ChemBioChem

Research Article
doi.org/10.1002/cbic.202200794

Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

Special Collection

Crosslinked Aggregates of Fusion Enzymes in Microaqueous Organic Media

Guillem Vernet,[a] Yu Ma,[a, b] Ningning Zhang,*[a] and Selin Kara*[a, b]

Baeyer-Villiger monooxygenases (BVMOs) are attractive for selectively oxidizing various ketones using oxygen into valuable esters and lactones. However, the application of BVMOs is restrained by cofactor dependency and enzyme instability combined with water-related downsides such as low substrate loading, low oxygen capacity, and water-induced side reactions. Herein, we described a redox-neutral linear cascade with in-situ cofactor regeneration catalyzed by fused alcohol dehydrogenase and cyclohexanone monooxygenase in aqueous and microaqueous organic media. The cascade conditions have been optimized regarding substrate concentrations as well as the amounts of enzymes and cofactors with the Design of Experiments (DoE). The carrier-free immobilization technique, crosslinked enzyme aggregates (CLEAs), was applied to fusion enzymes. The resultant fusion CLEAs were proven to function in microaqueous organic systems, in which the enzyme ratios, water contents (0.5–5 vol.%), and stability have been systematically studied. The fusion CLEAs showed promising operational (up to 5 cycles) and storage stability.

Introduction

Enzymatic processes have shown great potential for the sustainable production of chemicals, fulfilling most of the principles of *green chemistry*.[1–3] Plenty of biocatalytic processes can be found in the pharmaceutical and fine industries, especially in obtaining active pharmaceutical intermediates (APIs).[4–6] Among various biotransformations, the oxygenation reactions inter-cooperating molecular oxygen (O_2) to generate value-added chemicals have been of great interest in the biocatalysis community.[7–9] In this context, Baeyer-Villiger monooxygenases (BVMOs) are known to catalyze the regio- and enantioselective Baeyer-Villiger oxidations and sulfoxidations for the synthesis of a wide range of products, including bioactive compounds or chiral precursors.[10–14] Despite the extensive exploration of BVMOs, there are still several limitations linked to the practical use of BVMOs in aqueous media including i) enzyme instability, ii) nicotinamide cofactor dependence, iii) product auto-hydrolysis, iv) relatively low oxygen solubility, and v) the substrate and product inhibition present for most BVMOs.[15] In addition, the general limitations of using water as the solvent — low substrate loading and contaminated wastewater, exacerbate the environmental impact (E-factor) and lead to unsustainability.[16,17]

Using BVMOs in non-aqueous media is a straightforward solution to achieve high substrate loadings while sustaining improved oxygen supply. There are a few limitations to surmount to make it applicable. First, enzyme cofactor dependence can be overcome by using a self-sufficient redox-neutral cascade.[18–20] Especially, enzymatic regeneration approaches are preferred due to the growing emphasis on sustainable chemistry.[21] Therein, enzyme-coupled reactions are more frequently used than substrate-coupled systems because of their compatible reaction conditions and high efficiency.[22] This is exactly in consonance with the use of oxygenating enzymes that requires separate recycling enzymes. Alternatively, these multienzymatic cascades can be classified into four modes, i) linear, ii) orthogonal, iii) parallel, and iv) cyclic.[23] The most straightforward design is the linear cascade, where a single substrate is converted to a single product via an intermediate.[24–26] This kind of cascade largely promotes efficient and safe processes by circumventing the downstream processing, transforming intermediate spontaneously, and driving reversible reactions to completion.[27–29] Consequently, linear cascades have been established for various enzymes in aqueous systems including oxygenases.[28] However, applying oxygenases in linear cascade mode under non-aqueous conditions is still challenging. One reason is the necessity of a minimum amount of water for the diffusion of water-soluble nicotinamide cofactor between active sites of cascading enzymes. The other reason could be the relatively low stability of some oxygenases when exposed to high amounts of organic media.

Fusion approaches could enable the use of low water content in enzyme-coupled cofactor regeneration systems. The

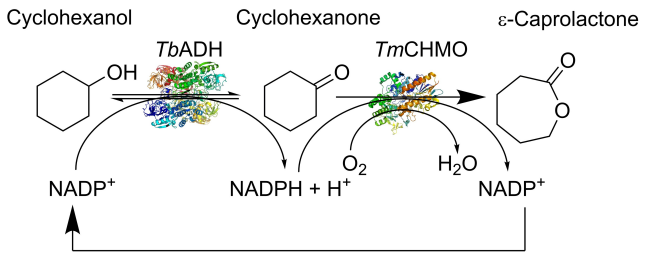
[a] G. Vernet, Y. Ma, Dr. N. Zhang, Prof. Dr. S. Kara
Institute of Technical Chemistry
Leibniz University Hannover
Callinstr. 5, 30167 Hannover (Germany)
E-mail: ningning.zhang@iftc.uni-hannover.de
selin.kara@iftc.uni-hannover.de
[b] Y. Ma, Prof. Dr. S. Kara
Biocatalysis and Bioprocessing Group
Department of Biological and Chemical Engineering
Aarhus University
Gustav Wieds Vej 10, 8000 Aarhus (Denmark)
E-mail: selin.kara@bce.au.dk

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202200794

This article is part of the Special Collection dedicated to the IBRPO2022 symposium. Please see our homepage for more articles in the collection.

fusion form of enzymes could shorten the transport distance of cofactors while diminishing the impact of organic solvents on unstable nicotinamide cofactors. Additionally, it can enhance the kinetics by faster provision of the reduced or oxidized nicotinamide cofactors.[30–32] Exemplarily, Huang et al.[31] reported the first use of fused type II flavin-containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) in microaqueous media (5 vol.% water). The results showed the decreased enzyme activity up to 1.7-fold while the K_i value towards diol substrate increased three-times showing reduced substrate inhibition of ADH upon fusion.[31] Furthermore, the identification of a highly stable cyclohexanone monooxygenase from *Thermocrispum municipale* DSM 44069 (*Tm*CHMO, EC 1.14.13.22)[33] offers the potential for using BVMOs in non-aqueous media.[34,35] Lately, Fraaije and coworkers reported the first use of the fusion protein of *Thermoanaerobacter brockii* (*Tb*) ADH and *Tm*CHMO (named Tb-Tm) in a linear cascade fashion to oxidize cyclohexanol (CHL) to synthesize ε-caprolactone (ECL), a precursor of nylon 6 (Scheme 1).[1,36] Therein, *Tm*CHMO exhibited approximately twofold higher oxidation activity when applied as a Tb-Tm fusion protein.[1,14,37] Despite that, there is no report of this fusion construct in microaqueous systems most probably due to the limited stability against organic solvents.

The stability of enzymes can be further optimized by "post-treatment". Especially, enzyme immobilization technique is a common strategy to mitigate the loss of stability under storage and operational conditions. In addition, it allows for the easy recovery and reusability of biocatalysts in batch or continuous operation systems while increasing stability against high temperatures and co-solvents.[38–43] Most isolated BVMOs have been immobilized by covalent binding to polymeric supports.[2] However, the chemical bond formed between enzymes and carriers might distort enzyme structures, leading to reduced activity, whilst the use of carriers is sometimes costlier.[44] Carrier-free strategies prevent the application of supports and thus overcome these demerits. It avoids adding inactive substances into reactors, thus can reduce costs and mass transfer limitations.[42] Crosslinked enzyme aggregate (CLEA) is one of the carrier-free immobilization approaches prevalently used for various enzymes. Enzymes are immobilized by a two-step procedure, precipitation with inorganic salts or organic solvents and crosslinking by reagents.[45,46] Recently, BVMOs were first immobilized in the form of CLEAs, offering an alternative to solid-support immobilization.[47] To the best of our knowledge, there is no report about immobilized fusion enzymes using CLEA technique and their consecutive use in organic media. This study systematically investigated the first use of CLEAs of the fusion enzyme *Tb*ADH-*Tm*CHMO (Tb-Tm) in linear redox-neutral cascades in aqueous and microaqueous organic media.



Scheme 1. Linear cascade reaction catalyzed by fused *Tb*ADH and *Tm*CHMO to convert cyclohexanol to ε-caprolactone with in-situ cofactor regeneration.

Results and Discussion

Cascade reaction

The reported linear cascade of the oxidation of CHL to ECL via the intermediate of cyclohexanone (CHO) was used as the model reaction (Scheme 1). The first step of the reaction is the oxidation of cyclohexanol (CHL) to cyclohexanone (CHO) by *Tb*ADH while reducing $NADP^+$ to NADPH. Then, the *Tm*CHMO can oxidize the intermediate CHO to ε-caprolactone (ECL), and NADPH to $NADP^+$ using molecular oxygen. Therefore, the sequential conversion of $NADP^+$ to NADPH and then back to $NADP^+$ achieved the "closed-loop" "self-sufficient" in-situ cofactor regeneration.[1,4,48] The optimal fusion protein (Tb-Tm) was well-characterized in a previous study and thus being initially explored here.[1] Prior to catalysis, the cell-free extract (CFE) of Tb-Tm was prepared by overexpression in *Escherichia coli* host and analyzed with SDS-PAGE (Figure S1 in the Supporting Information), accounting for a major proportion of the total proteins. First, the proof-of-concept has been demonstrated with Tb-Tm in the form of CFE in the aqueous medium (Figure 1). The cascade proceeds with sufficient initial reaction rates using only the fused enzyme, as after 1 hour, ECL can be detected. Knowing that CHMO being rate-limiting promoted the thought to circumvent the tie with an additional amount of Tm. An increase in reaction rate was clearly observed due to the refueling of Tm at 3 hours (green vertical dash line in Figure 1),

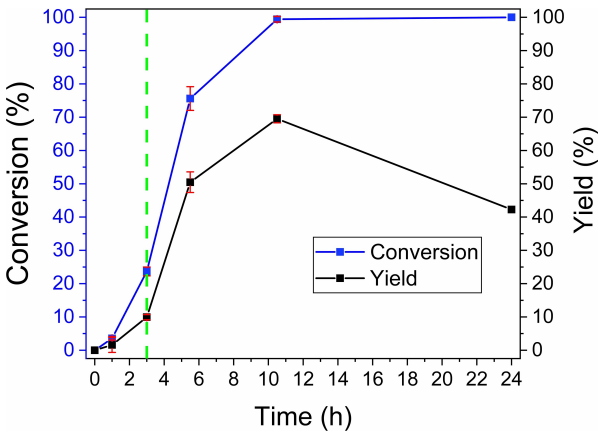


Figure 1. Progressive curve of the linear cascade catalyzed by fusion enzymes (Tb-Tm) with external addition of single *Tm*CHMO (Tm). Reaction conditions: 2 mL Tris·HCl buffer (50 mM at pH 7.6) containing 10 mM cyclohexanol, 1 mM $NADP^+$, 0.106 U Tb-Tm, 0.535 U Tm (added after 3 h), incubation at 37 °C and 150 rpm. Experiments were performed in duplicate.

and full conversion was achieved after 10 hours. Despite that, a relatively low yield was recorded due to the auto-hydrolysis of the ε-caprolactone,[49] which is an undesired major issue when performing the cascade in an aqueous medium (progressive curves and mass balance shown in Figure S2).

After proving the cascade reaction with the fusion enzyme, the optimal reaction conditions have been studied with the response surface methodology, central composite using the Design of Experiments (DoE). Wherein, the concentrations of CHL (ranging from 8 to 89 mM), $NADP^+$ (ranging from 0 to 1.0 mM), and fusion enzyme (CFE of Tb-Tm ranging from 0.06 to 0.49 U) were selected as variable parameters while the conversion to ECL was set as the main response (Table 1).

Table 1. Range of parameters and the target response studied in DoE.

	Input			Output
Range	[CHL] [mM]	[$NADP^+$] [mM]	[Tb-Tm] [U]	Conversion [%]
minimum	8.17	0.00	0.06	12
maximum	88.98	1.00	0.50	100

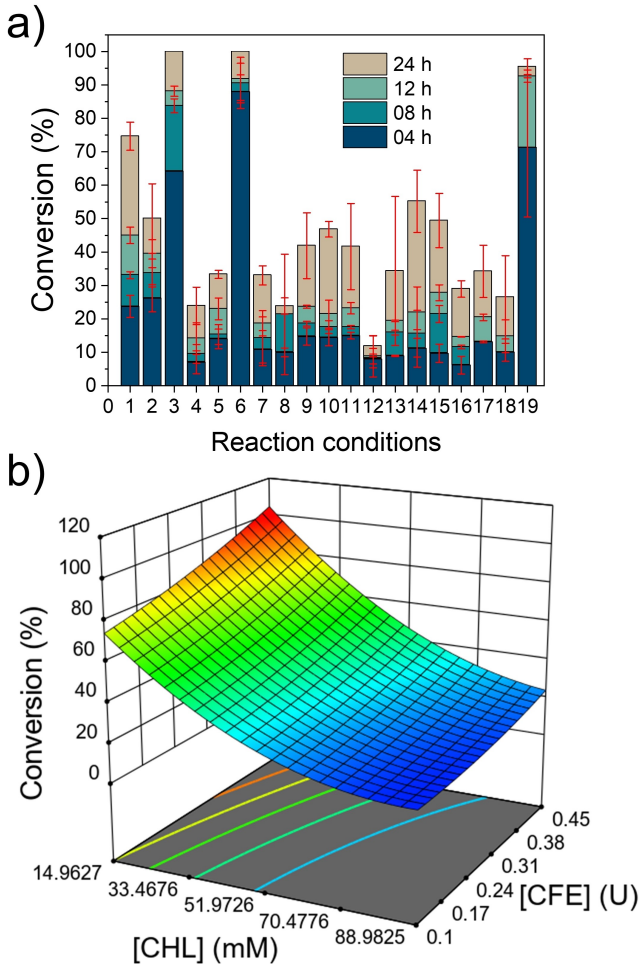


Figure 2. a) Conversion of CHL in the linear cascade catalyzed by a CFE of fusion enzyme Tb-Tm under various reaction conditions in DoE analysis. Reactions were performed in KPi buffer (50 mM at pH 8), at 30 °C, and 150 rpm. Experiments were performed in duplicate. b) 3D surface response map for reaction using 0.105 mM $NADP^+$ in DoE analysis.

According to previous studies[1,24] temperature was found as not a significant factor for this cascade and is therefore not included in the DoE analysis. Accordingly, 19 reaction conditions were set up by varying these parameters and the detailed information of each reaction is shown in Table S1.

Accordingly, a wide range of conversions was achieved, ranging from 12% to full conversion (Figure 2a). One interesting result was obtained from the reaction with the lower level of cofactor (No. 15 in Figure 2a) where there was no external $NADP^+$ added. The result constates the presence of $NADP^+$ within CFE is sufficient to promote the reaction.[50] The full conversion is reached with lower substrate loadings (8–18 mM; Table S1, reactions No. 3, 6, and 19), probably due to the alleviated substrate and product inhibition reported in previous studies.[48,49] As can be expected, increasing the concentration of the fusion enzyme at the same substrate and cofactor loadings results in higher conversion (reaction No. 14 vs. 17). The DoE analysis indicates, within the studied ranges, the concentration of CHL and the amount of enzyme are significant factors (Tables S2 and S3). Meanwhile, it can be considered the changes of $NADP^+$ are not prominent. Consequently, the analysis result of the 3D surface response map showed an increase in enzyme combined with a decrease in substrate concentration has a positive contribution to conversion when using constant amount of $NADP^+$ (Figure 2b). These results are in line with the expectation since most BVMOs,[10] including the *Tm*CHMO,[33] suffer severely from substrate inhibition. Given the benefits of using non-aqueous media, a relatively high concentration of 60 mM is chosen as the optimal concentration to be used in further experiments.

CLEA preparation and catalysis in aqueous media

Enzyme immobilization is well known to increase enzyme stability even though, some activity may be lost. In that sense, CLEA has shown to be a promising option as a cheap, carrier-free methodology.[47] The CLEA approach consists of two steps, precipitation and crosslinking.[45] In this study, we focused on the optimization of the glutaraldehyde-promoted crosslinking step because it is the step where the enzyme activity can be compromised.[51] The concentration of glutaraldehyde has been varying from 0.04% to 0.6% (w/v). Compared to a previous work where the authors reported a significant decrease of enzyme activity in higher amounts of glutaraldehyde than 0.12% (w/v),[47] the highest immobilization yield and enzyme activity were observed using 0.20% (w/v; Figure S3).

After the determination of the optimal condition for cross-linking step, the ratios of two enzymes within CLEAs have been optimized since the second step of the cascade is limiting the process.[52] In detail, the CFE of the fusion enzymes Tb-Tm and the individual Tm have been mixed in various volumetric ratios (Figure 3). Samples are named according to the amount of extra single Tm enzyme with varying values of 0, 10, 20, 40, 60, and 80 μL. For example, CLEA 8 consists of 100 μL CFE of fusion enzyme Tb-Tm, and 80 μL CFE of single Tm enzyme. The immobilization process was first evaluated by the immobiliza-

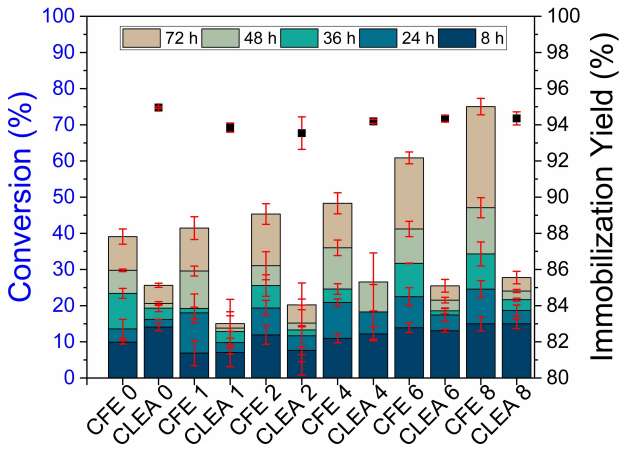


Figure 3. The immobilization yield of various CLEAs consisting of CFEs of Tb-Tm and extra Tm, and the progress curve of cascade catalyzed by CLEAs for up to 72 h. Experiments were performed in duplicate. Reactions were performed in KPi buffer (50 mM at pH 8) containing 60 mM cyclohexanol, and 0.1 mM $NADP^+$ at 30 °C, 150 rpm in a 40 mL glass vial with 2 mL reaction volume. The total units (U) of extra *Tm*CHMO used are 1.26, 1.30, 1.35, 1.44, 1.52, and 1.61.

tion yield. Near full immobilization yields are obtained for all ratios with the CLEA technique, signifying that nearly all proteins within the CFE are kept during the process (Figure 3). It's known the improved stability of enzymes can be obtained by immobilization at the cost of enzyme activity. The activity of these CLEAs was first studied in the linear cascade in aqueous media while the corresponding CFEs were used as comparisons. As expected, higher conversions were achieved with CFEs counterparts in 24 hours (Figure 3). The lower conversions from CLEAs can be attributed to the enzyme deactivation during immobilization or mass transfer limitations.

To further study the catalytic performance of CLEAs in the aqueous system, the reaction course was extended to 72 hours. It can be clearly seen the introduction of additional Tm helps to push the reaction to the product direction in the case of CFE, as expected (Figure 3). However, it does not improve the conversion significantly in the case of CLEAs, reinforcing the hypothesis of mass transfer limitation combined with potential enzyme deactivation owing to crosslinking. The second main observation from Figure 3 is the decrease in reaction rate after 8 hours. Especially in the case of CLEAs, most conversion was accomplished in the first 8 hours while the extension of the reaction contributed to the conversions at a slower pace. This result indicates the cascade might suffer more from mass transfer limitation than enzyme deactivation in the case of CLEAs when performing reactions in aqueous media. To further confirm that, a long-term reaction of 10 days was performed with the aim of reaching full conversion (Figure S4). However, after 5 days of the reaction course, the conversion did not significantly increase while a side product was observed in GC chromatography (Figure S5). To investigate that, the pH of the reaction system was measured at the end of the reaction, obtaining a value of 5.9. This acidification is presumably caused by the ring-opening of ECL in an aqueous environ, forming 6-hydroxyhexanoic acid. The dramatic decrease in pH can be a main cause of enzyme's malfunction and not reaching full conversion. This result propels the use of non-aqueous media to restrain auto-hydrolysis.

CLEA catalysis in a microaqueous organic system

Using organic solvent has been reported as a good alternative for process intensification of biocatalysis and can avoid water-related issues.[38] In particular, various studies have demonstrated that performing the established cascade in water-deficient media could largely minimize the auto-hydrolysis of ECL.[4,31,53] In this study, three organic solvents, 2-methyltetrahydrofuran (2-MeTHF), methyl *tert*-butyl ether (MTBE), and cyclopentyl methyl ether (CPME), have been selected because of their sustainability and reported prospective results.[54] The main physicochemical properties of these solvents are shown in Table S4.

The activation of enzymes in nonaqueous media demands the least water to maintain sufficient enzyme hydration.[55] Consequently, the first attempt was carried out in organic solvents with different water contents. Due to the addition of $NADP^+$ dissolved in buffer, 0.5 vol.% of water content was involved necessarily. In the case of using CFEs, 5 vol.% water was compulsorily added. For this reason, CLEA has been first tested with 0.5 vol.% water (in the form of cofactor stock solution) and 5.5 vol.% water to be able to fairly compare with the CFE. As expected, the formation of 6-hydroxyhexanoic acid from auto-hydrolysis was suppressed in microaqueous organic systems and not observed in GC analysis (Figure S6). Intriguing results are obtained with CPME and MTBE while no conversion was observed in 2-MeTHF. For the first time, the CLEAs of fusion enzymes worked in organic solvents despite the relatively low conversion compared to CFEs (Figure S7). The CLEAs might suffer a lot from the impact of 2-MeTHF as a drastic color change was observed compared to the other two solvents. Similar results were reported with ADHs using this solvent.[55]

After proving that CLEA and CFE can function in CPME and MTBE, medium engineering is performed to assess the influence of water addition on reaction efficiency and identify the optimal water concentration. Three water contents, 0.5, 2.5, and 5 vol.%, were selected within the range of microaqueous systems. For both solvents, the higher water content is more favorable, leading to relatively higher conversions (Figure 4). For CPME, there is no significant difference between 2.5 and 5 vol.%. The best water content for MTBE is 5 vol.% despite not much difference at the beginning observed. This is also consistent with other works showing promising results with MTBE.[31,53] For a fair comparison, several control experiments were performed with the CFE of Tb-Tm, the CFE combination of two single enzymes, CLEAs of two single enzymes (Combi-CLEA), and CLEA combination of two single enzymes (Multi-CLEA). The results show the CFE of Tb-Tm outperformed all other CLEAs in 24-hour reactions (Figure S8). A possible explanation is the mass transfer limitation within the concrete CLEAs compared to the free enzymes. The preliminary results showed that an increase

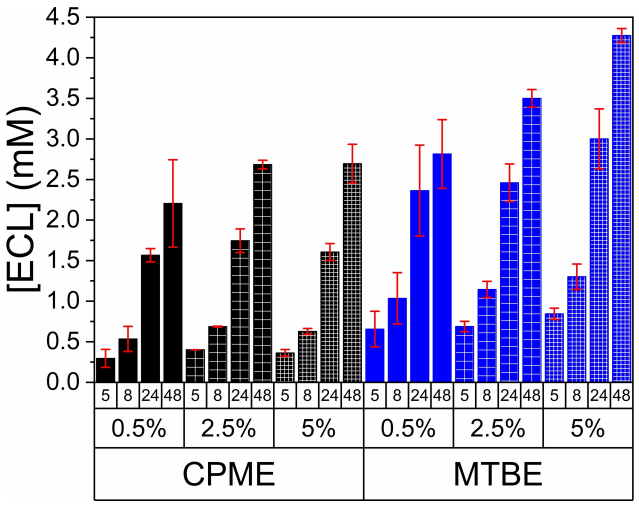


Figure 4. Progressive curves of the cascade performed in CPME and MTBE with different water content (0.5, 2.5, and 5.0 vol.%) and sampling at different reaction times (5, 8, 24, and 48 h). Reactions were performed at 30 °C, 160 rpm in a 40 mL glass vial with 2 mL reaction volume containing 60 mM cyclohexanol, 0.1 mM $NADP^+$, and 2 U of immobilized Tb-Tm. All reactions were performed in triplicate.

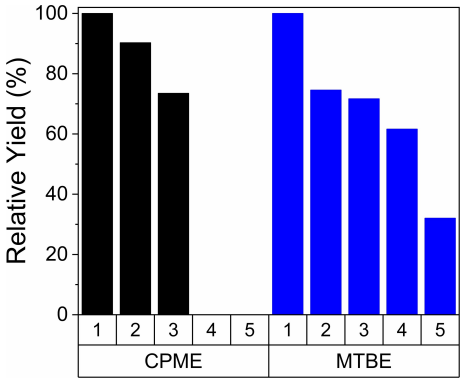


Figure 5. Operational stability and reusability of fusion CLEAs in two organic solvents, CPME and MTBE with 0.5 vol.% water content. Reactions performed at 30 °C, 160 rpm in a 40 mL glass vial with 2 mL reaction volume for 4 h each cycle using 60 mM cyclohexanol, 0.1 mM $NADP^+$.

of shaking speed can to some extent overcome the mass transfer limitation.

CLEA operational and storage stability

The main purpose of enzyme immobilization is to gain stability while minimizing the loss of activity. Besides, from the process point of view, enzyme immobilization allows for easy recovery and reuse of enzymes. For this reason, the reusability of CLEAs under operational conditions has been studied in both organic solvents by repeating a 4-hour short-term reaction course. To avoid possible enzyme loss, the residual solution was carefully removed and the new reaction solution containing the substrate, cofactor, and water was added to reach the same conditions as before for a new cycle. In the case of CPME, the CLEA of Tb–Tm can be recycled for three times with 10% and 26% loss of enzyme activities in the second and third cycles (Figure 5). However, no product was detected in the next two cycles. For MTBE, a bigger decrease in production of 25% was observed for the first cycle but five cycles were sustained with a 32% conversion for the last one.

To investigate if the enzyme is deactivated after five reuses, the recovered CLEAs were applied for a 72-hour reaction under the same conditions leading to 34% relative residual yield for MTBE but ninefold higher relative yield for CPME. This result further demonstrated the operational stability of CLEAs in organic solvents. Not only the operational stability is important for the process, but also the storage steadiness. The longer the storage time of immobilized enzymes, the easier it is to implement CLEA on a technical scale.[56] The CLEA of fusion enzymes has shown excellent storage stability for more than 20 days without obvious activity loss. These preliminary results demonstrate the reusability of CLEAs in microaqueous organic systems.

Conclusion

In summary, this study presents the pioneering generation and application of the crosslinked enzyme aggregates of a fusion enzyme, Tb-Tm, in aqueous and microaqueous organic systems. The model linear cascade was established by using the cell-free extract of Tb-Tm to synthesize valuable lactones, while the reaction conditions have been optimized with DoE, revealing significant parameters for substrate and enzyme concentrations. A CFE of fusion Tb-Tm was demonstrated to effectively catalyze the self-sufficient linear cascade with a minimum amount of cofactor. The concept of CLEAs was first combined with fusion approaches. Both CFEs and CLEAs show moderate activity in aqueous and microaqueous systems. Pleasingly, CLEAs displayed strong stability after being recycled 3–5 times while remaining active for more than 20 days under storage conditions. This success offers the potential for fusion CLEAs to be combined with flow catalysis or pack bed reactors for process intensification.

Experimental Section

Enzyme production: In a 1-L autoclaved Erlenmeyer, a small amount of glycerol stock of *E. coli* strain containing the plasmid of the fusion enzyme (Tb-Tm) is inoculated into 100 mL LB medium with 50 $\mu g\,mL^{-1}$ ampicillin. The pre-culture is incubated at 37 °C and 100 rpm overnight. In a 5-L autoclaved Erlenmeyer, 100 mL pre-culture is inoculated into 1000 mL TB media in a ratio of 1 to 10 with 0.02% (w/v) L-arabinose and 50 $\mu g\,mL^{-1}$ ampicillin. The obtained main culture is incubated at 24 °C and 80 rpm for 48 h. Afterward, the cells were harvested by centrifugation at 4 °C, 8000 rpm for 15 min. The cell-free extract (CFE) is prepared first by resuspending and washing the whole cells in KPi buffer (50 mM at pH 8), followed by centrifugation at 4 °C, 13000 rpm for 15 min. The cells are resuspended again in 0.7 g wet cells per mL buffer. The

cell solution is sonicated using MS73 probe and 50% amplitude for 6 cycles with each cycle of 2 min (2 s on and 8 s off). The solution is cooled down on ice for 2 min in between of each cycle. Finally, the solution is centrifuged at 4 °C, 13000 rpm for 45 min to obtain the clear CFE.

SDS-PAGE analysis is performed to visualize the fusion proteins are over-expressed in the *E. coli* Top 10 cells. The samples are prepared by mixing loading buffer RunBblue 4x with diluted CFE, and then heating at 90 °C for 10 min. The samples were spun down at 13000 rpm before being added to the 12% SDS-PAGE gel. PageRuler™, Prestained, Protein Ladder stems were purchased from Thermo Fisher Scientific. ExpressPlus™ 12% BT gel and SDS running buffer were purchased from GenScript (Piscataway, USA).

Specific activity assay: Kinetic measurements are done by following the depletion of NADPH at 340 nm using Agilent Cary 60 spectrophotometer. The final 1 mL reaction system contains 0.25 mM thioanisole and 0.2 mM NADPH in KPi buffer (50 mM at pH 8) for the confirmation of *Tm*CHMO activity in the Tb-Tm fused enzymes. The reaction is monitored by measuring the change of absorbance for 1 min at 25 °C while the linear slope of the first 20 s is used to calculate the volumetric specific activity (U mL^{-1}). For evaluating the activity of the *Tb*ADH, 1 mL reaction system containing 10 mM CHL and 0.2 mM $NADP^+$ is monitored by measuring the change of absorbance for one minute at 25 °C while the linear slope of the 60 s is used to calculate the volumetric specific activity (U mL^{-1}).

Protein concentration measurement: The concentration of protein is determined by Bradford assay using bovine serum albumin as the standard protein.[57] The analysis is performed at 25 °C by measuring the absorbance at 595 nm with the ThermoScientific Multiskan Sky.

Water content in organic solvents: The water content in organic solvents was measured using the Karl Fischer titrator TitroLine 7750 from SI Analytics. The measurements were done following the protocol from the company in sextuplicate.

Biotransformation-cascade reaction: Unless otherwise noted, all reactions were performed in a 40 mL glass vial with 2 mL total reaction volume at 30 °C and 150 rpm. In the case of the using aqueous reaction media, ethyl acetate including internal standard was used for liquid–liquid extraction. When the biotransformation was performed in organic medium, the sample was added with one fourth of the organic solvent with internal standard. The extracts were analyzed by gas chromatography (Shimadzu GC-2030, GC-fid), using a GC column CP-Chirasil-Dex CB (25 m × 0.25 mm × 0.25 μm), inlet temperature of 250 °C, 0.70 mL min^{-1} column flow rate for 12 min. 30 mL min^{-1} to 3.00 mL min^{-1} hold 3.52 min., FID temperature at 250 °C, column at 130 °C hold 12 min., 100 °C min^{-1} to 190 °C hold 3 min (total program time 15.60 min).

Crosslinked enzyme aggregates (CLEA) preparation: The protein was precipitated out by adding 80 vol.% saturated solution of ammonium sulfate. Secondly, the CFE and 80 vol.% saturated $(NH_4)_2SO_4$ are mixed in a ratio of 1 fraction of CFE and 9 fractions of precipitation reagent in a 2 mL Eppendorf. The solution was shaken at 4 °C and 1000 rpm for 30 min to ensure all proteins get precipitated. After mixing, glutaraldehyde was added to obtain a final concentration of 0.20% (w/v). The solution is reposed for 120 min at room temperature to ensure all the aggregates are crosslinked. The CLEAs are collected by centrifuging at 4 °C, 500 g for 30 min, and washed twice with KPi buffer (50 mM at pH 8) followed by centrifuging with the same conditions as before. The protein concentration of the first supernatant (SN), and the two washing fractions (W_1 and W_2) are measured to determine the immobilization yield [Eq. (1)].

$$\text{Immobilization yield (\%)} = \frac{\text{CFE (mg)} - \text{SN (mg)} - W_1\text{(mg)} - W_2\text{(mg)}}{\text{CFE (mg)}} \quad (1)$$

Acknowledgements

The Deutsche Forschungsgemeinschaft (DFG; grant no. KA 4399/3-2) and China Scholarship Council (CSC; grant no. 202108610066) are acknowledged for their kind financial support.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: biotransformations · crosslinked enzyme aggregates · cyclohexanone monooxygenase · enzyme immobilization · fusion enzymes

[1] F. S. Aalbers, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 7557–7565.
[2] M. A. F. Delgove, D. Valencia, J. Solé, K. V. Bernaerts, S. M. A. De Wildeman, M. Guillén, G. Álvaro, *Appl. Catal. A* **2019**, *572*, 134–141.
[3] T.-L. Chen, H. Kim, S.-Y. Pan, P.-C. Tseng, Y.-P. Lin, P.-C. Chiang, *Sci. Total Environ.* **2020**, *716*, 136998.
[4] S. Schmidt, C. Scherkus, J. Muschiol, U. Menyes, T. Winkler, W. Hummel, H. Gröger, A. Liese, H.-G. Herz, U. T. Bornscheuer, *Angew. Chem. Int. Ed.* **2015**, *54*, 2784–2787; *Angew. Chem.* **2015**, *127*, 2825–2828.
[5] D. L. Hughes, *Org. Process Res. Dev.* **2022**, *26*, 1878–1899.
[6] K. Hecht, H.-P. Meyer, R. Wohlgemuth, R. Buller, *Catalysts* **2020**, *10*, 1420.
[7] A. Hoschek, B. Bühler, A. Schmid, *Angew. Chem. Int. Ed.* **2017**, *56*, 15146–15149; *Angew. Chem.* **2017**, *129*, 15343–15346.
[8] R. H. Ringborg, A. Toftgaard Pedersen, J. M. Woodley, *ChemCatChem* **2017**, *9*, 3285–3288.
[9] A. Al-Shameri, L. Schmermund, V. Sieber, *Curr. Opin. Green Sustain. Chem.* **2022**, *40*, 100733.
[10] M. Bučko, P. Gemeiner, A. Schenkmayerová, T. Krajčovič, F. Rudroff, M. D. Mihovilovič, *Appl. Microbiol. Biotechnol.* **2016**, *100*, 6585–6599.
[11] H. Leisch, K. Morley, P. C. K. Lau, *Chem. Rev.* **2011**, *111*, 4165–4222.
[12] N. M. Kamerbeek, D. B. Janssen, W. J. H. van Berkel, M. W. Fraaije, *Adv. Synth. Catal.* **2003**, *345*, 667–678.
[13] H. R. Mansouri, O. Gracia Carmona, J. Jodlbauer, L. Schweiger, M. J. Fink, E. Breslmayr, C. Laurent, S. Feroz, L. C. P. Goncalves, D. V. Rial, M. D. Mihovilovic, A. S. Bommarius, R. Ludwig, C. Oostenbrink, F. Rudroff, *ACS Catal.* **2022**, *12*, 11761–11766.
[14] D. E. Torres Pazmiño, H. M. Dudek, M. W. Fraaije, *Curr. Opin. Chem. Biol.* **2010**, *14*, 138–144.
[15] L. Sellés Vidal, C. L. Kelly, P. M. Mordaka, J. T. Heap, *Proteins Proteomics* **2018**, *1866*, 327–347.
[16] F. Tieves, F. Tonin, E. Fernández-Fueyo, J. M. Robbins, B. Bommarius, A. S. Bommarius, M. Alcalde, F. Hollmann, *Tetrahedron* **2019**, *75*, 1311–1314.
[17] D. Holtmann, F. Hollmann, *J. Mol. Catal.* **2022**, *517*, 112035.
[18] S. Kara, J. H. Schrittwieser, F. Hollmann, M. B. Ansorge-Schumacher, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1517–1529.
[19] W. Hummel, H. Gröger, *J. Biotechnol.* **2014**, *191*, 22–31.
[20] A. I. Benítez-Mateos, D. Roura Padrosa, F. Paradisi, *Nat. Chem.* **2022**, *14*, 489–499.

[21] X. Wang, T. Saba, H. H. P. Yiu, R. F. Howe, J. A. Anderson, J. Shi, *Chem* **2017**, *2*, 621–654.
[22] N. Zhang, B. Müller, T. Ø. Kirkeby, S. Kara, C. Loderer, *ChemCatChem* **2022**, *14*, e202101625.
[23] E. Ricca, B. Brucher, J. H. Schrittwieser, *Adv. Synth. Catal.* **2011**, *353*, 2239–2262.
[24] J. Engel, K. S. Mthethwa, D. J. Opperman, S. Kara, *J. Mol. Catal.* **2019**, *468*, 44–51.
[25] J. H. Schrittwieser, J. Sattler, V. Resch, F. G. Mutti, W. Kroutil, *Curr. Opin. Chem. Biol.* **2011**, *15*, 249–256.
[26] V. K. Sharma, J. M. Hutchison, A. M. Allgeier, *ChemSusChem* **2022**, *15*, e202200888.
[27] S. Gandomkar, A. Żądło-Dobrowolska, W. Kroutil, *ChemCatChem* **2019**, *11*, 225–243.
[28] J. H. Schrittwieser, S. Velikogne, M. Hall, W. Kroutil, *Chem. Rev.* **2018**, *118*, 270–348.
[29] E. García-Junceda, I. Lavandera, D. Rother, J. H. Schrittwieser, *J. Mol. Catal. B* **2015**, *114*, 1–6.
[30] Y. Ma, N. Zhang, G. Vernet, S. Kara, *Front. Bioeng. Biotechnol.* **2022**, *10*, 944226.
[31] L. Huang, F. S. Aalbers, W. Tang, R. Röllig, M. W. Fraaije, S. Kara, *ChemBioChem* **2019**, *20*, 1653–1658.
[32] A. Kokorin, P. D. Parshin, P. J. Bakkes, A. A. Pometun, V. I. Tishkov, V. B. Urlacher, *Sci. Rep.* **2021**, *11*, 21706.
[33] E. Romero, J. R. G. Castellanos, A. Mattevi, M. W. Fraaije, *Angew. Chem.* **2016**, *128*, 16084–16087; *Angew. Chem. Int. Ed.* **2016**, *55*, 15852–15855.
[34] H. Ramesh, T. Mayr, M. Hobisch, S. Borisov, I. Klimant, U. Krühne, J. M. Woodley, *J. Chem. Technol. Biotechnol.* **2016**, *91*, 832–836.
[35] A. Lorente-Arevalo, M. Ladero, J. M. Bolivar, *Curr. Opin. Green Sustain. Chem.* **2021**, *32*, 100544.
[36] A. Gran-Scheuch, F. Aalbers, Y. Woudstra, L. Parra, M. W. Fraaije in *Methods in Enzymology, Vol. 647* (Eds.: M. Merkx), Elsevier, **2021**, pp. 107–143.
[37] F. S. Aalbers, M. W. Fraaije, *ChemBioChem* **2019**, *20*, 51–56.
[38] G. Vernet, M. Hobisch, S. Kara, *Curr. Opin. Green Sustain. Chem.* **2022**, *38*, 100692.
[39] N. R. Mohamad, N. H. C. Marzuki, N. A. Buang, F. Huyop, R. A. Wahab, *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 205–220.
[40] J. M. Bolivar, J. M. Woodley, R. Fernandez-Lafuente, *Chem. Soc. Rev.* **2022**, *51*, 6251–6290.
[41] A. Illanes, L. Wilson, C. Vera, *Problem Solving in Enzyme Biocatalysis* Wiley, **2013**.
[42] P. De Santis, L.-E. Meyer, S. Kara, *React. Chem. Eng.* **2020**, *5*, 2155–2184.
[43] L.-E. Meyer, M. Hobisch, S. Kara, *Curr. Opin. Biotechnol.* **2022**, *78*, 102835.
[44] U. Roessl, J. Nahálka, B. Nidetzky, *Biotechnol. Lett.* **2010**, *32*, 341–350.
[45] R. A. Sheldon, *Catalysts* **2019**, *9*, 1–31.
[46] R. Noori, M. Perwez, M. Sardar in *Biocatalysis* (Eds.: Q. Husain, M. Ullah), Springer International, Cham, **2019**, pp. 83–112.
[47] J. Zhu, Q. Geng, Y.-Y. Liu, J. Pan, H. L. Yu, J.-H. Xu, *Org. Process Res. Dev.* **2022**, acs.oprd.1c00382.
[48] S. Wedde, P. Rommelmann, C. Scherkus, S. Schmidt, U. T. Bornscheuer, A. Liese, H. Gröger, *Green Chem.* **2017**, *19*, 1286–1290.
[49] C. Scherkus, S. Schmidt, U. T. Bornscheuer, H. Gröger, S. Kara, A. Liese, *ChemCatChem* **2016**, *8*, 3446–3452.
[50] J. London, M. Knight, *J. Gen. Microbiol.* **1966**, *44*, 241–254.
[51] S. Velasco-Lozano, F. López-Gallego, J. C. Mateos-Díaz, E. Favela-Torres, *Biocatalysis* **2016**, *1*, 166–177.
[52] H. Mallin, H. Wulf, U. T. Bornscheuer, *Enzyme Microb. Technol.* **2013**, *53*, 283–287.
[53] Á. Mourelle-Insua, F. S. Aalbers, I. Lavandera, V. Gotor-Fernández, M. W. Fraaije, *Tetrahedron* **2019**, *75*, 1832–1839.
[54] P. Domínguez de María, in *Biocatal. Green Solvents*, Elsevier, **2022**, pp. 511–527.
[55] N. Zhang, J. P. Bittner, M. Fiedler, T. Beretta, P. D. de María, S. Jakobtorweihen, S. Kara, *ACS Catal.* **2022**, *12*, 9171–9180.
[56] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J. M. Woodley, *Org. Process Res. Dev.* **2011**, *15*, 266–274.
[57] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

Manuscript received: December 29, 2022
Revised manuscript received: February 6, 2023
Accepted manuscript online: February 7, 2023
Version of record online: March 22, 2023