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Immobilized Synthetic Pathway for Biodegradation of Toxic Recalcitrant Pollutant 1,2,3-Trichloropropane

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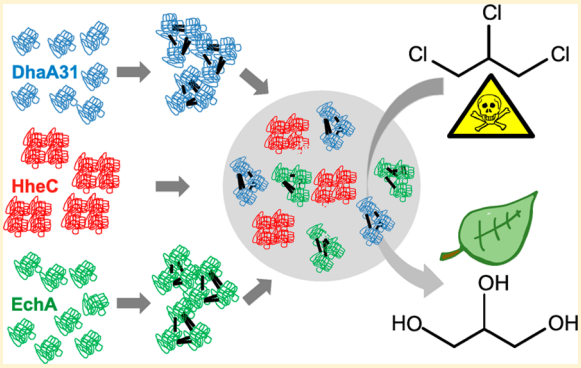
Supporting Information

ABSTRACT: The anthropogenic compound 1,2,3-trichloropropane (TCP) has recently drawn attention as an emerging groundwater contaminant. No living organism, natural or engineered, is capable of the efficient aerobic utilization of this toxic industrial waste product. We describe a novel biotechnology for transforming TCP based on an immobilized synthetic pathway. The pathway is composed of three enzymes from two different microorganisms: engineered haloalkane dehalogenase from *Rhodococcus rhodochrous* NCIMB 13064, and haloalcohol dehalogenase and epoxide hydrolase from *Agrobacterium radiobacter* AD1. Together, they catalyze consecutive reactions converting toxic TCP to harmless glycerol. The pathway was immobilized in the form of purified enzymes or cell-free extracts, and its performance was tested in batch and continuous systems. Using a packed bed reactor filled with the immobilized biocatalysts, 52.6 mmol of TCP was continuously converted into glycerol within 2.5 months of operation. The efficiency of the TCP conversion to the intermediates was 97%, and the efficiency of conversion to the final product glycerol was 78% during the operational period. Immobilized biocatalysts are suitable for removing TCP from contaminated water up to a 10 mM solubility limit, which is an order of magnitude higher than the concentration tolerated by living microorganisms.



INTRODUCTION

1,2,3-trichloropropane (TCP) is an anthropogenic compound recently recognized as an emerging contaminant of groundwater.[1,2] TCP is produced worldwide in quantities reaching 50 000 t annually and used by chemical companies as a solvent, precursor of soil fumigants, and building block for synthesis of other chemicals, e.g., dichloropropene or polysulfone liquid polymers.[3] TCP is also formed as a byproduct during the synthesis of epichlorohydrin. Due to its massive production, TCP can often be found at industrial and hazardous waste sites.[4] Recent incidents with drinking water sources in California polluted by TCP emphasized the need to develop efficient technologies for removing this toxic and carcinogenic compound from the environment.[5]

Conventional remediation techniques are relatively inefficient, due to the physical and chemical properties of the compound.[6] The exception is promising reductive conversion by zerovalent zinc.[6,7] In addition to abiotic transformations, biotransformations are extensively studied for their ability to decontaminate sites polluted with chemical contaminants.[8] Recently, isolated bacterial strains were found to transform TCP under anaerobic conditions.[9,10] However, these biotransformations often result in products toxic for surrounding environments, and due to the low efficiency can be applied only at limited (<1 mg·L^{-1}) TCP concentrations.[11] No organism capable of aerobic TCP biodegradation has yet been isolated, probably due to the anthropogenic nature of TCP and its recent introduction into the environment.

The absence of natural catabolic pathways for aerobic TCP utilization was addressed by Bosma and co-workers.[12,13] The authors assembled a synthetic metabolic pathway with the heterologous expression of haloalkane dehalogenase DhaA from *Rhodococcus* sp. in the natural host *Agrobacterium radiobacter* AD1, capable of utilization of haloalcohols. Engineered AD1 strain, expressing 5-times more active variant of haloalkane dehalogenase, showed a slow growth on 1 mM TCP in minimal medium. However, practical utility of this construct is limited by the toxicity of TCP for bacterial cells above 1 mM concentration, isufficient activity of engineered haloalkane dehalogenase, accumulation of toxic intermediates, and legislative barriers on the use of genetically modified microorganisms.[13] We recently addressed the problems of the low catalytic efficiency of the first dehalogenation step and

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accumulation of intermediates limiting the productivity of the pathway in vivo by combination of protein and metabolic engineering.[14]

In this study, we report the application of an immobilized enzymatic pathway for a complete five-step degradation of TCP to the harmless product glycerol (GLY; Figure 1). We

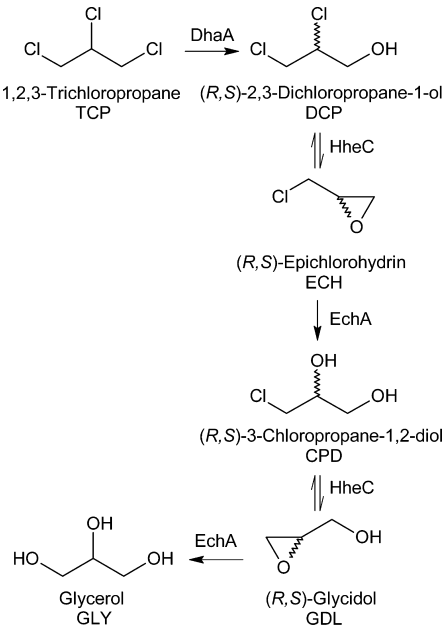

Figure 1. Scheme of the five-step synthetic metabolic pathway for biotransformation of 1,2,3-trichloropropane to glycerol. Abbreviations used for individual metabolites are shown. DhaA, haloalkane dehalogenase from *Rhodococcus rhodochrous* NCIMB 13064; HheC, haloalcohol dehalogenase; and EchA, epoxide hydrolase, both from *Agrobacterium radiobacter* AD1.

immobilized engineered 32-times more active haloalkane dehalogenase DhaA31 from *Rhodococcus rhodochrous* NCIMB 13064,[15] the wild-type haloalcohol dehalogenase HheC and the wild-type epoxide hydrolase EchA from *A. radiobacter* AD1 into cross-linked enzyme aggregates (CLEAs)[16] and poly(vinyl alcohol) (PVA) LentiKats lenses.[17] The immobilized biocatalysts were used to convert TCP into the desirable commodity chemical GLY in both batch and continuous systems. A comparison of the dynamic behavior of the complex multienzyme system in both soluble and immobilized forms is provided. To the best of our knowledge, this is the first report on the use of an immobilized synthetic metabolic pathway employing engineered enzyme for the biotransformation of an environmental pollutant. The established immobilization strategy is robust and suitable for scale-up. The developed biocatalyst is recyclable, resistant to biodegradation, compatible with high input loads of TCP, and can operate under mild nonsterile conditions. Moreover, the possibility to operate the process without the use of genetically modified microorganisms makes this biotechnology suitable for environmental applications.

EXPERIMENTAL SECTION

Reagents. TCP, 2,3-dichloropropane-1-ol (DCP), epichlorohydrin (ECH), 3-chloropropane-1,2-diol (CPD), glycidol (GDL), and GLY standards were purchased from Sigma-Aldrich (USA). All of the chemicals used in this study were of analytical grade. Bovine serum albumin (BSA) for preparation of CLEAs was purchased from Sigma-Aldrich (USA). PVA and poly(ethylene glycol) of M_W 1000 were provided by LentiKat's a.s. (Czech Republic). The solution of the cross-linker dextran polyaldehyde (DPA) was prepared according to a procedure described elsewhere.[18] A Free Glycerol Assay Kit was purchased from BioVision (USA). The work with toxic compounds was conducted in a fume hood and with protective equipment to minimize safety risks.

Preparation of Enzymes. The genes of DhaA, DhaA31, HheC, and EchA were commercially synthesized (Geneart, Germany). Synthetic genes were subcloned into pET21b (*dhaA*, *dhaA*31, and *hheC*), and pET28b (*echA*). The constructs were transformed into *E. coli* DH5α for plasmid propagation and into *E. coli* BL21(DE3) for enzyme expression. The recombinant His-tagged DhaA, DhaA31, and EchA were purified from cell-free extracts using 5 mL Ni-NTA Superflow column (Qiagen, Germany). Wild-type HheC was purified using Econo-Column (Bio-Rad, USA) packed with 25 mL of Q Sepharose Fast Flow (GE Healthcare, USA). Details of the expression and purification protocols are provided in Section 1 of the Supporting Information (SI).

Preparation of CLEAs. CLEAs of DhaA31 and EchA were prepared by dissolving 25 mg of enzyme and 25 mg of BSA in 1 mL of 10 mM phosphate buffer (pH 7.5). The solution was added to 9 mL of saturated ammonium sulfate (pH 8.0). After 1 h of incubation in an ice bath with stirring, 1.3 mL of DPA was added and cross-linking occurred for another 1 h. The resulting suspension was centrifuged at 4000*g* for 15 min at 4 °C. The supernatant was stored for the determination of residual enzymatic activity. CLEAs were resuspended in 20 mL of saturated sodium hydrogen carbonate and incubated with stirring in an ice bath for 30 min. The suspension was centrifuged at 4000*g* for 15 min at 4 °C and the supernatant was removed. CLEAs were washed with a 50 mM phosphate buffer (pH 7.5) and stored in 1 mL of this buffer at 4 °C before further use.

Encapsulation of Enzymes into PVA Particles Lenti-Kats. PVA (0.56 g) and poly(ethylene glycol) (0.34 g) were mixed with 3.7 mL of distilled water and heated at 98 °C until PVA dissolved completely. The liquid was cooled to 35 °C, and 10 mg of free HheC in a PD buffer (50 mM phosphate buffer of pH 7.5 with 2 mM dithiothreitol) or 1 g of CLEAs of EchA or DhaA31 was added and mixed thoroughly. Small droplets (3−4 mm) of mixture were dripped on plastic plates and incubated at 37 °C until LentiKats lost 70% of their initial wet weight. Dried LentiKats were soaked in 50 mL of 0.1 M sodium sulfate for 2 h to reswell. HheC LentiKats were washed with a PD buffer and stored in the same buffer at 4 °C. LentiKats of EchA and DhaA31 were washed with a 50 mM phosphate buffer (pH 7.5) and stored at 4 °C.

Enzyme Assays. Specific activities of soluble and immobilized DhaA31 with TCP and HheC with DCP and CPD were assayed in 10 mL of a 50 mM Tris-SO_4 buffer (pH 8.5) at 37 °C with a 10 mM substrate. The concentration of the reaction product (chloride ions) was measured by the method of Iwasaki[15,19] The specific activity of EchA was assayed by following the substrate depletion in 10 mL of a 50 mM Tris-SO_4 buffer (pH 8.5) at 37 °C with 5 mM ECH or 10 mM GDL. Samples of the reaction mixture were mixed with acetone (1:1) with internal standard hexanol and analyzed by gas chromatography (GC) as described in Section 11 of the SI. The same GC method was used for the quantitative analyses of

all metabolites of the TCP pathway except for GLY. The rates of abiotic conversions of all metabolites at selected time intervals were negligible.

Multienzyme Conversion of TCP in Batch System. The multienzyme conversion of TCP to GLY was assayed in 15 mL of a 50 mM Tris-SO_4 buffer (pH 8.5) in gastight glass vials (Sigma-Aldrich, USA) incubated at 37 °C. The reaction was initiated by adding 1 mg of DhaA31, HheC, and EchA in a soluble or immobilized form, into the reaction mixture with 5 mM TCP. The samples withdrawn from the reaction mixture (0.5 mL for soluble enzymes or CLEAs and 0.1 mL for LentiKats) were mixed with acetone (1:1) containing hexanol as an internal standard and analyzed by GC. Selected samples were analyzed by gas chromatograph and mass spectrometer (GC-MS) (Agilent, USA) for identification of metabolites otherwise routinely detected by GC (SI Section 11). The concentration of GLY was determined spectrophotometrically by the Free Glycerol Assay Kit. Samples of the reaction mixture (0.1 mL) were heated at 95 °C for 5 min, centrifuged at 18 000*g* for 1 min, diluted in an assay buffer, and analyzed according to the manufacturer's protocol. Concentrations of GLY were calculated from absorbance at 570 nm.

Multienzyme Conversion of TCP in Continuous System. Glass column 1 (28 cm high, 1.5 cm internal diameter, 50 mL working volume) with 100 mg of DhaA31 immobilized in 47 g of wet LentiKats and glass column 2 (25 cm high, 2.5 cm internal diameter, 100 mL working volume) with 100 mg of both HheC and EchA immobilized in 45 and 43 g of LentiKats, respectively, were used for the removal of TCP in a 10-week continuous operation of a packed bed reactor placed in fume hood at 22 ± 2 °C. A feed vessel contained TCP dissolved under stirring in 1 L of distilled water buffered with 0.1 M Tris-SO_4 (pH 8.2) to a final theoretical concentration of 5 mM (week 1) or 10 mM (weeks 2−10). The experimental concentrations of TCP, ranging from 2.25 to 7.97 mM, for evaluation of system efficiency were determined in the input of column 1 (SI Figure S8 and Table S6). The operation conditions for the packed bed reactor are summarized in SI Table S1. Influent and effluent lines were constructed from polytetrafluoroethylene tubing. Samples from the feed vessel, inlet of column 1, and effluent vessels 1 and 2 were withdrawn periodically and analyzed by GC and the Free Glycerol Assay Kit. A new cycle of operation was started whenever the content of the feed vessel was completely transferred to the effluent vessel 2. To evaluate possible leaching of the immobilized enzymes from the reactor, samples from effluent vessel 2 were withdrawn, concentrated 25 times using stirred ultrafiltration cells (Millipore, USA), and analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

RESULTS AND DISCUSSION

Multienzyme Conversion of TCP Using Free Enzymes. We initially tested the ability of an in vitro assembled pathway to fully convert 5 mM TCP to GLY in one-pot reaction at a time interval of 30 h. Purified DhaA, HheC, and EchA were mixed in the mass ratio of 1:1:1 mg and incubated with TCP. Because the molecular weights of DhaA, HheC, and EchA are similar (34, 29, and 35 kDa, respectively), the proposed 1:1:1 ratio corresponded closely with the same molar ratio of enzymes. The pH 8.5 and temperature 37 °C were selected to approach the reaction optima of all three enzymes.[20−22] Using wild-type enzymes, TCP and the intermediates DCP and GDL were degraded from 73% within 30 h of the reaction (Figure 2A and SI Table S2). The percentage of degradation correlated well with amount of GLY produced from TCP during the same time interval.

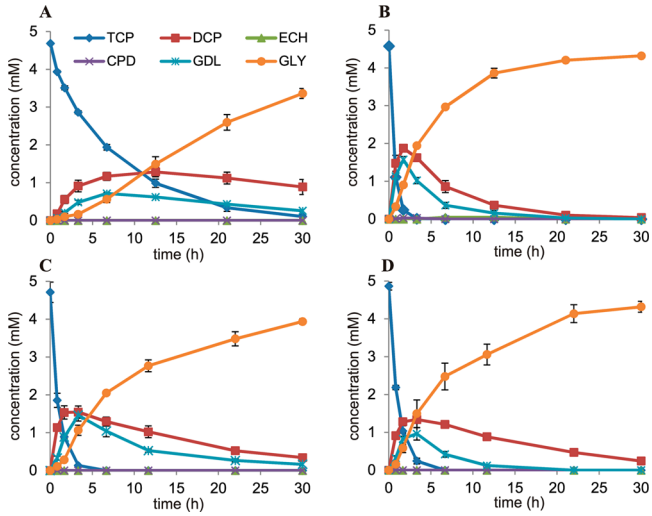

Figure 2. Time courses of multienzyme conversions of 1,2,3-trichloropropane with free and immobilized enzymes: (A) free purified enzymes DhaAwt, HheC, and EchA; (B) free purified enzymes DhaA31, HheC, and EchA; (C) immobilized purified enzymes DhaA31, HheC, and EchA; (D) immobilized cell-free extracts containing DhaA31, HheC, and EchA. All reactions were performed using enzymes of mass ratio of 1:1:1 mg in 15 mL of reaction mixture. TCP, 1,2,3-trichloropropane; DCP, 2,3-dichloropropane-1-ol; ECH, epichlorohydrin; CPD, 3-chloropropane-1,2-diol; GDL, glycidol; GLY, glycerol. Each data point represents the mean value ± standard deviation from three independent experiments.

The time course of the reaction showed the major bottleneck of the pathway: slow consumption of TCP by DhaA. Additionally, significant accumulations of two intermediates, DCP and GDL, were observed. The accumulation of DCP was caused by the high enantioselectivity ($E \geq 100$) of HheC.[23] Nonselective DhaA converts the prochiral TCP into both enantiomers of DCP in an almost equimolar ratio. Because HheC prefers (*R*)-DCP, (*S*)-DCP tends to accumulate (SI Section 2). The specific activities of EchA with ECH and GDL (29.5 and 6.5 μmol·min^{-1}·mg^{-1}, respectively) in combination with the previously reported kinetic parameters indicate that ECH is a much better substrate for EchA than GDL.[24] Therefore, the substrate specificity of EchA is probably the main cause of GDL accumulation during the multienzyme conversion of TCP.

To overcome the limitation of the first reaction step, the wild-type DhaA (k_{cat} = 0.04 s^{-1}, k_{cat}/K_m = 40 s^{-1}·M^{-1}) was substituted with the mutant DhaA31 (k_{cat} = 1.26 s^{-1}, k_{cat}/K_m = 1050 s^{-1}·M^{-1}), constructed in our laboratory using a computer-assisted design.[15] The selectivity of DhaA31 with TCP remained unchanged. The benefit of engineered DhaA31 in the multienzyme conversion of TCP was verified during the second experiment with free enzymes. The time course of the reaction shows that TCP was completely converted into its metabolites within the first 200 min of the reaction, and the degradation of TCP, DCP, and GDL reached 99% of the theoretical maximum within 30 h of the reaction (Figure 2B and SI Table S3). The conversion of TCP to GLY reached 95% of the theoretical maximum. We conclude that complete in

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vitro biodegradation of TCP and its biotransformation to the final product GLY is possible despite the suboptimal stereochemistry and specificity in the pathway resulting in the accumulation of DCP and GDL in the initial phase of reaction.

Immobilization of DhaA31, HheC, and EchA. Application of free enzymes in biotransformation processes is not practical due to their complicated recycling, limited use in bioreactors, and low stability in harsh process conditions such as elevated temperatures or the presence of organic cosolvents.[25] We therefore immobilized DhaA31, HheC, and EchA to avoid such limitations. Various strategies for immobilization of individual biocatalysts are available.[26] Multienzyme systems also benefit from immobilization, but development of joint immobilization protocols for all employed catalysts is challenging. There is currently no protocol available for immobilization of DhaA, HheC, and EchA. Therefore, we tested enacapsulation of three enzymes into PVA, which has previously proven its utility in immobilization of haloalkane dehalogenase LinB, close relative of DhaA.[27]

The encapsulation of biocatalysts into PVA hydrogel is a widely used immobilization technique.[28,29] Lens-shaped PVA hydrogel particles, known as LentiKats, are promising matrices for biocatalysis due to their low cost, resistance to biodegradation, and good mechanical properties.[17] Their favorable geometry (thickness 200−400 μm) allows better mass transfer than spherical microbeads.[30,31] The biotechnology based on LentiKats has already been used in large scale applications, e.g., synthesis of beta-lactam antibiotics or denitrification in wastewater treatment. The size of LentiKats allows easy separation from the reaction mixture and is suitable for application in packed bed reactors, which can suffer from large pressure drops over the column when packed with particles of inadequate size.[32] LentiKats provide a hydrophilic environment with the pores sufficiently small to entrap whole cells, cross-linked enzymes,[30] cross-linked enzyme aggregates (CLEAs),[27,33] or free enzymes of molecular weights higher than 80 kDa.

In contrast to the HheC tetramer with a molecular weight of 116 kDa, smaller monomeric molecules of DhaA31 and EchA are not suitable for direct encapsulation in LentiKats. Therefore, aggregation and cross-linking of DhaA31 and EchA was carried out to produce CLEA particles. Immobilization in CLEAs is a straightforward technique which has been used for many enzymes, including hydrolases.[16,34,35] The protocols for preparation of CLEAs from LinB and for miscroscopic monitoring of immobilized biocatalyst were previously established in our laboratory.[27,36] Here we expand these protocols also for immobilization of DhaA31 and EchA. To enable detailed characterization of assembled pathway, immobilization was initially performed separately for each of the three purified enzymes. CLEAs of DhaA31 and EchA were prepared by mixing an enzyme solution with lyophilized bovine serum albumin, serving as a proteic feeder.[37] The protein mixture was precipitated using ammonium sulfate, which is widely used for preparing CLEAs due to its low cost and easy treatment.[38] The suspension of aggregated enzymes was cross-linked using DPA, which had been shown to have a less detrimental effect on enzymatic activity than the widely used glutaraldehyde.[39] CLEAs and free HheC were labeled by fluorescent dyes to allow microscopic monitoring of biocatalysts during the immobilization procedure (Figure 3). Free HheC and wet CLEAs of DhaA31 and EchA were separately encapsulated in LentiKats in mass ratio of 1:4 (enzyme or CLEAs:PVA gel).

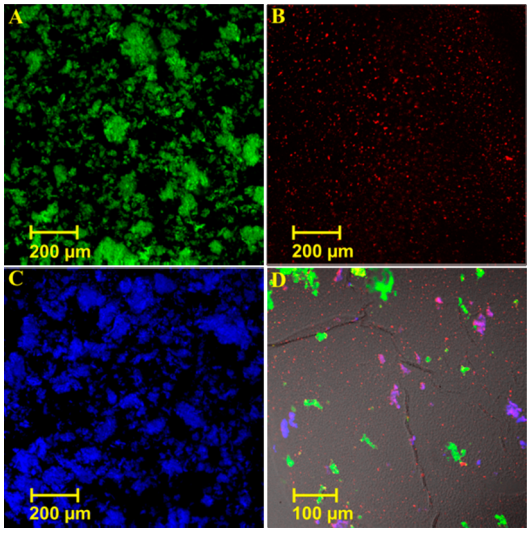

Figure 3. Confocal microscopy of immobilized enzymes from the synthetic pathway for biodegradation of 1,2,3-trichloropropane: (A) Cross-linked enzyme aggregates (CLEAs) of haloalkane dehalogenase DhaA31 labeled with fluorescein 5(6)-isothiocyanate; (B) free haloalcohol dehalogenase HheC labeled with X-rhodamine-5(6)-isothiocyanate; (C) CLEAs of epoxide hydrolase EchA labeled with Pacific Blue succinimidyl ester; (D) section through combi-LentiKat containing CLEAs of DhaA31, EchA, and free HheC. Visible fissures are caused by the fragility of PVA after freezing and appear after treatment with Leica Cryocut 1800 Cryostat (Leica Microsystems, Germany).

Characterization of Immobilized Enzymes. Immobilized enzymes were characterized in terms of their activity, storage and operational stability, and distribution in PVA matrix. Immobilization resulted in the decrease of specific activities of all three enzymes with their five corresponding substrates (SI Figure S2). The catalytic performance of CLEAs of DhaA31 was 80% of the initial activity of the soluble enzyme. CLEAs of EchA showed reduced activity with both GDL (50%) and ECH (37%). The encapsulation of free HheC and CLEAs of DhaA31 and EchA into PVA hydrogel led to activity retention of approximately 73/69% with DCP/CPD, 54% with TCP, and 17/36% with ECH/GDL, respectively. Observed activity retentions for immobilized DhaA and HheC are comparable with studies describing formation of CLEAs or LentiKats using other hydrolases, such as penicillin acylase, acetyl xylan esterase, or naringinase.[30,33,40] Some epoxide hydrolases have been immobilized with high activity retention using alternative methods, e.g., interaction with carrier through His-tag or covalent binding.[41,42] We verified these methods with EchA using Ni-NTA Agarose and activated CH Sepharose 4B as carriers, achieving 93% and 68% activity retention with ECH (SI Section 4). Nevertheless, high activity retention in these matrices is compromised by higher price and lower mechanical stability, which makes them less suitable for full-scale processes. The specific activity of soluble EchA with ECH (29.5 μmol·min^{-1}·mg^{-1}) is 1 order of magnitude higher than the specific activity of DhaA31 with TCP (1.1 μmol·min^{-1}·mg^{-1}) and HheC with DCP (1.6 μmol·min^{-1}·mg^{-1}) and CPD (3.0 μmol·min^{-1}·mg^{-1}). Despite the reduction of the catalytic performance of EchA after immobilization, the pattern of

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specific activities of three enzymes remained unchanged (SI Figure S3). We therefore concluded that even 80% loss of the activity of EchA toward ECH after immobilization in CLEAs and LentiKats would not result in the accumulation of intermediates in the TCP pathway and the analogous immobilization strategy was maintained also for this enzyme.

No enzymatic activities were detected in the supernatants obtained during the preparation of CLEAs and LentiKats, besides low activity of HheC corresponding to approximately 10% loss of enzyme during immobilization. Therefore, we expect that the decreased activities of DhaA31, HheC, and EchA are predominantly due to the partial deactivation caused by aggregation, cross-linking, or encapsulation in PVA hydrogel, rather than due to the leaching of enzymes during immobilization process.

The storage stability of LentiKats of DhaA31 and EchA in a phosphate buffer without additives at 4 °C was assayed during a 3-month period and compared with the storage stability of free enzymes (SI Section 5). After this period, changes in the activity of immobilized and free enzymes correlated for both DhaA31 and EchA. Free and immobilized DhaA31 retained 93% and 86% of its initial activity with TCP, respectively. EchA showed 121% and 122% of its initial activity in free and immobilized form, respectively. The increase was statistically significant for free EchA (t test, $p < 0.05$). Similar moderate increases in specific activity during storage were reported also for some other enzymes.[27,30]

HheC showed significantly lower storage stability than DhaA31 and EchA. Free HheC stored in a phosphate buffer at 4 °C lost all its activity with DCP within 2 months (data not shown). Tang and co-workers previously suggested that inactivation of HheC is caused by the monomerization of the enzyme and intramolecular disulfide bond formation under oxidizing conditions.[43] Confirming this suggestion, the stability of HheC was significantly improved by its storage in a buffer containing a reducing agent (SI Figure S4A). Free and immobilized HheC retained 63% and 49% of its initial activity, respectively, after 2 months of storage in the presence of 2 mM dithiothreitol. The lower activity of HheC immobilized in LentiKats is due to partial leaching (SI Figure S4B). The storage stability of free enzymes was tested also at room temperature (22 ± 2 °C), which was later applied during the continuous removal of TCP in a packed bed reactor (SI Section 5). In contrast to DhaA31 and EchA, HheC showed better storage stability at room temperature than at 4 °C (SI Figures S4 and S5). The enzyme retained 70% of its initial activity after 10 weeks of storage at room temperature in phosphate buffer without any additive.

Multienzyme Conversion of TCP Using Immobilized Enzymes. Numerous studies describe the immobilization of individual enzymes or whole cells, but reports on the effects of immobilization on the performance of complete biochemical pathways are scarce.[44] For synthetic biodegradation pathways employing engineered enzymes such reports are to the best of our knowledge missing. We applied TCP pathway immobilized in LentiKats in a one-pot multienzyme reaction to study how the modified activities of individual immobilized enzymes affected the time course of TCP biodegradation. Immobilized enzymes were mixed in a ratio 1:1:1 mg and incubated with 5 mM TCP under the same conditions as had been used for soluble enzymes (Figure 2C and SI Table S4). Despite the fact that activity of individual enzymes after immobilization was reduced by 27−83%, the efficiency of the whole pathway was lower only about 11% when compared to the conversion with free enzymes. The conversion of TCP to GLY after 30 h reached 84% and conversion of all intermediates reached 89% of the theoretical maximum. We also studied the reusability of the immobilized TCP pathway in a batch system (SI Section 6). The immobilized pathway retained 77% of its initial efficiency of TCP conversion to GLY after 10 successive cycles of batch operation.

The application of purified enzymes immobilized individually provides better control over the reaction by enabling the tuning of enzyme stoichiometry and by compensating for the activity loss of individual enzymes. However, the purification and separate treatment of all three enzymes increases the cost of the biocatalyst. We therefore demonstrated that cell-free extracts obtained from *E. coli* BL21(DE3) cells expressing DhaA31, HheC, or EchA can be utilized. For the preparation of the LentiKats, cell-free extracts were mixed to provide a ratio of enzymes corresponding to 1:1:1 (SI Section 7). The combi-LentiKats containing approximately 1 mg of each of the three enzymes were used for the biodegradation of 5 mM TCP. The degradation of TCP and the intermediates DCP and GDL reached 95% of the theoretical maximum within 30 h of reaction (Figure 2D, SI Table S5). The overall reaction profile was very similar to the profile obtained with purified immobilized enzymes (Figure 2C). The conversion of TCP to GLY reached 89% of the theoretical maximum. Thus, the coimmobilization of enzymes and their improved proximity resulted in conversion comparable by efficiency with free enzymes. The proximity and homogeneous distribution of DhaA31, HheC, and EchA in combi-LentiKats was verified by confocal microscopy (Figure 3D).

Multienzyme Conversion of TCP Using Packed Bed Reactor. The performance of immobilized pathway was also tested under continuous operation using a packed bed reactor composed of two columns (SI Figure S8). Column 1 was packed with LentiKats of DhaA31, and column 2 was packed with a mixture of LentiKats of HheC and EchA. The total mass ratio of enzymes 1:1:1 corresponded to the previous batch experiments. DhaA31 was separated from HheC and EchA in order to prevent the inhibition of the last step in the pathway, the conversion of GLD to GLY by TCP (K_i = 0.21 mM). EchA was combined with HheC in column 2 in order to prevent a reverse reaction and push the reaction equilibrium during the conversion of DCP and CPD by HheC toward the product (SI Figure S1).[23] The separation of enzymes into two columns also enabled better control over the individual reaction steps and evaluation of their efficiency.

The packed bed reactor was operated at room temperature 22 ± 2 °C and pH 8.2 for 10 weeks under the conditions described in SI Table S1. During that period, the reactor with an effective volume of 0.15 L processed 11 L of contaminated water. Experimental concentrations of TCP for evaluating column 1 efficiency were determined in the input of the column 1 due to the leakage of TCP from the pumping system before reaching the column (SI Figure S8 and Table S6). The average leakage caused by hydrophobic nature of TCP (log P = 2.24) and its tendency to penetrate through rubber tubing of peristaltic pump was approximately 29%. The experimental concentrations of TCP in the column 1 input for each of 10 weeks of operation were 2.25, 3.03, 5.91, 6.54, 7.97, 6.31, 7.94, 6.65, 6.80, and 7.02 mM. The levels of residual TCP and produced DCP were determined in the effluent of column 1. The levels of residual DCP and produced GDL and GLY were

determined in the effluent of column 2. These values were used to evaluate the efficiency of both columns and the efficiency of GLY production from TCP (Figure 4). No leakage of intermediates was observed. Neither ECH nor CPD was detected in the system.

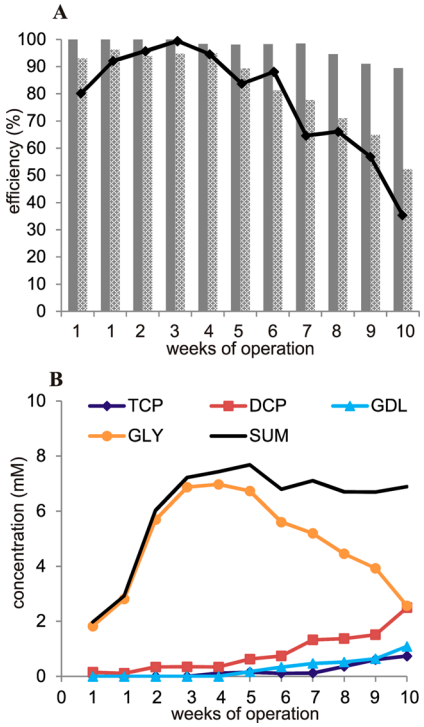

Figure 4. (A) Efficiency of column 1 (filled) and column 2 (cross-hatched) of the packed bed reactor during the continuous biodegradation of 1,2,3-trichloropropane (TCP). Black diamonds with a solid line show the overall efficiency of TCP conversion to glycerol by immobilized biocatalysts in the two-step packed bed reactor. Efficiency was calculated from the concentration of TCP measured in the inlet of column 1 and the concentration of glycerol measured in effluent vessel 2. (B) Residual concentrations of the metabolites from the synthetic pathway for biodegradation of TCP detected in effluent vessel 1 (TCP) and effluent vessel 2 (2,3-dichloropropane-1-ol, DCP; glycidol, GDL; glycerol, GLY) during 10 weeks of operating the packed bed reactor.

The efficiencies of column 1 and 2 were higher than 95% during the first 8 and 4 weeks of operation, respectively (Figure 4 and SI Table S6). The efficiency of the overall conversion of TCP to GLY decreased from values above 90% observed during the first 4 weeks to values lower than 60% at the end of 2 months of operation. The decreased reactor efficiency accompanied by an accumulation of unreacted TCP, DCP, and GDL in effluent vessels could be attributed to (i) slow enzyme inactivation caused by oxidation of HheC and thermal unfolding of DhaA31 and EchA and (ii) slow leaching of enzymes from the reactor, which resulted in about 10% loss of HheC activity and about 5% loss of DhaA31 and EchA activities during the operation (Figure 4B and SI Figure S9). In total, 65.5 mmol of the 67.7 mmol of TCP that entered the packed bed reactor during 10 weeks of operation was converted to intermediates (efficiency 97%), and 52.6 mmol of GLY was produced (efficiency 78%).

Perspectives on In Vitro Biotransformation of TCP. Recent studies on bacterial utilization of 1,3-dichloroprop-1-ene and chlorinated ethenes suggest that the biodegradation of chlorinated aliphatic pollutants is associated with the accumulation of reactive intermediates and strong oxidative stress, representing a significant barrier for the evolution of corresponding aerobic metabolic pathways in vivo.[45,46] The absence of natural microorganisms carrying aerobic pathways for the biodegradation of TCP and experiences gained during attempts to engineer microorganisms growing on this toxic compound seem to support this view.[11−14] Engineering synthetic pathway for converting toxic TCP to the harmless GLY in vitro is an alternative approach, which does not suffer from the limitations of engineered bacterium. It is now widely accepted that in vitro multienzyme systems represent an emerging field of biocatalysis due to their simplicity, predictability, and controllability.[47,48] This study shows that an in vitro assembly of natural or synthetic enzymatic pathways can be a promising concept for the biodegradation of environmental pollutants and can provide promising results especially when combined with protein engineering techniques.

Developed biotechnology requires further validation before it can be scaled-up and used in real conditions. The performance of the immobilized biocatalyst should be tested in real water samples contaminated with TCP. Pretreatment of the contaminated water by adjusting high salinity or eliminating possible enzyme-inhibiting constituents might be necessary. Conversion of TCP to GLY was demonstrated to be efficient at 22 ± 2 °C and is also possible in broad pH range 7−10 (SI Section 10). Yet, buffering of the contaminated water to pH close to the reaction optima of enzymes can be beneficial to achieve maximal efficiency of the process. The favorable features of the presented biotechnology are (i) degradation of TCP using immobilized cell-free extracts instead of purified enzymes is possible, (ii) material used for immobilization of enzymes is affordable, safe, and nonbiodegradable, (iii) protocol for disposal of used LentiKats by burning is well established, and (iv) the amount of enzymes necessary to degrade almost 10 g of TCP during the operation of reactor can be obtained from less than 2 L of cell culture yet without optimized cultivation conditions. An immobilized synthetic pathway works at TCP concentrations that are close to the water solubility limit of TCP (10 mM), which is 1 order of magnitude higher than concentrations tolerated by engineered microorganisms.[13] At the same time, the system has the potential to cope with significantly lower concentrations of TCP. Kinetic parameters of DhaA31 for TCP (k_{cat} = 1.26 s^{-1}, k_{cat}/K_m = 1050 $s^{-1}{\cdot}M^{-1}$) are of the same order of magnitude as those of the haloalkane dehalogenase DhlA for 1,2-dichloroethane (k_{cat} = 3.3 s^{-1}, k_{cat}/K_m = 6200 $s^{-1}{\cdot}M^{-1}$), which has already proven its utility in a groundwater purification plant treating micro- to nanomolar concentrations of the pollutant.[49]

The remaining bottlenecks of the pathway are (i) lower activity of HheC with nonpreferred (*S*)-DCP, (ii) accumulation of GDL due to the substrate preference of EchA, and (iii) gradual inactivation of HheC. These important, but not critical, limitations can be overcome by another round of protein engineering or modification of the immobilization protocol. The flux of intermediates through the immobilized pathway can be further tuned using kinetic modeling and optimization of enzyme stoichiometry, which is an objective of our future research.

■ ASSOCIATED CONTENT

Supporting Information

Details on the preparation of enzymes, the activity, storage stability, and recycling of immobilized enzymes, the preparation of combi-LentiKats from cell-free extracts, construction and operational conditions of packed bad reactor and the analysis of enzyme leaching, multienzyme conversion of TCP at pH 7 and 10 at 20 °C, and some analytical procedures (e.g., GC, chiral GC, and GC/MS) together with supporting tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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