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Whole-Cell Teabag Catalysis for the Modularisation of Synthetic Enzyme Cascades in Micro-Aqueous Systems

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Combining enzymes to form multi-step enzyme cascades has great potential to replace existing chemical routes with high atom-efficient and eco-efficient synthesis strategies as well as to grant access to new products, especially those with multi-stereogenic centres. However, easy solutions and tools for setting up appropriate reaction conditions and process modes are hardly available. The utilisation of teabags filled with whole cells has several advantages, such as 1) simplified handling and recovery of catalyst, 2) easy combination of various catalysts from catalyst toolboxes, 3) fast testing of different operating modes during cascadation and 4) simplified downstream processing. One of the main advantages is that lyophilised whole-cell catalysts can be applied in micro-aqueous media, allowing high substrate loads (also of poorly water-soluble substrates) and concomitantly enabling high catalyst stability. This was demonstrated herein for a synthetic two-step cascade towards chiral 1,2-diols starting from cheap aldehydes. The carboligation of two aldehydes using *Pseudomonas fluorescens* benzaldehyde lyase and subsequent oxidoreduction with *Ralstonia* sp. alcohol dehydrogenase yielded 1-phenylpropane-1,2-diol [(1*R*,2*R*)-PPD] in concentrations of up to 339 mM and excellent enantiomeric and diastereomeric excesses >99%. Therefore, the combination of whole-cell catalysis and teabag modularisation allows cheap, easy-to-apply and efficient catalyst preparation to test enzyme combinations and optimal reaction conditions up to the preparative scale. By circumventing catalyst purification and immobilisation, and enabling high substrate loadings compared to those in aqueous systems, efficient production of a chiral diol with extraordinarily high product concentrations can be achieved.

Introduction

Enantiopurity is often a prerequisite for the use of molecules in the fine-chemical industry or as building blocks for pharmaceuticals. Chiral diols are one example of synthons for the further synthesis of bioactive compounds.[1,2] Owing to their extraordinarily high regio-, chemo- and especially stereoselectivity, enzymes are ideal catalysts for manufacturing these building blocks.[3] Numerous enzymes of different classes have been identified. Due to the ability to change enzyme properties by protein, reaction or solvent engineering,[4–7] enzyme toolboxes, encompassing catalysts performing the same reaction type but having different substrate spectra or chemo-/stereoselectivity, allow single biocatalytic reactions to be combined in synthetic enzyme cascades.[8–12] By a modular combination, a broad platform of products with multi-chiral centres is available in a highly selective manner. Still, appropriate reaction conditions for all applied catalysts have to be found to gain optimal selectivity and activity. To supply researchers with easy solutions for setting up such multi-parameter systems and optimising synthetic enzyme cascades, we propose a simple modularisation of single biocatalytic steps, requiring the retention or immobilisation of the catalyst. Enzyme purification and immobilisation techniques are often expensive, labour-intensive and suffer from activity reductions. Recombinant whole cells, for example, in a lyophilised form, represent an alternative, inexpensive and easy-to-handle biocatalyst source, in which the enzyme is naturally retained inside the cell. Furthermore, lyophilised cells usually still contain enzyme cofactors. The addition of a second enzyme or co-substrate instead of the expensive enzyme cofactor itself allows in situ cofactor regeneration, hence cutting costs.[13] As a further advantage, the residual cell membrane protects the enzyme from the surrounding reaction environment, enabling catalysis with large amounts of unconventional solvents up to neat substrate systems. Such solvent-engineered systems enable increased substrate loads for sparingly water-soluble educts and thus provide conditions for reaching high product concentrations (>100 g L^{-1}).[14–16] Furthermore, simplified downstream processing is possible by solvent evaporation.

To further facilitate product workup by plain catalyst removal without the need for filtration or centrifugation, retention in an easily manageable module is proposed. Teabag-like containers have already proven their applicability for example in peptide synthesis, the recycling of dendrimer-immobilised copper catalysts and the retention of biocatalytic immobilisate particles.[17–19] Herein, we retained a lyophilised whole-cell catalyst

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in teabags made from polyvinylidene fluoride (PVDF, see Figure 2). Bags with a cut-off of 0.2 μm were tested for their ability to retain active catalyst if used in micro-aqueous systems. The aim was to circumvent possible cross-reactivity between the single reaction steps of a cascade and to simplify product workup with teabag removal. Additionally, if teabag-entrapped catalysts were removed, they were tested for recyclability in repeated batch experiments.

As a model reaction, benzaldehyde lyase from *Pseudomonas fluorescens* (BAL, EC 4.1.2.38)[20] was used for the asymmetric carboligation of cheap aldehyde substrates to the chiral α-hydroxyketone (*R*)-2-hydroxy-1-phenylpropanone [(*R*)-HPP].[21] Subsequent oxidoreduction with an alcohol dehydrogenase from *Ralstonia* sp. DSM 6428 (RADH) introduces a second chiral centre to yield (1*R*,2*R*)-1-phenylpropane-1,2-diol [(1*R*,2*R*)-PPD] with an excellent diastereomeric excess (*de* > 99%) in buffered systems.[22] For alternative chemical routes, diol synthesis is a challenging task. Chemical methods towards diols include 1) the reduction of α-hydroxyketones or α-diketones employing hydride transfer agents, 2) the Sharpless dihydroxylation of alkenes, 3) the hydrolysis of epoxides and 4) asymmetric aldol condensation. Despite this variety, all methods suffer from one or more of the following drawbacks: low stereoselectivity, low yields, expensive or toxic catalysts and/or limited access to all stereoisomers of a target compound.[1,29–36] In comparison to such chemical synthesis, the biocatalytic route provides access to all isomers with excellent stereoselectivity under mild reaction conditions.[37]

During the approach presented here, the NADPH cofactor was recycled in a substrate-coupled cofactor regeneration by adding cyclohexanol (see Scheme 1).[38] All reaction steps were performed in a micro-aqueous system with methyl *tert*-butyl ether (MTBE) as the organic phase and no more than 7.5 vol% buffer phase, with both catalysts employed as lyophilised recombinant *E. coli* cells.[39]

This work proves that teabags containing whole cells are effective in setting up appropriate reaction conditions and process modes, as well as in testing enzyme combinations in multi-step enzyme cascades. In addition, they provide access to high product concentrations and easy downstream processing from laboratory to preparative scale.

Results and Discussion

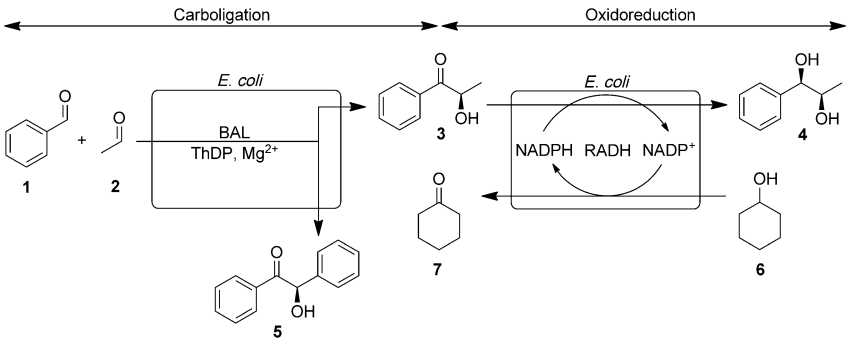
Optimisation of the teabag reaction setup

To identify appropriate reaction conditions for operating reactions with a teabag-entrapped catalyst, different mixing modes, teabag geometries, gas–liquid volume ratios and catalyst loadings were tested as described in the Supporting Information (S1). Most variations of the tested parameters (e.g., the type of mixing) had none or only few effects. Only changes in the filling volume of the catalyst inside the teabag led to prominent differences in the reaction rate (S1F/S1G). To allow proper mass transfer inside the teabag, the bags should be loosely filled. Optimally the final catalyst-to-teabag-surface ratio should not exceed 31.25 mg of catalyst per cm^2 of teabag material. This corresponds to bags that are approximately half-full. Exceeding this ratio with the further addition of catalyst does not lead to any increase in the reaction rate, given the chosen reaction parameters.

Catalyst retention in teabags

An effective retention method is characterised by its ability to prevent catalyst leakage from the retention compartment. Therefore, a solvent-resistant PVDF membrane[40] with a cut-off of 0.2 μm was chosen to entrap the lyophilised whole-cell catalyst. Tightness of membranes, particularly at seams, and resistance against mechanical forces caused by shaking were major concerns. To investigate these aspects, the carboligation of benzaldehyde and acetaldehyde catalysed by BAL cells, which represents the first step of the cascade reaction (Scheme 1), was tested as an example. Having performed the carboligation reaction (for product formation see the Supporting Information, plot S2), teabags were removed from the reaction bulk at different time points to terminate the biocatalytic reaction. Afterwards, new substrate was supplied and additional (side-) product formation owing to possible catalyst leakage was monitored. With tight membranes no conversion should be visible after teabag removal.

As shown in Figure 1, teabag removal and substrate pulse after 24 h resulted in hardly any changes in analyte concentrations, indicating that no active catalyst leaked from the teabag. After removal of the teabag-entrapped catalyst after 48 h (Figure 1 B), a minimal (*R*)-HPP concentration increase of 0.5 mM h^{-1} occurred, but also benzaldehyde concentration increased slightly (0.4 mM h^{-1}). Thus, changes in analyte concentration are more likely to be caused by a regular analytics error than by residual catalyst activity in the reaction. The positive control without teabag removal (Figure 1 C) revealed very clear accumulation



Scheme 1. Synthetic enzyme cascade of mixed carboligation and oxidoreduction with substrate-coupled regeneration of NADPH using lyophilised whole cells. **1**: benzaldehyde, **2**: acetaldehyde, **3**: (*R*)-HPP, **4**: (1*R*,2*R*)-PPD, **5**: (*R*)-benzoin, **6**: cyclohexanol, **7**: cyclohexanone.

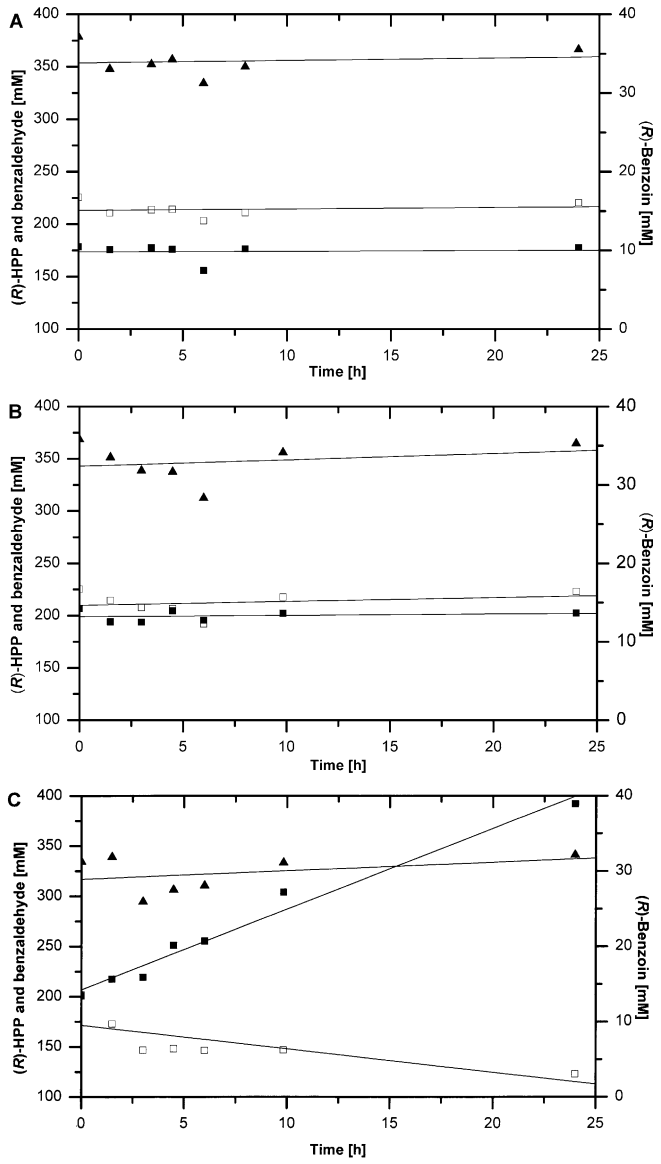


Figure 1. Benzaldehyde (□), (*R*)-benzoin (■) and (*R*)-HPP (▲) concentrations upon removal of teabags and the substrate pulse after A) 24 h and B) 48 h. C) Positive control with no teabag removal after 48 h. The time scale refers to time points after teabag removal and substrate pulse. For product formation of the carboligation reaction conducted prior to these leaching experiments, see the Supporting Information (S2).

of (*R*)-HPP and (*R*)-benzoin, while the substrate benzaldehyde was consumed. This verified the experimental setup, as the catalyst exhibited activity if exposed to MTBE for 48 h. Visual inspection revealed that the reaction medium had turned a slightly brownish colour (see the Supporting Information, S3). Based on the results obtained, this colouration cannot be attributed to active catalyst particles, but it may be caused by cell debris or residues of the cell fermentation broth before lyophilisation. This issue was addressed in more detail in the catalyst recycling experiment.

The leakage experiment demonstrates that the combination of a whole-cell catalyst in a micro-aqueous reaction system entrapped in PVDF teabags successfully allows the modularisation of enzyme cascades because the teabags are tight.

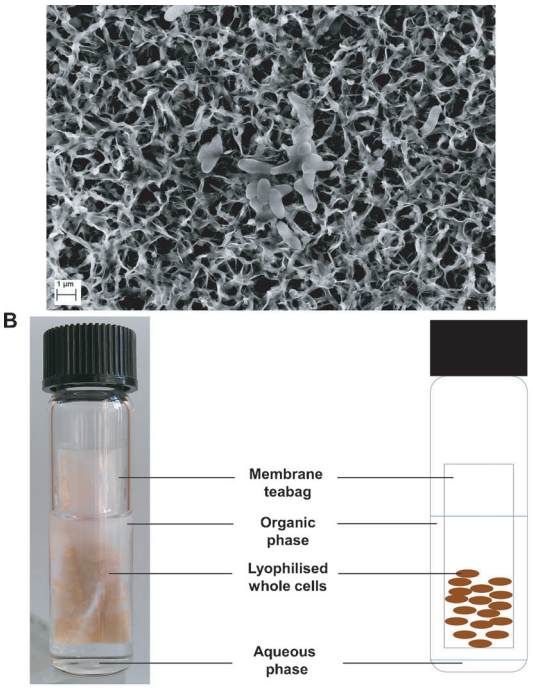


Figure 2. A) Scanning electron micrograph of lyophilised *E. coli* whole cells on teabag membrane material. B) Photograph and schematic view of a teabag reaction setup.

SEM analysis of whole cells and teabags

To visualise size ratios between teabag pores and lyophilised cells, and to gain information on the outer appearance of lyophilised whole cells under the chosen micro-aqueous conditions, SEM analysis was conducted (Figure 2).The SEM analysis revealed that the membrane pores (cut-off 0.2 μm) were significantly smaller than the lyophilised cells, thus providing sufficient retention of the whole-cell catalyst. In comparison to pictures of growing cells in the literature,[41] these cells exhibited a slightly compressed morphology of a less pronounced rod shape. They were reduced in length from 1–2 μm (living cells) to <1 μm (lyophilised cells). Holes in the cell membrane (see the Supporting Information S4) were only rarely seen, so that a protective effect against the organic compounds can be assumed.

Recyclability of teabag-entrapped catalyst

Recyclability was investigated in multiple repeated fed-batch experiments with intermediate washing steps. First, only carboligation of aldehydes was tested in three consecutive batches. The effect of different buffer volumes and acetaldehyde concentrations on the catalyst stability was investigated. All reactions were monitored for 12 h, even if maximum product concentrations were reached earlier (Figure S5). Maximal conversions during reaction progression are shown in Figure 3.

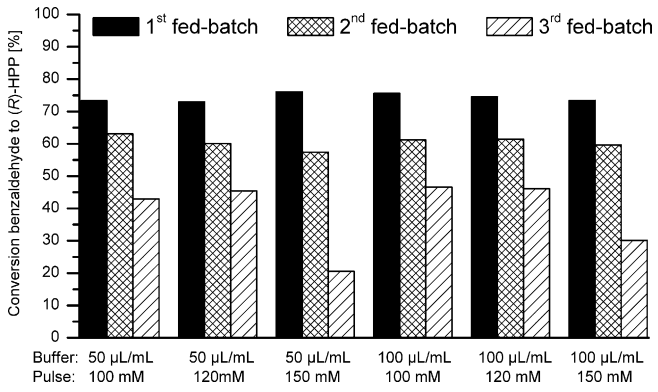


Figure 3. Maximal conversions of benzaldehyde to (*R*)-HPP during repeated fed-batch experiments with varying amounts of aqueous phase and different acetaldehyde pulsing strategies. Injections were given every 90 min until 600 mM was applied in total.

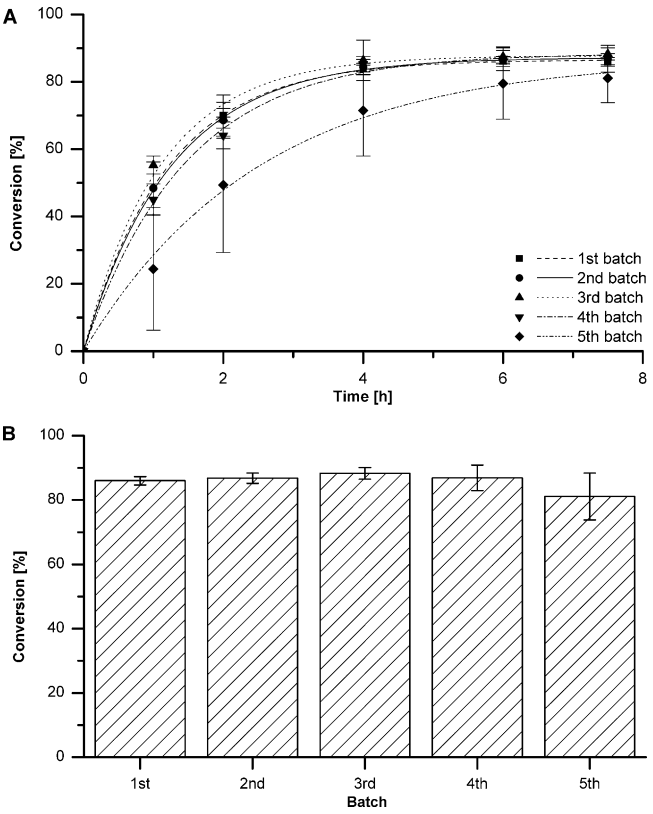


Figure 4. A) Conversion curves and B) final conversion during RADH recycling in repeated batches.

In all reaction conditions applied, conversions dropped by 28–56% during the first three fed-batches indicating gradual catalyst inactivation. During the third fed-batch, both reactions fed with four injections of 150 mM acetaldehyde exhibited significantly decreased activity, most likely attributable to acetaldehyde-induced toxicity.[42] Although these acetaldehyde injections were better tolerated during the first two batches, they seemed to impair catalyst longevity by inactivation. Reactions fed with 100 mM and 120 mM acetaldehyde only slightly differed in loss of conversion over three batches. However, reactions fed with 150 mM acetaldehyde injections exhibited less reduced activity if the volume of the buffer phase was increased. These results not only reveal reduced product yields in recycling experiments with acetaldehyde concentration >120 mM, but also prove that acetaldehyde-induced toxicity is lowered by a high water content, which is most likely caused by the preferred partition of acetaldehyde into the aqueous phase.[43]

As the stability of the BAL whole-cell catalyst is heavily challenged by the toxicity of acetaldehyde if cells were exposed to high acetaldehyde concentration for a long time, we tested how recyclable a teabag-entrapped catalyst is under less harsh conditions. The oxidoreduction reaction with RADH whole cells (see Scheme 1) was therefore tested in a separate recycling trial. As shown in Figure 4, RADH cells in the micro-aqueous reaction medium were recyclable in four consecutive batches without any loss of conversion. Therewith high recyclability is possible with substrates less toxic than acetaldehyde. Only from the fourth to the fifth batch, 6% of conversion capacity was lost.

A major advantage of the teabag approach is that handling the teabags is extremely straightforward. The catalyst can be recovered from the reaction bulk simply by teabag removal and rinsing with MTBE. Brown colouration of the reactions (probably occurring from lyophilised growth medium) can be prevented by thoroughly washing the teabags prior to the reactions, as demonstrated by the clarification of reactions during repeated batches of the recycling experiments (see the Supporting Information, S3). Thus, although the BAL catalyst in particular may not show very good recycling potential in the micro-aqueous reaction system owing to acetaldehyde-induced instabilities, the teabag system has proven its excellent applicability in catalyst recycling.

Operational flexibility of teabags for process-mode investigations

To demonstrate the versatility of teabags for investigating different operation modes, the two-step cascade from benzaldehyde and acetaldehyde to (1*R*,2*R*)-PPD combining an enzymatic carboligation and oxidoreduction step was run sequentially, simultaneously and in a mixed-mode cascade.

To regenerate the NADPH cofactor for the oxidoreduction step, cyclohexanol was added in molar excess to drive the reaction towards the production of diol (Scheme 1). Optimal reaction parameters for the operation of BAL and RADH cells in a micro-aqueous reaction medium (choice of organic solvent and co-substrate, substrate concentrations, buffer conditions) were previously identified.[39]

In the sequential reaction mode (Figure 5 A), in which RADH was added after completion of the first carboligase reaction step, the accumulation of the intermediate (*R*)-HPP and its subsequent consumption during oxidoreduction to the final diol (1*R*,2*R*)-PPD can clearly be seen. The diol product accumulates

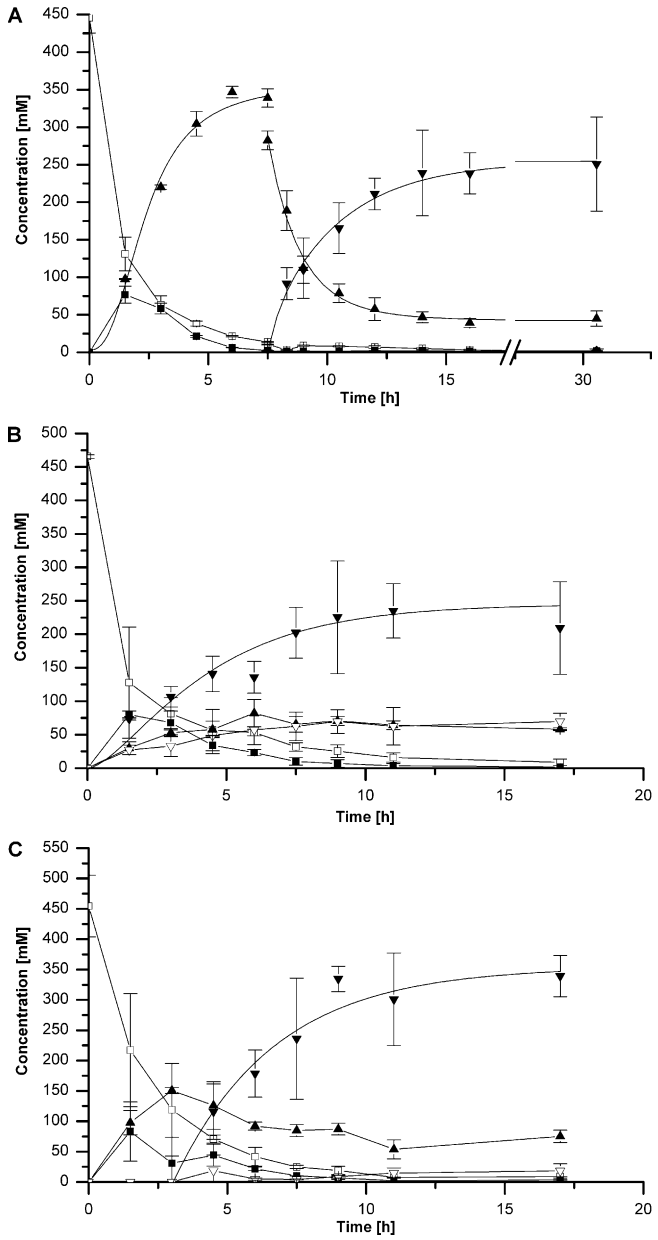


Figure 5. Concentration curves of benzaldehyde (□), (*R*)-benzoin (■), benzyl alcohol (▽), (*R*)-HPP (▲) and (1*R*,2*R*)-PPD (▼) during cascadation of fed-batch carboligation and oxidoreduction in A) sequential, B) simultaneous and C) mixed operation mode. In all three processes, acetaldehyde was fed by four injections of 100 mM each at 0 h, 1.5 h, 3 h and 4.5 h, respectively. RADH was added after A) 7.5 h or C) 3 h, respectively.

to a concentration of 238 mM within 16 h. The absence of benzyl alcohol in the products (see Table 1) reveals that competition between both enzymes for the conversion of benzaldehyde was successfully circumvented by the chosen reaction mode.[21,22] As (*R*)-HPP and (*R*)-benzoin were equally formed at the beginning, accumulation of acetaldehyde caused by the fed-batch strategy led to benzoin cleavage and subsequent (*R*)-HPP formation and thus to side-product decay.[39]

During the simultaneous mode (Figure 5B), in which both enzymes of the cascade where added directly at the beginning, (*R*)-benzoin was accumulated and cleaved, similarly to the reaction in sequential mode. (*R*)-HPP and benzyl alcohol accumulated and remained at a constant concentration until the end of the reaction. Unwanted benzyl alcohol production, caused by the reduction of benzaldehyde by RADH, was promoted in this operation mode (final concentration 70 mM). In contrast to these drawbacks, the space–time yield for (1*R*,2*R*)-PPD increased by 43.8%.

Taking full advantage of both strategies and the use of the teabag-entrapped catalyst, mixed-mode cascade operation was established, adding the second teabag containing RADH cells after 3 h of carboligation with BAL cells. As expected, this mode of operation led to high reaction rates at the beginning of the oxidoreduction and lowered production of the by-product benzyl alcohol (18 mM), because RADH holds much higher activity for (*R*)-HPP than for benzaldehyde.[38] In the mixed mode, product concentrations of up to 339 mM could be reached (Table 1). After RADH cells were added, the intermediate product (*R*)-HPP was quickly converted to the corresponding diol. Hence, the equilibrium of the carboligation reaction was shifted towards the product side by this operation mode.

In comparison, the sequential mode is characterised by very low side-product concentrations, as the reduction of benzaldehyde is circumvented by the belated addition of RADH cells. In contrast, the space–time yield was low compared to that in the other operation modes (Table 1). The simultaneous mode gave the highest space–time yields, but also the highest accumulation of the by-product benzyl alcohol. The mixed-mode operation of the cascade led to only 22 mM side products in total, yielding significantly more product than the sequential and simultaneous modes.

Regardless of the chosen operating mode, (1*R*,2*R*)-PPD was produced with an excellent diastereomeric ratio and excess (Table 1). The results make the use of teabag-entrapped catalysts particularly interesting for the targeted realisation of synthetic enzyme cascade operation modes towards chiral prod-

Table 1. Performance parameters of different operating modes during cascadation of carboligation and oxidoreduction.

Mode	STY[a] [g L^{-1} h^{-1}]	(1*R*,2*R*)-PPD[a] [mM]	(*R*)-Benzoin[a] [mM]	Benzyl alcohol[a] [mM]	Diastereomeric product distribution[b] [%] (1*R*,2*R*)-PPD	(1*S*,2*S*)-PPD	(1*R*,2*S*)-PPD	(1*S*,2*R*)-PPD
Sequential	2.26	238 ± 27.3	2 ± 0	**0**	**98.2 ± 0.4**	0.0 ± 0.0	0.2 ± 0.4	1.6 ± 0.1
Simultaneous	**3.25**	235 ± 41	4 ± 4	63 ± 28	**98.7 ± 0.1**	0.0 ± 0.0	0.6 ± 0.0	0.7 ± 0.2
Mixed	3.03	**339** ± 34	4 ± 1	18 ± 12	**98.3 ± 0.0**	0.0 ± 0.0	0.8 ± 0.1	0.9 ± 0.1

[a] Space–time yield determined for points of most feasible reaction termination (16 h in sequential mode, 11 h in simultaneous mode and 17 h for mixed mode). [b] Calculated for the last time points plotted.

ucts by plain teabag addition. Furthermore, extremely high product concentrations are accessible under micro-aqueous conditions with recombinant whole cells.

Stereoselective diol synthesis on a preparative scale

To prove the scalability of teabag biocatalysis for synthetic enzyme cascades from the laboratory to the preparative scale, the synthesis of (1*R*,2*R*,)-PPD using eight teabags in each reaction step was conducted on the scale of 32 mL. To circumvent side-product formation, a sequential reaction mode was chosen. The reaction progress was qualitatively similar to that described before for the sequential mode (see Figure 5 A; see the Supporting Information S6), although a higher final product concentration of 294.8 mM was reached within the organic phase. Finally, besides the organic phase also the aqueous phase, as well as the whole-cell catalyst, were analysed for the presence of (1*R*,2*R*)-PPD. Calculated from the analysis data, a total of 137 mg diol product (representing 7.6% of the total isolated yield) was extracted from the aqueous phase, whereas only 5 mg (< 1% of isolated yield) was gained from the cell extract. Thus, it appears worthwhile to extract the buffered phase, whereas the cells can simply be discarded or reused after the process. Alternatively, for further teabag-applications, the amount of added aqueous phase might be reduced. After combining the extracted aqueous fraction, the organic fraction, and cell extract, the raw product was concentrated by evaporation and purified by flash chromatography. The isolated yield was 75.7% (1.8 g) with a diastereomeric excess of > 99% and a diastereomeric ratio of 1:55.3. Product identity was confirmed by NMR analysis, revealing only minor impurities (see the Supporting Information S6).

In conclusion, the sequential cascade reaction was easily scaled up to gram-scale production of stereopure (1*R*,2*R*)-PPD. The catalyst was simply separated from the reaction by removing the teabag. Downstream processing was further greatly facilitated by the use of the micro-aqueous reaction medium, leaving the raw product after extraction of the aqueous phase and subsequent rotary evaporation.

Conclusions

The use of lyophilised whole-cell catalysts in combination with a micro-aqueous reaction system allows high substrate loadings, even with toxic aldehyde substrates, thus resulting in high product concentrations. Entrapping the catalyst in teabags enables simplified handling and thus the easy recovery of both the catalyst and product from the reaction bulk. Furthermore, single catalytic steps were easily combined in modularised synthetic enzyme cascades.

The use of teabag entrapment can generally be performed with any lyophilised whole-cell catalyst and may be a helpful method for testing single-step and multi-step biotransformations in a very fast and easy-to-apply manner. The teabags were shown to be recyclable from laboratory-scale reaction optimisations up to preparative-scale approaches. In a volume of 32 mL, more than 1 g product yield and excellent stereoselectivity were afforded. Therefore, a new tool is now available for setting up optimal process modes, screening for suitable reaction conditions or easily combining various biocatalysts in multistep cascades.

Experimental Section

Chemicals

All chemicals were purchased in high chemical grade. Aldehydes and MTBE were purchased from Carl Roth (Karlsruhe, Germany). Racemic benzoin was purchased from Fluka (Buchs, Switzerland). Racemic HPP for calibration of HPLC analytics was synthesised as described elsewhere.[44]

Preparation of lyophilised whole cells

Lyophilised cells containing BAL were prepared from frozen cell pellets, produced by high-cell-density fermentation in a 40 L Techfors reactor (Infors AF) as described elsewhere.[45,46] Recombinant cells with RADH were prepared as described by Kulig et al.[22] Frozen pellets were lyophilised for two to four days (Martin Christ Gefriertrocknungsanlagen GmbH). Lyophilised cells were carefully ground into crude particles by using a spatula and stored at −20 °C.

Teabag preparation

Teabags of polyvinylidene fluoride membrane were prepared from western blotting membranes with a cut-off of 0.2 μm (Bio-Rad Laboratories GmbH). Material of 8×1.6 cm was cut, folded in half and sealed by three seams. The bags were sealed by using a Polystar 100 GE sealing unit and Polystar tong sealers for 5 s per seam (Rische + Herfurth GmbH).

Setup of leakage studies

Four identical reactions with a total volume of 4 mL were set up with a final concentration of 500 mM benzaldehyde in MTBE (3.8 mL), supplemented with 1 M triethanolamine (TEA) buffer (50 μL mL^{-}, pH 9.0). Teabags were filled with lyophilised BAL cells (50 mg mL^{-1}). Acetaldehyde (final concentration 150 mM) was injected at 0 h, 1.5 h, 3 h and 4.5 h by using an ice-cold syringe. Vials were shaken overhead (Scientific Industries, Inc.) at 30 °C and 30 rpm. Teabags were removed after 9 h, 24 h and 48 h, respectively. After removal, a benzaldehyde injection of 61 μL (≈150 mM) was given to test the activity of supposedly leached catalyst. The positive control was supplied with benzaldehyde without removing the teabag.

Recyclability studies

For the recycling of the BAL catalyst, six identical reactions each with a total volume of 4 mL were set up with a final concentration of 500 mM benzaldehyde in MTBE with volumes of 3.6 or 3.8 mL, respectively, supplemented with 1 M triethanolamine buffer (pH 9.0) at a volume fraction of 50 or 100 μL mL^{-1}. Teabags were filled with lyophilised BAL cells (50 mg mL^{-1}). Acetaldehyde injections of 4×150 mM, 5×120 mM, or 6×100 mM, respectively, were given every 90 min using an ice-cold syringe. Vials were shaken overhead (Scientific Industries, Inc.) at 30 °C and 30 rpm. Reactions were run for 12 h, even if maximum (*R*)-HPP concentrations were

reached before this. After the reaction, teabags were washed four times with MTBE to avoid analyte carryover between batches.

For the recycling of the RADH catalyst, each batch was set up in triplicate. Each reaction consisted of 1 M TEA buffer (100 μL, pH 9.0), cyclohexanol (295 μL) and a MTBE stock solution including approximately 400 mM (*R*)-HPP (1605 μL). Vials were shaken overhead (Scientific Industries, Inc.) at 30 °C and 30 rpm. Reactions were monitored by HPLC occasionally monitored during the first 7.5 h. Subsequently, teabags were removed, washed twice for 15 min with MTBE, once for 15 min with 100 mM TEA buffer (pH 9.0), and once (30 min to overnight) with a mixture of 5 vol% 1 M TEA buffer (pH 9.0) in MTBE. Datasets shown above (Figure 4) were derived from triplicates, with exception of the fifth batch, from which one outlying run was removed.

Setup of operation-mode investigation

For the sequential reaction, reactions with a total volume of 4 mL were prepared, containing a final concentration of 500 mM benzaldehyde in MTBE, supplemented with 25 μL mL^{-1} 1 M TEA buffer (pH 9.0). Teabags were filled with 25 mg mL^{-1} lyophilised BAL cells. Acetaldehyde injections of 4×100 mM were applied every 90 min by using an ice-cold syringe. Vials were shaken overhead (Scientific Industries, Inc.) at 30 °C and 30 rpm. After carboligation (if no further increase in the HPP concentration was determined), cyclohexanol (602 μL) was added, together with RADH cells (200 mg) and another 200 μL volume of 1 M TEA buffer (pH 9.0).

The simultaneous reaction was prepared in duplicates with a total volume of 4 mL each. The reactions were composed of cyclohexanol (590 μL), 1 M TEA-buffer (75 μL mL^{-1}, pH 9.0) and benzaldehyde in a final concentration of 500 mM in MTBE (3.1 mL). RADH cells (50 mg mL^{-1}) and BAL cells (25 mg mL^{-1}) were each entrapped in a separate teabag and added. Acetaldehyde was injected 4× 100 mM every 90 min by using an ice-cold syringe. Vials were shaken overhead (Scientific Industries, Inc.) at 30 °C and 30 rpm.

The mixed-mode reaction was set up in exactly the same manner as the simultaneous-mode reaction, with the exception that the teabag containing RADH cells was not added until after 3 h of the carboligation reaction.

Setup of preparative scale

The preparative production of (1*R*,2*R*)-PPD was performed in the same way as the sequential reaction described above, but scaled up to a total volume of 32 mL in a 50 mL glass bottle and sealed with a PTFE gasket. The reaction was set up in duplicate: one to be monitored by analytics, the other one to be processed for product purification. For each part of the two-step reaction, eight teabags were each filled with BAL (100 mg) or RADH (200 mg) containing cells, respectively. The carboligation reaction comprised MTBE (31.2 mL) including benzaldehyde in a final concentration of 500 mM and 1 M TEA buffer (0.8 mL, pH 9.0). Feeding proceeded with 5×100 mM acetaldehyde. Incubation was performed under continuous shaking (220 rpm) at 30 °C (Infors AG). After carboligation, the BAL catalyst was removed and cyclohexanol (3.65 mL) was added together with 1 M TEA buffer (1.6 mL, pH 9.0) and eight teabags with RADH containing lyophilised whole cells. At the end of the reaction, teabags were cut open and washed with MTBE, which was combined with the reaction bulk. The cells were pooled, resuspended in MTBE and lysed by sonication (Hielscher Ultrasonics GmbH), before samples were taken for chromatographic analysis. The buffered reaction phase was extracted twice with ethyl acetate and the extract was sampled for chromatographic analysis before combining it with the reaction's organic phase. The combined organic fraction was concentrated by rotary evaporation and further purified by flash chromatography with a mobile phase of petrol ether and ethyl acetate mixed in a 7:3 ratio. The product fraction was again concentrated by rotary evaporation and subjected to high vacuum. Product identity was confirmed by ^{1}H and ^{13}C NMR analysis (600 MHz, $CDCl_3$; see the Supporting Information S6).

HPLC Analytics

As most of the product (>90%) accumulated in the organic phase (as demonstrated by preparative scale results), only this phase was sampled for analytics in all 4 mL-scale reactions (in the preparative-scale reaction, both liquid phases and the cell extract were analysed). Reactions were monitored by chiral HPLC by using a Dionex Gina 50 autosampler, a Dionex UVD170U detector (Thermo Fisher Scientific, USA) coupled with a Gynkotek high-precision pump model 480, and a Gynkotek Degasys DG 1310 (Thermo Fisher Scientific, USA). The Chiralpack IC column (4.6×250 mm, 5 μm particle size; Daicel Chemical IND., LTD) was operated with an analytical-grade mobile phase of 70 vol% *n*-heptane and 30 vol% 2-propanol at a flow of 1 mL min^{-1} at 25 °C. 2-Hydroxyacetophenone was used as an internal standard. Samples were diluted 100- or 500-fold, depending on the expected concentrations. Approximate retention times were 5.0 min for benzaldehyde, 5.8 min for (*R*)-HPP, 6.3 min for (*S*)-HPP, 6.7 min for (*R*)-benzoin, and 7.2 min for (*S*)-benzoin.

GC analytics

Diol formation was monitored by using chiral-phase GC analytics. Samples taken from the reaction were diluted 30-fold in ethyl acetate supplemented with 1-dodecanol as an internal standard. 1 μL of these samples was analysed by using a chiral CP-Chirasil-DEX CB column (Varian; 25 m×0.25 mm×0.25 μm) at a temperature of 140 °C, run for 30 min. Detection proceeded by means of a flame ionisation detector using hydrogen as a carrier gas. Retention times were 4.2 min for benzyl alcohol, 24.1 min for (1*S*, 2*S*)-PPD, 25.9 min for (1*R*,2*R*)-PPD, 27.4 min for (1*S*,2*R*)-PPD, and 28.3 min for (1*R*, 2*S*)-PPD.[22]

Sample preparation for SEM analysis

Samples were washed in 0.1 M sodium phosphate buffer (pH 7.2) before dehydration performed by a graded series of ethanol in water (30, 50, 70, 95 and 100 vol%; 15–30 min). After overnight drying, microscopic investigation was performed by the Laboratory for Electron Microscopy, Karlsruhe Institute of Technology.

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[1] H. C. Kolb, M. S. Vannieuwenhze, K. B. Sharpless, *Chem. Rev.* **1994**, *94*, 2483 – 2547.
[2] R. N. Patel, *Coord. Chem. Rev.* **2008**, *252*, 659 – 701.
[3] J. Pan, H. L. Yu, J. H. Xu, G. Q. Lin in *Asymmetric Catalysis from a Chinese Perspective, Vol. 36* (Eds.: M. Beller, J. M. Brown, P. H. Dixneuf, A. Fürstner, L. J. Gooßen, L. S. Hegedus, P. Hofmann, T. Ikariya, L. A. Oro, Q.-L. Zhou), Springer, **2011**, pp. 67 – 103.
[4] U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185 – 194.
[5] T. Gerhards, U. Mackfeld, M. Bocola, E. von Lieres, W. Wiechert, M. Pohl, D. Rother, *Adv. Synth. Catal.* **2012**, *354*, 2805 – 2820.
[6] A. Illanes, A. Cauerhff, L. Wilson, G. R. Castro, *Bioresour. Technol.* **2012**, *115*, 48 – 57.
[7] F. van Rantwijk, R. A. Sheldon, *Chem. Rev.* **2007**, *107*, 2757 – 2785.
[8] D. Gocke, G. Kolter, M. Muller, M. Pohl, *Chem. Ing. Tech.* **2010**, *82*, 81 – 86.
[9] P. A. Santacoloma, G. Sin, K. V. Gernaey, J. M. Woodley, *Org. Process Res. Dev.* **2011**, *15*, 203 – 212.
[10] J. H. Schrittwieser, J. Sattler, V. Resch, F. G. Mutti, W. Kroutil, *Curr. Opin. Chem. Biol.* **2011**, *15*, 249 – 256.
[11] T. Sehl, R. C. Simon, H. C. Hailes, J. M. Ward, U. Schell, M. Pohl, D. Rother, *J. Biotechnol.* **2012**, *159*, 188 – 194.
[12] S. Shanmuganathan, D. Natalia, L. Greiner, P. Domínguez de María, *Green Chem.* **2012**, *14*, 94 – 97.
[13] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J. M. Woodley, *Org. Process Res. Dev.* **2011**, *15*, 266 – 274.
[14] A. Jakoblinnert, R. Mladenov, A. Paul, F. Sibilla, U. Schwaneberg, M. B. Ansorge-Schumacher, P. D. de Maria, *Chem. Commun.* **2011**, *47*, 12230 – 12232.
[15] R. León, P. Fernandes, H. M. Pinheiro, J. M. S. Cabral, *Enzyme Microb. Technol.* **1998**, *23*, 483 – 500.
[16] H. Pfruender, R. Jones, D. Weuster-Botz, *J. Biotechnol.* **2006**, *124*, 182 – 190.
[17] M. Gaab, S. Bellemin-Laponnaz, L. H. Gade, *Chemistry* **2009**, *15*, 5450 – 5462.
[18] R. A. Houghten, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 5131 – 5135.
[19] D. Okrob, M. Paravidino, R. V. A. Orru, W. Wiechert, U. Hanefeld, M. Pohl, *Adv. Synth. Catal.* **2011**, *353*, 2399 – 2408.
[20] B. Gonzalez, R. Vicuna, *J. Bacteriol.* **1989**, *171*, 2401 – 2405.
[21] A. S. Demir, M. Pohl, E. Janzen, M. Muller, *J. Chem. Soc. Perkin Trans. 1* **2001**, 633 – 635.
[22] J. Kulig, R. C. Simon, C. A. Rose, S. M. Husain, M. Hackh, S. Ludeke, K. Zeitler, W. Kroutil, M. Pohl, D. Rother, *Catal. Sci. Technol.* **2012**, *2*, 1580 – 1589.
[23] T. H. Johnson, K. C. Klein, *J. Org. Chem.* **1979**, *44*, 461 – 462.
[24] R. J. Mcmahon, K. E. Wiegers, S. G. Smith, *J. Org. Chem.* **1981**, *46*, 99 – 101.
[25] R. Noyori, I. Tomino, Y. Tanimoto, *J. Am. Chem. Soc.* **1979**, *101*, 3129 – 3131.
[26] K. B. Sharpless, W. Amberg, Y. L. Bennani, G. A. Crispino, J. Hartung, K. S. Jeong, H. L. Kwong, K. Morikawa, Z. M. Wang, D. Q. Xu, X. L. Zhang, *J. Org. Chem.* **1992**, *57*, 2768 – 2771.
[27] M. Tokunaga, J. F. Larrow, F. Kakiuchi, E. N. Jacobsen, *Science* **1997**, *277*, 936 – 938.
[28] Z. Wang, Y. T. Cui, Z. B. Xu, J. Qu, *J. Org. Chem.* **2008**, *73*, 2270 – 2274.
[29] G. Guillena, M. D. Hita, C. Najera, *Tetrahedron: Asymmetry* **2006**, *17*, 1027 – 1031.
[30] W. Notz, B. List, *J. Am. Chem. Soc.* **2000**, *122*, 7386 – 7387.
[31] S. B. Bowlus, J. A. Katzenellenbogen, *J. Org. Chem.* **1974**, *39*, 3309 – 3314.
[32] S. M. Husain, T. Stillger, P. Dunkelmann, M. Lodige, L. Walter, E. Breitling, M. Pohl, M. Burchner, I. Krossing, M. Muller, D. Romano, F. Molinari, *Adv. Synth. Catal.* **2011**, *353*, 2359 – 2362.
[33] T. Ikariya, A. J. Blacker, *Acc. Chem. Res.* **2007**, *40*, 1300 – 1308.
[34] J. A. Katzenellenbogen, S. B. Bowlus, *J. Org. Chem.* **1973**, *38*, 627 – 632.
[35] K. Murata, K. Okano, M. Miyagi, H. Iwane, R. Noyori, T. Ikariya, *Org. Lett.* **1999**, *1*, 1119 – 1121.
[36] T. Nakata, T. Tanaka, T. Oishi, *Tetrahedron Lett.* **1983**, *24*, 2653 – 2656.
[37] D. Kihumbu, T. Stillger, W. Hummel, A. Liese, *Tetrahedron: Asymmetry* **2002**, *13*, 1069 – 1072.
[38] J. Kulig, A. Frese, W. Kroutil, M. Pohl, D. Rother, *Biotechnol. Bioeng.* **2013**, *110*, 1838 – 1848.
[39] A. Jakoblinnert, D. Rother, unpublished results.
[40] H. Fröhlich, L. Villian, D. Melzner, J. Strube, *Chem. Ing. Tech.* **2012**, *84*, 905 – 917.
[41] M. Hartmann, M. Berditsch, J. Hawecker, M. F. Ardakani, D. Gerthsen, A. S. Ulrich, *Antimicrob. Agents Chemother.* **2010**, *54*, 3132 – 3142.
[42] B. Franken, T. Eggert, K. E. Jaeger, M. Pohl, *BMC Biochem.* **2011**, *12*, DOI: 10.1186/1471-2091-12-10.
[43] E. Silva Souza, L. Zaramello, C. A. Kuhnen, B. da Silva Junkes, R. A. Yunes, V. E. Fonseca Heinzen, *Int. J. Mol. Sci.* **2011**, *12*, 7250 – 7264.
[44] T. Cheng, Y. Zhao, X. Li, F. Lin, Y. Xu, X. Zhang, Y. Li, R. Wang, L. Lai, *J. Chem. Inf. Model.* **2007**, *47*, 2140 – 2148.
[45] D. J. Korz, U. Rinas, K. Hellmuth, E. A. Sanders, W. D. Deckwer, *J. Biotechnol.* **1995**, *39*, 59 – 65.
[46] M. Schwarz, dissertation thesis, HHU Düsseldorf (Jülich), **2010**.

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