (5 mL, 200 mM) was added to start the reaction. If "teabags" were used (filled with 51.5 mg catalyst), benzaldehyde/MTBE solution (4.95 mL, 202 mM) was added into 8 mL glass vials and mixed with TEA buffer (51.4 μL, 1 M, pH 10.0). If freely suspended cells were used, lyophilized cells (51.5 mg) were put into the 8 mL glass vial and reaction solution (5 mL) composed of benzaldehyde, MTBE, and buffer was added to start the reaction as described for the use of catalytic "teabags". All reactions were incubated with overhead shaking (30 rpm, 30 °C).

Benzoin condensation on 140 mL scale by using the SpinChem reactor

For alginate-bead-catalyzed reactions, each compartment of the SpinChem (type S4530) was filled with beads (1.75 g in each compartment, in total corresponding to a dry catalyst load of 10.3 mg mL^{-1} reaction volume). Substrate stock solution (benzaldehyde in MTBE; 140 mL, 200 mM) was put into the prewarmed SpinChem reactor (type R100), and the reaction was incubated while stirring (500 rpm, 30 °C; IKA stirrer RW20.n). During the investigation of the impact of the stirring speed on the reaction rate, the beads were recycled twice between the benzoin condensation reactions. To avoid product carryover, the immobilizate was washed (3×5 min) in water-saturated MTBE after each reaction, and excess MTBE was removed by spinning the chamber above the liquid for

2 min. For reactions that involved a pre-incubated "teabag" ring, the ring was placed in the SpinChem and rotated (10 min, 500 rpm) in TEA buffer (1 M, pH 10.0) to allow the cells to re-hydrate. Subsequently, the SpinChem was raised above the liquid surface and stirred for another 2 min to remove excess buffer. The outer surface of the chamber was wiped with a paper towel before the SpinChem was placed in the reaction solution, which was composed of substrate stock solution (138 mL, 202 mM) supplemented with TEA buffer (1.4 mL; 1 M, pH 10.0). In cases in which the "teabag" ring was not pre-wetted, the dry ring was placed directly in the reaction solution.

Benzoin condensation on 140 mL scale by using a stirred-tank reactor

A 250 mL closed round-bottomed flask equipped with a rounded-down stirrer bar served as a model reactor. The temperature (30 °C) in the double-walled flask was controlled by using an attached water bath. All reactions were stirred (700 rpm) with a magnetic stir bar. For reactions that employed alginate beads, the beads (7 g, which corresponds to a dry cell load of 10.3 mg mL^{-1} reaction volume) were put into the flask before the addition of the substrate stock solution (benzaldehyde in MTBE; 140 mL, 200 mM). When "teabags" were used, four identical "teabags" of 3.1×2.6 cm dimension (comparable to the dimension of the "teabag" ring, filled with dry catalyst load of 10.3 mg mL^{-1} reaction volume) could move independently in the stirred-tank reactor (without pre-incubation in buffer). The reaction medium was composed of substrate stock solution (138 mL, 202 mM) and TEA buffer (1.4 mL, 1 M, pH 10.0). The reaction was started by the addition of the "teabag". When free cells were used, the reaction medium was set up as described for single "teabags" (see above). To start the reaction, lyophilized cells (1.4 g) were added without pre-incubation.

One-pot two-step diol synthesis on a 140 mL scale using a catalytic "teabag" ring inside the SpinChem reactor

To catalyze the first step of the reaction, the carboligation towards HPP, a "teabag" ring was manufactured as described above and filled with lyophilized cells (360 mg per compartment). Benzaldehyde (303 mM) was diluted in MTBE (to give a final concentration of 299 mM after buffer addition). The ring was pre-incubated as described above for the SpinChem approach. The reaction mixture was composed of benzaldehyde reaction solution in MTBE (138 mL), TEA buffer (1.4 mL; 1 M, pH 10.0), and acetaldehyde (320 μL) that was introduced ice-cold with a pre-cooled, gas-tight glass syringe. Additional pulses of acetaldehyde (320 μL each) were added manually after 2.5 and 4.5 h. At 7 h of reaction, automated feeding was initiated, which added 1280 μL of dilute acetaldehyde (1:4 dilution in MTBE) per pulse by using a Landgraf LA 160 syringe pump system equipped with four gas-tight glass syringes. Automated pulses were given at 7, 10, 13, 16, 19, and 22 h of reaction (approximately 369 mM acetaldehyde was applied in total). After 24.8 h, the first "teabag" was replaced by a "teabag" that contained RasADH-inclosing cells (4×360 mg lyophilized cells) and which had been pre-incubated in buffer as described above. Simultaneously, TEA buffer (1.4 mL; 1 M, pH 10.0) and 2-propanol (13.72 mL) as co-substrate (1.1 M; approximately fivefold excess over (*R*)-HPP) were added. The one-pot two-step reaction was incubated while stirring (500 rpm, 30 °C) and followed by HPLC and GC.

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