Organic solvent stability and long-term storage of myoglobin-based carbene transfer biocatalysts

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Abstract

Recent years have witnessed a rapid increase in the application of enzymes for chemical synthesis and manufacturing, including the industrial-scale synthesis of pharmaceuticals using multienzyme processes. From an operational standpoint, these bioprocesses often require robust biocatalysts capable of tolerating high concentrations of organic solvents and possessing long shelflife stability. In this work, we investigated the activity and stability of myoglobin (Mb)-based carbene transfer biocatalysts in the presence of organic solvents and after lyophilization. Our studies demonstrate that Mb-based cyclopropanases possess remarkable organic solvent stability, maintaining high levels of activity and stereoselectivity in the presence of up to 30%–50% (v/v) concentrations of various organic solvents, including ethanol, methanol, *N,N*-dimethylformamide, acetonitrile, and dimethyl sulfoxide. Furthermore, they tolerate long-term storage in lyophilized form, both as purified protein and as whole cells, without significant loss in activity and stereoselectivity. These stability properties are shared by Mb-based carbene transferases optimized for other type of asymmetric carbene transfer reactions. Finally, we report on simple protocols for catalyst recycling as whole-cell system and for obviating the need for strictly anaerobic conditions to perform these transformations. These findings demonstrate the robustness of Mb-based carbene transferases under operationally relevant conditions and should help guide the application of these biocatalysts for synthetic applications.
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1. Introduction

Over the past two decades, biocatalysis has covered an increasingly important role in organic synthesis and in the chemical manufacturing of drugs, advanced pharmaceutical intermediates, food additives, biofuels, and other important chemical products [1-6]. Attractive features of enzymes in this regard include their potentially high degrees of chemo-, regio-, and stereoselectivity [1-3], which can be altered and tuned by protein engineering and directed evolution [7-9]. As a prominent example, a multienzyme cascade process involving a total of nine enzymes, five of which were modified via protein engineering, was recently implemented by Merck for the large-scale synthesis of the anti-HIV drug islatravir, providing a much shorter and more efficient route for the production of this molecule compared with previously reported synthetic routes [10]. In addition to the desired activity and selectivity, an important requirement for the application of enzymes in industrial and large-scale processes is an inherent (or acquired) stability in the presence of organic solvents, since high concentration of organic co-solvents are often required to solubilize lipophilic substrates and/or achieve high substrate loadings [2,4,11-14]. For example, a recently implemented industrial-scale biocatalytic process for the production of the antidiabetic drug sitagliptin involved an engineered transaminase capable of operating in the presence of 50% (v/v) dimethyl sulfoxide (DMSO), which was necessary to permit the use of high substrate loadings (100 g/L) [15]. Since most proteins and enzymes are readily denatured by organic solvents, significant

Abbreviations: *ACN, Acetonitrile; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; EDA, ethyl diazoacetate; EtOH, ethanol; Mb, myoglobin; MeOH, methanol; OD, optical density; TON, turn over number; v/v, volume/volume.*

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Myoglobin-based carbene transferases are shown to maintain high levels of activity and stereoselectivity in the presence of up to 30-50% (v/v) concentrations of various organic solvents including methanol, dimethylsulfoxide, dimethylformamide, and acetonitrile. Furthermore, they are amenable to long-term storage in lyophilized form, both as purified protein and as whole cells, without showing significant loss in activity and stereoselectivity.

protein engineering/directed evolution efforts are typically required to equip biocatalysts with sufficient activity and stability properties to fit the target manufacturing processes [2,16]. For example, the aforementioned engineered transaminase was obtained after 11 rounds of directed evolution [15], and similar efforts (18 rounds of engineering/screening) were required for engineering a halohydrin dehalogenase biocatalyst for the manufacturing of the cholesterol-lowering drug atorvastatin (Lipitor) [17]. Along with organic solvent stability, shelflife stability constitutes another highly desirable feature for enzymes to be employed for organic synthesis [2,4,11].

Although a number of natural enzyme classes represent key components of the biocatalytic toolbox for organic synthesis (e.g., hydrolases, ketoreductases, transaminases) [1,4,18,19], we and others have reported that heme-containing enzymes and proteins are functional biocatalysts for "abiological" carbene transfer reactions, including olefin cyclopropanation [20-29], Y–H carbene insertion (where Y = N, S, B, or Si) [30-36], C–H functionalization [37-41], aldehyde olefination [42,43], and others [44, 45]. In particular, engineered myoglobins (Mbs) have emerged as particularly selective and versatile scaffolds for promoting a variety of carbene-mediated transformations useful for the selective construction of new carbon–carbon and carbon–heteroatom bonds [23-29,31,32,35,36,38,39,42,44,46]. In the interest of further exploring the utility of these Mb-based carbene transferases for practical synthesis, we investigated here the activity and stability of these biocatalysts in the presence of organic solvents, after lyophilization, and under other operationally relevant conditions.

2. Materials and Methods

2.1. Experimental procedures

General information

All the chemicals and reagents were purchased from commercial suppliers (Sigma–Aldrich (St. Louis, MO, USA)), Alfa Aesar (Haverhill, MA, USA), Acros Organics (Fair Lawn, NJ, USA), MilliporeSigma (Burlington, MA, USA)) and used without any further purification. All dry reactions were carried out under argon in flame-dried glassware with magnetic stirring using standard gas-tight syringes, cannula, and septa. ^{1}H and ^{13}C NMR spectra were measured on Bruker DPX-500 (operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C) or Bruker DPX-400 (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C). Tetramethylsilane served as the internal standard (0 ppm) for ^{1}HNMR and $CDCl_3$ was used as the internal standard (77.0 ppm) for ^{13}CNMR. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 230–400 mesh. Thin-layer chromatography (TLC) was carried out using Merck Millipore (Sigma) TLC silica gel 60 F254 glass plates.

Product analysis

The reactions were analyzed by adding 20 µL of internal standard (1,3-benzodioxole, 50 mM in ethanol) to each reaction mixture, followed by extraction with 400 µL of dichloromethane (CH_2Cl_2) and analyzed by GC-FID using a Shimadzu GC-2010 gas chromatograph equipped with an FID detector and a Cyclosil-B column (30 m × 0.25 mm × 0.25 µm film). Calibration curves for quantification of all reactions were constructed using authentic standards produced synthetically as described in section *Synthetic Procedures* and analyzed by GC-FID. Separation method: 1 µL injection, injector temperature: 250 °C, detector temperature: 300 °C, column temperature set at 120 °C for 3 Min, then to 140 °C at 0.85 °C/Min, then to 245 °C at 30 °C/Min. Total run time was 31.03 min. Enantiomeric excess of cyclopropane **3** was determined by chiral GC-FID using the previously described method. Enantiomeric excess of rearrangement product **10** and cyclopropane **11** was determined by supercritical fluid chromatography (SFC) analysis, using a JASCO analytical and semireparative SFC instrument equipped with a column oven (35 °C), a photodiode array detector, a backpressure regulator (12.0 MPa), a carbon dioxide pump, and a sample injection volume of 3 µL. Daicel Chiralpak IB or IC (0.46 cm ID × 25 cm L) was used for separation of enantiomers. All samples were eluted using an isocratic solvent system with isopropyl alcohol as the modifier solvent in liquid CO_2 at an elution rate of 4 mL/Min and detected at $\lambda = 220$ nm. Total run time was 10.2 min.

Synthetic procedures

Procedures for the synthesis of reagents and authentic standards are provided as Supporting Information.

Protein expression and purification

Engineered Mb variants were expressed in *E. coli* BL21(DE3) cells as described previously [23]. Briefly, cells were grown in terrific broth medium (ampicillin, 100 mg L^{-1}) at 37 °C (150 rpm) until OD_{600} reached 0.9–1.2. Cells were then induced with 0.25 mM β-D-1-thiogalactopyranoside (IPTG) and 0.3 mM δ-aminolevulinic acid (δ ALA). After induction, cultures were shaken at 180 rpm and 27 °C and harvested after 18–20 hours by centrifugation at 4 °C. After cell lysis by sonication, the proteins were purified by Ni-affinity chromatography. The lysate was transferred to a Ni-NTA column equilibrated with Ni-NTA lysis buffer. The resin was washed with 50 mL of Ni-NTA lysis buffer and then 50 mL of Ni-NTA wash buffer (50 mM KPi, 250 mM NaCl, 20 mM imidazole, pH 8.0). Proteins were eluted with Ni-NTA elution buffer (50 mM KPi, 250 mM NaCl, 250 mM histidine, pH 7.0). After elution, the proteins were buffer exchanged against 50 mM potassium phosphate

(KPi) buffer (pH 7.0 or 8.0) using 10 kDa Centricon filters. Mb concentration was determined using an extinction coefficient $\varepsilon_{410} = 157\ mM^{-1}\ cm^{-1}$.

Catalyst recovery after lyophilization
Samples of purified Mb(H64V,V68A) or whole cells expressing Mb(H64V,V68A) in KPi buffer solution (50 mM, pH 7.0) were flash-frozen in dry ice and lyophilized using a LabConco Freeze-dryer Freezone for 24 hours. The lyophilized sample was resuspended in the original volume of Milli-Q water and analyzed via UV–vis spectroscopy. For the purified protein, the percent protein recovery (Table S2) was determined by comparison of the protein concentration prior and after lyophilization based on the Soret bands at 408, 436, and 425 nm for the ferric, ferrous, and CO-bound form of the hemoprotein, respectively. For the whole cell catalyst, optical density at 600 nm (OD_{600}) was measured prior and after lyophilization to measure percent cell recovery. To determine protein recovery, the whole cells were lysed by sonication and the concentration of Mb was measured from the cell lysate via UV–vis spectroscopy.

Anaerobic protocol for protein reactions via cannulation
In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0) was purged by bubbling argon into the mixture for 3 min in a sealed vial. A buffered solution containing Mb (20 μM final concentration) was carefully degassed via its headspace in a similar manner in a separate sealed vial equipped with a stir bar. The two solutions were then mixed together via cannulation. Reactions were initiated by addition of organic solvent (if applicable), olefin (10 mM final concentration), and α-ethyl diazoacetate (EDA, 20 mM final concentration) bringing the mixture to a final volume of 400 μL. The reaction mixture was stirred for 16 hours at room temperature.

Anaerobic protocol for protein reactions via anaerobic chamber
In a typical procedure inside an anaerobic chamber, a solution of sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0), organic solvent (if applicable), olefin (10 mM final concentration), and EDA (20 mM final concentration) was added to a reaction vessel equipped with a stir bar. Reactions were initiated by addition of Mb (20 μM final concentration) to the vessel bringing the mixture to a final volume of 400 μL. The reaction vessel was capped and stirred inside the chamber for 16 hours at room temperature. Reaction work up was performed outside the anaerobic chamber. All buffered solutions and organic solvents were purged with Ar prior to entering the anaerobic chamber.

Microaerobic reaction setup
In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0) was added to a reaction vessel equipped with a stir bar. The vessel was sealed, and the solution was purged by bubbling Ar to the solution for 3 min. Reactions were initiated by the addition of Mb (20 μM final concentration), olefin (10 mM final concentration), and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μL. The reaction mixture was stirred for 16 hours at room temperature.

Semiaerobic reaction setup
In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0) and Mb (20 μM final concentration) was added to a reaction vessel equipped with a stir bar. The vessel was sealed, and the headspace was purged with Ar for 3 min. Reactions were initiated by the addition of olefin (10 mM final concentration) and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μL. The reaction mixture was stirred for 16 hours at room temperature.

Aerobic protocol for protein reactions
In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0), olefin (10 mM final concentration), and EDA (10 or 20 mM final concentration) was added to a reaction vessel equipped with a stir bar. Reactions were initiated by addition of Mb (20 μM final concentration) to the vessel bringing the solution to a final volume of 400 μL in open air. The reaction mixture was capped and stirred for 16 hours at room temperature.

Initial rates measurements
In a typical procedure inside an anaerobic chamber, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0), organic solvent, olefin (10 mM final concentration), and EDA (20 mM final concentration) were added to a vessel equipped with a stir bar. The reaction vessel was sealed and brought out of the anaerobic chamber. Reactions were immediately initiated by addition of Mb (5 μM final concentration, 2 mL final volume) and the resulting mixture was left stirring for 1 min. An aliquot (400 μL) was extracted via syringe and quenched by placing it in an Eppendorf tube containing 5 M HCl (100 μL), CH_2Cl_2 (400 μL), and internal standard (20 μL, 2.5 mM final concentration). The tube was immediately vortexed for 30 seconds, centrifuged at 13,000 × *g* for 5 min, and the CH_2Cl_2 layer was extracted for GC analysis.

Anaerobic protocol for whole cells reactions
In a typical procedure, a suspension of Mb(H64V,V68A)-expressing cells was added to a reaction vessel equipped with a stir bar containing potassium phosphate buffer (50 mM, pH 7.0). The vessel was sealed, and the headspace purged with Ar for 3 min. Reactions were initiated by addition of olefin (10 mM final concentration) and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μL. The reaction mixture was stirred for 16 hours at room temperature under positive argon pressure. The turnover number (TON) for whole cell reactions was calculated based on Mb concentration in

the reaction mixture as measured via UV–vis spectroscopy (ε_{410} = 157 mM^{-1} cm^{-1}) after cell lysis.

Anaerobic protocol for whole cells reactions via anaerobic chamber

In a typical procedure inside an anaerobic chamber, a suspension of Mb(H64V,V68A)-expressing cells was added to a reaction vessel equipped with a stir bar containing potassium phosphate buffer (50 mM, pH 7.0). Reactions were initiated by addition of olefin (10 mM final concentration) and EDA (20 mM final concentration) bringing the solution to a final volume of 400 µL. The vessel was capped and stirred inside the chamber for 16 hours at room temperature. Reaction work up was performed outside the anaerobic chamber. The TON for whole-cell reactions was calculated based on the Mb concentration in the reaction mixture as measured via UV–vis spectroscopy (ε_{410} = 157 mM^{-1} cm^{-1}) after cell lysis. All buffered solutions and organic solvents were purged with Ar prior to entering the anaerobic chamber.

Whole-cell biocatalyst recycling experiments

All reactions were carried out using the anaerobic protocol for whole-cell reactions, except that each set of reactions were left stirring at room temperature under positive Ar pressure for 30 min. After a reaction cycle was completed, 20 µL of internal standard (50 mM stock solution, 2.5 mM final conc.) was added to the vial. The reaction mixture was transferred to an Eppendorf tube and centrifuged at 13,000 × *g* for 5 min. The supernatant was removed and transferred to another Eppendorf tube. After centrifugation, the cell pellet was resuspended in 400 µL of a 1.5% (v/v) Tween 20 solution in potassium phosphate buffer (50 mM, pH 7.0). The centrifugation and supernant transfer step were repeated two additional timesand all the supernatants were combined. An aliquot of 400 µL of the combined supernatant solution was extracted with DCM for GC analysis. After the last extraction of supernatant, the cell pellet was resuspended in 400 µL of potassium phosphate buffer (50 mM, pH 7.0) and transferred to a reaction vial to begin the next cycle of catalysis.

3. Results and Discussion

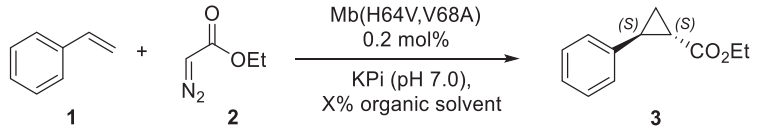
3.1. Effect of organic solvents on activity and stability of Mb-based cyclopropanase

To assess the impact of organic solvents on the function of Mb-based carbene transferases, we analyzed the catalytic activity and selectivity of sperm whale Mb variant Mb(H64V,V68A) in the presence of various concentrations of five water-miscible organic solvents, which are commonly used in biocatalysis, namely ethanol (EtOH), methanol (MeOH), *N,N*-dimethylformamide (DMF), acetonitrile (ACN), and DMSO. Mb(H64V,V68A) was previously developed to catalyze the highly stereoselective cyclopropanation of styrene and other vinylarenes in the presence of ethyl α-diazoacetate (EDA, **2**) as the carbene donor with a high degree of diastereo- and enantioselectivity [23,24]. Consistent with previous reports [23,24], this Mb variant catalyzes the cyclopropanation of styrene (**1**) with EDA (**2**) to give the *trans*-(1*S*,2*S*) cyclopropane **3** in 99% *de* and 99% *ee* (Fig. 1). In addition to the solvent effect on activity, we envisioned the stereoselectivity of this enzyme could be used to gauge the effect of the applied reaction conditions on the catalytic properties of the biocatalyst.

Initially, we examined the activity and selectivity of Mb(H64V,V68A) in the styrene cyclopropanation reaction in the presence of EtOH from 6% to 60% vol/vol (v/v) in potassium phosphate buffer (KPi) under standard reaction conditions (10 mM substrate, 0.2 mol% catalyst). As shown in Fig. 1A and Table S1, the Mb catalyst was found to maintain high levels of activity (>99% yield) and stereoselectivity (>99% *de* and *ee*) up to 30% (v/v) of EtOH. Upon increasing the concentration of organic cosolvent beyond this point, both the activity and stereoselectivity decrease resulting in 45% yield, 95% *de*, and 67% *ee* at 40% (v/v) EtOH and 22% yield and 88% *de*, and 32% *ee* at 60% (v/v) EtOH. Styrene cyclopropanation with EDA catalyzed by free hemin in buffer produces **3** in 79% *de (trans)* as a racemate (0% *ee*) [23]. A reduction in % *ee* can thus be derived from solvent-induced alteration of the enzyme active site and/or contribution from background reaction from hemin released upon denaturation of the hemoprotein. The experiments above thus showed that the Mb biocatalyst tolerates an EtOH concentration as high as 30% (v/v) without any deleterious effect on its catalytic function and stereoinduction properties.

Similar experiments were then extended to the other organic solvents (Figs. 1B–1E). In the presence of methanol, the Mb(H64V,V68A) catalyst was found to show no noticeable decrease in both activity and stereoselectivity (>99% *de* and *ee*) from 6% up to 40% (v/v) (Fig. 1B). At 50% (v/v), only a slight reduction in stereoselectivity was observed (99% *de*, 96% *ee*), while maintaining high activity (77% yield) and no decrease in relative activity compared with the "no-solvent" reference conditions (=6% (v/v)). At 60% (v/v), although the relative activity of the enzyme decreases to about 35%, stereoselectivity remains high (96% *de*, 89% *ee*). These results thus revealed a remarkable robustness of the Mb biocatalyst against denaturation in the presence of methanol (i.e., up to 50–60% (v/v)) as well as its superior stability in this solvent compared with ethanol (Fig. 1A vs. 1B).

The experiments with DMF and ACN show an overall similar behavior of the biocatalyst in the presence of these organic solvents (Figs. 1C and 1D). In both cases, no deleterious effect on the diastereo- and enantioselectivity of the catalyst was observed up to 40% (v/v) of the organic cosolvent, under which conditions, Mb(H64V,V68A) produced **3** with excellent stereoselectivity (>99% *de* and *ee*). With ACN, only a small reduction in diastereoselectivity was observed at 40% (v/v) (96% *de* and >99% *ee*). Within this concentration range, the catalytic activity of Mb(H64V,V68A) also remained largely unaffected in the presence of DMF, whereas a twofold reduction in relative activity was observed upon transitioning from 30% to 40% ACN, thus indicating a slightly higher robustness of



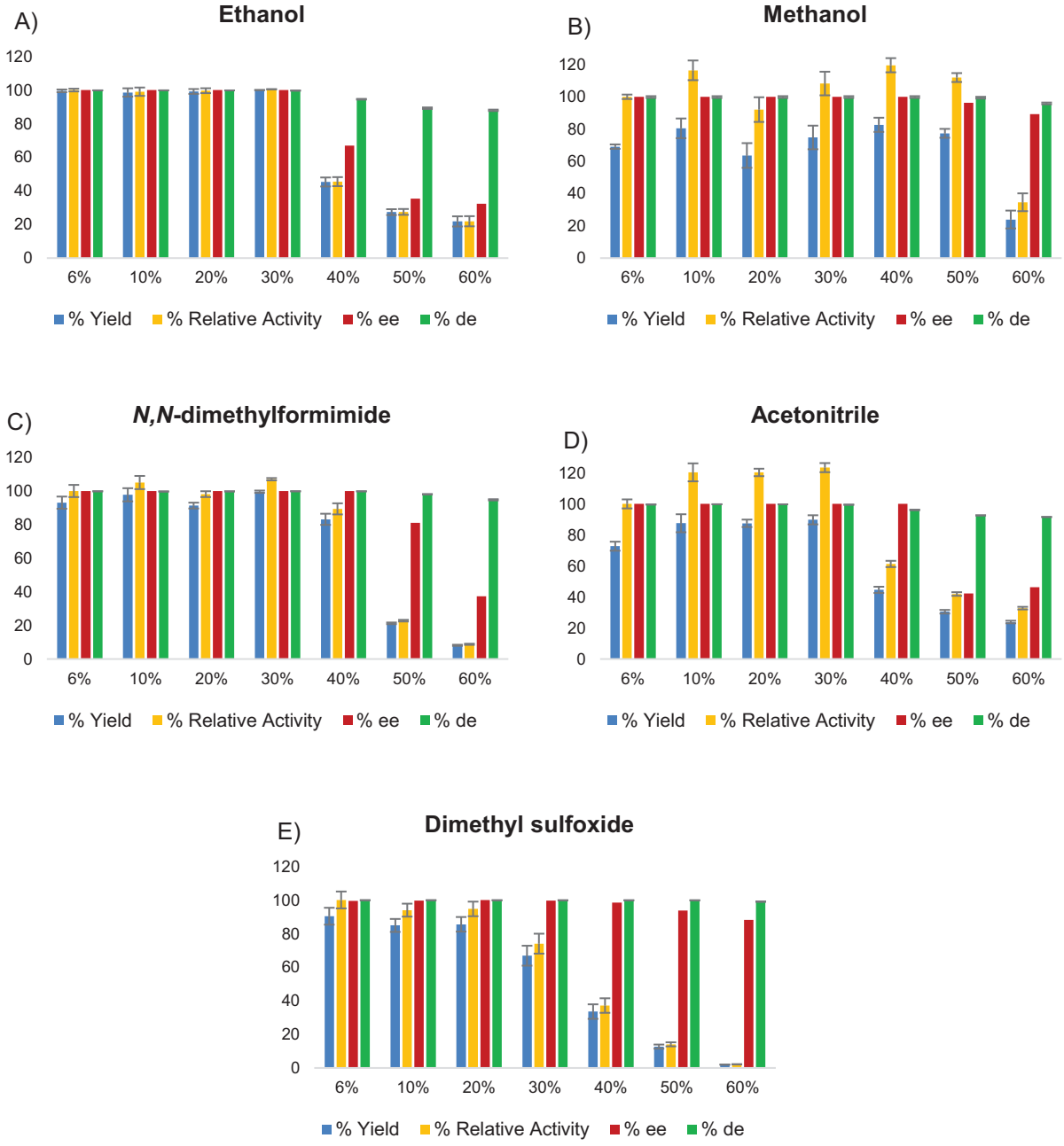


FIG. 1

*Product yield, relative activity, and stereoselectivity of Mb(H64V,V68A) for the conversion of styrene and EDA into **3** in the presence of the indicated organic solvent at varying concentrations. Relative activity corresponds to the yields normalized to the yield at the minimal solvent concentration (=6% (v/v)), which corresponds to 100% for EtOH, 69% for MeOH, 93% for DMF, 73% for ACN, and 90% for DMSO. Reaction conditions: 10 mM styrene, 20 mM EDA, 10 mM $Na_2S_2O_4$, 20 μM Mb(H64V,V68A) in 50 mM potassium phosphate buffer (pH 7.0), anaerobic, R.T., 16 hours. Product yield, diastereomeric excess (%* de*), and enantiomeric excess (%* ee*) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.*

the catalyst toward DMF over ACN. Interestingly, a small but significant increase in relative activity (+20%) was consistently observed between 10% and 30% (v/v) of ACN, indicating a beneficial effect of this solvent on catalytic activity. With both solvents, a noticeable reduction in activity and stereoselectivity occurred by increasing the organic solvent concentration to 50% and 60% (v/v), with the biocatalyst providing 8%–30% yields with 37%–81% *ee* (Figs. 1C and 1D). This notwithstanding, the Mb(H64V,V68A)-catalyzed reaction was found to maintain a significant degree of stereoselectivity in the presence of a 1:1 mixture of buffer:DMF (98% *de*, 81% *ee*).

In DMSO, the cyclopropanation biocatalyst showed no to little reduction in relative activity (74%–100% relative activity, corresponding to 67%–90% yield) observed up to 30% (v/v) of the organic cosolvent, after which point a progressive decrease in activity accompanied an increase in the cosolvent concentration (Fig. 1E). Under the applied conditions, the maximal cosolvent concentration for detectable activity was 50% (v/v) DMSO, corresponding to a 13% yield in the cyclopropanation reaction. Interestingly, in the presence of this solvent, only excellent stereoselectivity (99% *de* and 98–99% *ee*) was displayed up to 40% (v/v) and high stereoselectivity (99% *de* and 88–94% *ee*) was maintained at the high organic solvent concentrations of 50% and 60% (v/v) (Fig. 1E). Thus, although the concentration-dependent effect of DMSO on enzymatic activity is similar to that of ethanol and ACN, the enzyme's robustness toward this solvent in terms of stereoselectivity is similar to its robustness in methanol and, to a lesser extent, in DMF.

Altogether, these studies show that the Mb-based cyclopropanation biocatalyst retains high levels of activity and stereoselectivity in up to 40%–50% (v/v) of various organic cosolvents. Such organic solvent stability is remarkable considering that most enzymes, and in particular cofactor-dependent enzymes [15], are readily denatured under these conditions [14]. For example, cytochromes P450, which also contain a heme cofactor, are readily inactivated by low concentrations (<5%–10% vol/vol) of organic solvents such as ethanol, DMF, or ACN [47-50]. On balance of both activity and stereoselectivity, the Mb(H64V,V68A) biocatalyst was found to display the following order of organic solvent tolerance: MeOH > DMSO > DMF ~ ACN > EtOH. This order shows only a modest correlation with the dielectric constant of these solvents (ε = 32, 47, 36, 37, and 24, respectively), indicating that multiple factors are at play in influencing the solvent effect on activity and stereoselectivity. With the least polar solvent, ethanol, a decrease in activity is accompanied by a decrease in stereoselectivity, suggesting that protein denaturation could be occurring at high solvent concentrations. For DMSO and DMF, high stereoselectivity is maintained at high solvent concentrations (50% and 40% v/v, respectively), despite a decrease in the product yield and thus in catalytic activity. This suggests an inhibitory effect of the solvent on the enzyme activity without involving denaturation of the hemoprotein. Coordination of DMSO to the heme iron has been proposed to represent a key mechanism of inactivation of P450s in this solvent [50,51], and a similar phenomenon could be at the basis of the reduced cyclopropanation activity of the Mb catalyst at high concentrations of DMSO. A similar phenomenon could be invoked for DMF, which can also bind metals. As noted earlier, the enzyme shows the highest stability toward methanol, in which it maintains high activity and stereoselectivity up to 50% of co-solvent (v/v), this indicating both a lack of solvent-induced protein denaturation and activity inhibition.

3.2. Effect of organic solvents on cyclopropanation rate

To further assess the effect of the different solvents on the catalytic properties of the Mb-based carbene transferase, we measured initial rates of product formation in the presence of 30% (v/v) organic cosolvent using 0.05 mol% catalyst. As summarized in Table 1, these experiments showed that the Mb-catalyzed cyclopropanation reaction proceeds with faster rates in the presence of ethanol and methanol compared with the reactions carried out in the presence of ACN, DMF, and DMSO. The measured cyclopropanation rates in the presence of the alcohols correspond to 195–227 turnovers Min^{-1}, which are about fivefold lower than that measured in the absence of organic solvent (1,000 turnovers Min^{-1}) [23]. Compared with the alcohol-based solvents, twofold to fivefold slower cyclopropanation rates were observed in the presence of ACN (148 TON Min^{-1}), DMF (105 TON Min^{-1}), and DMSO (44 TON Min^{-1}). The more pronounced effects of the latter solvents on the catalytic rates can be explained on the basis of their Lewis basicity and thus their potential ability to coordinate the heme-Fe when present at high concentrations (30% (v/v) corresponds to 5.7 M ACN, 3.9 M DMF, and 4.2 M DMSO). Reasonably, this process would compete for the interaction of the heme cofactor with EDA, as required for carbene transfer catalysis [52], resulting in a slowing down of the cyclopropanation rate.

3.3. Biocatalytic activity under microaerobic and semiaerobic conditions

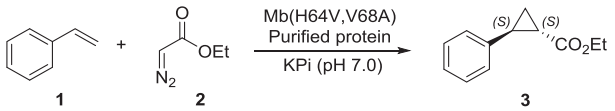
Hemoprotein-catalyzed carbene transfer reactions show optimal performance under anaerobic conditions due to the inhibitory effect of O_2 on carbene transferase activity resulting from O_2 binding to the heme cofactor [20,23,53]. For our Mb-catalyzed carbene transfer reactions, anaerobic conditions are typically achieved by performing these reactions in an anaerobic chamber or through cannula transfer methods. Since these equipment and techniques may not be available in all laboratories, we wished to develop operationally simpler protocols to conduct these biocatalytic reactions. To this end, we tested an alternative protocol, referred here as "microaerobic," which requires simple purging of the vial headspace and buffer solution containing the reductant with argon (using a balloon) followed by addition of the protein, substrate, and diazo reagent via a syringe. This protocol removes the need for the use of cannula transfer techniques. As shown in Table 2, under these microaerobic conditions, the Mb(H64V,V68A)

TABLE 1 *Initial rates of Mb(H64V,V68A) for the cyclopropanation of styrene and EDA in various organic solvents at 30% (v/v)[a]*

	Solvent				
	Ethanol	*Methanol*	N,N-*dimethylformamide*	*Acetonitrile*	*Dimethyl sulfoxide*
Rate (TON Min^{-1})	195 ± 37	227 ± 41	105 ± 16	148 ± 4.0	44 ± 18

[a]Reaction conditions: 10 mM styrene, 20 mM EDA, 10 mM $Na_2S_2O_4$, 30% organic co-solvent (v/v), 5 μM Mb(H64V,V68A), potassium phosphate buffer (50 mM, pH 7.0), anaerobic, R.T., 1 min. Product yield and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

TABLE 2 *Catalytic activity and selectivity of freshly prepared and lyophilized Mb(H64V,V68A) toward styrene cyclopropanation with ethyl diazoacetate under various atmospheric conditions[a]*



1 2 3

Entry	*Catalyst*	*Condition*	*Yield*	*TON*	de	ee
1	Solution	Anaerobic	>99%	500	>99%	>99%
2	Solution	Aerobic	4%	19	>99%	>99%
3	Solution	Microaerobic	>99%	500	>99%	>99%
4	Solution	Semiaerobic	>99%	500	>99%	>99%
5	Lyophilized	Anaerobic	>99%	500	>99%	>99%
6	Lyophilized	Anaerobic[b]	>99%	2,000	>99%	>99%
7	Lyophilized	Microaerobic	>99%	500	>99%	>99%
8	Lyophilized	Semiaerobic	>99%	500	>99%	>99%
9	Lyophilized	Aerobic	7%	37	>99%	>99%

[a]Reaction conditions: 10 mM styrene, 20 mM EDA, 10 mM $Na_2S_2O_4$, 8% EtOH (v/v), 20 μM Mb(H64V,V68A), potassium phosphate buffer (50 mM, pH 7.0), various atmospheric conditions, room temperature, 16 H.

[b]5 μM protein. Product yield, diastereomeric excess (% *de*), enantiomeric excess (% *ee*), and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

biocatalyst performed with the same level of activity and stereoselectivity as under strictly anaerobic conditions (Entry 3 vs. 1), providing full conversion of styrene to the *trans*-(1*S*,2*S*) cyclopropane **3** with >99% *de* and *ee*. This is in contrast to the reaction carried out under aerobic conditions, which showed a significantly reduced yield as expected (Table 2, Entry 2). Encouraged by these results, we tested an even simpler "semiaerobic" protocol, in which the protein is simply added to a sealed vial containing a nondegassed buffered solution with the protein and reductant, followed by filling of

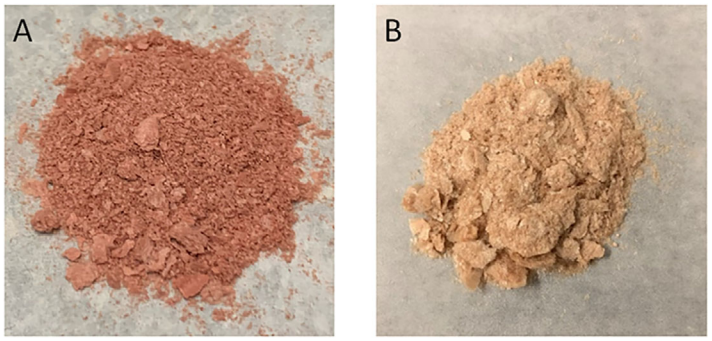


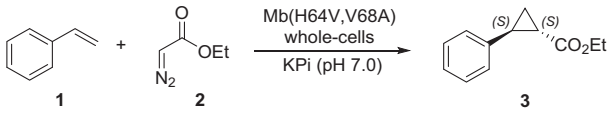
FIG. 2 *Lyophilized Mb(H64V,V68A) variant (A) and lyophilized* E. coli *cells containing Mb(H64V,V68A) (B).*

the head space with argon, and addition of the substrate and diazo reagent. Under these conditions, quantitative yield of **3** was obtained with excellent diastereo- and enantioselectivity (>99% *de* and *ee*), thus demonstrating the functionality of these simplified microaerobic and semiaerobic protocols for performing Mb-catalyzed carbene transfer reactions.

3.4. Reactions with lyophilized proteins and whole cells

Long shelflife stability is a highly desirable feature for enzymes to be applied in biocatalysis [2,4,11], but cofactor-dependent enzymes such as heme enzymes often tend to lose catalytic activity upon long-term storage in solution or after lyophilization due to cofactor loss and/or denaturation [54,55]. To investigate this aspect, the Mb(H64V,V68A) variant was lyophilized via a freeze-dry cycle (Fig. 2A) and reconstituted in buffer after storage as lyophilized powder at room temperature. UV–vis absorption spectroscopy experiments showed nearly quantitative recovery of the Mb variant (94%–99%) after reconstitution in buffer, as determined based on the intensity of the Soret bands in its ferric, ferrous, and CO-bound form (Table S2). To further assess the impact of lyophilization on the catalytic properties of the Mb variant, the resolubilized hemoprotein was tested for activity in the model cyclopropanation reaction with styrene and EDA. Importantly, under standard anaerobic conditions, the lyophilized protein showed identical levels of high activity

TABLE 3 *Catalytic activity of freshly prepared and lyophilized whole cells expressing Mb(H64V,V68A) for styrene cyclopropanation with ethyl diazoacetate*[a]



1 2 3

Entry	*Catalyst*	*Yield*	*TON*	de	ee
1	Fresh	>99%	363	>99%	>99%
2	Lyophilized	>99%	n.d.	>99%	>99%
3	Fresh[b]	44%	402	>99%	99%
4	Lyophilized[b]	62%	560	>99%	>99%
5	Lyophilized[c]	71%	n.d.	>99%	98%

[a]Reaction conditions: 10 mM styrene, 20 mM EDA, 8% EtOH (v/v), OD_{600} = 40, potassium phosphate buffer (50 mM, pH 7.0), anaerobic, R.T., 16 hours.

[b]OD_{600} = 20.

[c]OD600 = 20, lyophilized cells stored at room temperature for 6 months. Product yield, diastereomeric excess (% *de*), enantiomeric excess (% *ee*), and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards. n.d. = not determined.

(>99% yield) and excellent stereoselectivity as the control (nonlyophilized) protein (Table 2, Entry 5 vs. 1). At lower catalyst loading of 0.05 mol%, the resolubilized Mb variant was found to provide quantitative yields of the enantiopure cyclopropanation product, supporting 2,000 TON (Table 2, Entry 6). These results thus demonstrate that the Mb-based cyclopropanase is able to tolerate well the lyophilization process without exhibiting loss in catalytic activity or stereoselectivity. Upon testing the lyophilized protein under the microaerobic and semiaerobic protocols described above, excellent levels of activity and stereoselectivity were found in both cases (Table 2, Entries 7–8), further demonstrating the functionality and catalytic efficiency of the resolubilized protein in combination with these operationally simpler reaction setups. Although various additives [54,55] were also tested during the lyophilization process (e.g., sucrose, glucose, and PEG 4000), the results above demonstrated that additives were not required for full recovery of the protein and its activity.

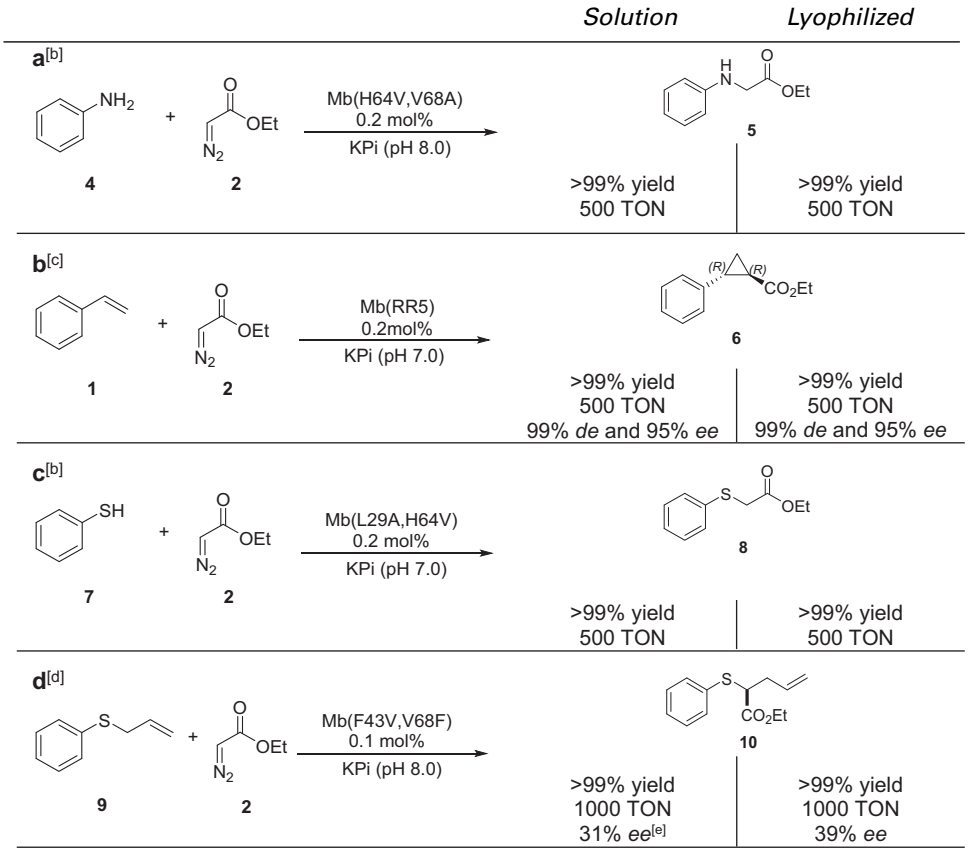
Encouraged by the results with the lyophilized protein, we investigated the effect of lyophilization on *E. coli* whole-cells expressing Mb(H64V,V68A). Similarly to the control reactions with freshly prepared cells, cyclopropane **3** could be obtained in quantitative yields and with excellent stereoselectivity (99% *de* and *ee*) using resolubilized Mb(H64V,V68A)-containing *E. coli* cells at a cell density (OD_{600}) of 40 (Table 3, Entry 2 vs. 1). The experiments were then repeated under catalyst-limited conditions (OD_{600} = 20) to better assess the impact of lyophilization on the activity of the intracellular biocatalyst. Surprisingly, the resolubilized Mb(H64V,V68A)-containing cells provided an even higher yield of the cyclopropanation product compared with fresh cells (62% vs. 44%), while maintaining excellent stereoselectivity (Table 3, Entries 4 vs. 3). Furthermore, the lyophilized cells could be utilized after storage on a shelf at room temperature for over 6 months (Fig. 2B) without exhibiting any loss in catalytic activity and stereoselectivity (Table 3, Entry 5). Overall, these experiments demonstrate the robustness of the Mb catalyst to lyophilization both in the form of purified protein and in whole cells as well as its long shelflife stability in lyophilized form. As noted above, the observation of the higher catalytic activity of the lyophilized cells compared with the fresh cells was a positive yet unexpected outcome of these experiments. Although further studies are required to elucidate the basis of this phenomenon, a possible explanation is that the lyophilization process may increase the permeability of the cells to the reagents and/or contribute to reduce cellular components that interfere with the reaction in the intracellular settings.

3.5. Other Mb-based carbene transferases

Having established the ability of Mb(H64V,V68A) cyclopropanase to maintain activity and stereoselectivity after lyophilization, further experiments were carried out to determine the generality of this property across other Mb(H64V,V68A)-catalyzed reactions (Table 4, a) as well as across other Mb-based carbene transferases (Table 4, b–d). Mb(H64V,V68A) was previously reported to catalyze the N–H insertion of amines in the presence of diazo compounds [31]. In a model reaction with aniline (**4**) and EDA (**2**), lyophilized Mb(H64V,V68A) was found to produce the N–H insertion product **5** in quantitative yield and support 500 TON (Table 4, a), thus displaying comparable activity to the nonlyophilized control. Next, we tested the catalytic activity of lyophilized Mb(L29T,F43W,H64V,V68F) (a.k.a. Mb(RR5)), which was previously developed to catalyze the cyclopropanation of vinylarenes with EDA with high *trans*-(1*R*,2*R)* selectivity, that is, with opposite enantioselectivity compared with Mb(H64V,V68A) (Table 4, b) [24]. After lyophilization, this variant produced cyclopropane **6** in quantitative yield and high stereoselectivity (99% *de*, 95% *ee*), thus exhibiting no reduction in activity and selectivity compared with the nonlyophilized protein. Similar experiments were carried with Mb(L29A,H64V), which was previously optimized to catalyze S–H insertion reactions with thiols and diazoacetates [32]. Also in this case, the Mb variant was found to maintain its catalytic activity after lyophilization, resulting in the quantitative yield of the desired S–H insertion product **8** from the reaction with thiophenol (**7**) and EDA (**2**) (Table 4, c).

Lastly, we tested Mb(F43V,V68F), a Mb-based catalyst previously optimized for catalyzing asymmetric Doyle–Kirmse reactions with allenyl sulfides and EDA [44]. In a model reaction with allyl phenyl sulfide (**9**) and EDA (**2**), the lyophilized Mb variant was able to support 1,000 TON and produce the [2,3]-sigmatropic rearrangement product **10** in quantitative yield

TABLE 4 *Catalytic activity and selectivity of engineered Mb catalysts for different carbene transfer reactions before and after lyophilization[a]*



Product yield, diastereomeric excess (% *de*), enantiomeric excess (% *ee*), and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

[a]Reaction conditions: 10 mM substrate, 10 or 20 mM EDA, 10 mM $Na_2S_2O_4$, 10% EtOH, potassium phosphate buffer (50 mM, pH 7.0 or 8.0), anaerobic, R.T., 16 hours.

[b]20 mM EDA and pH 7.0.

[c]10 mM EDA and pH 8.0.

[d]20 mM EDA and pH 8.0.

[e]According to Ref. [44].

and 39% *ee*, thus mirroring the results obtained with the same protein prior to lyophilization. Altogether, these results combined demonstrate that robustness to lyophilization is shared by different Mb variants and across different types of Mb-catalyzed carbene transfer reactions.

3.6. Recyclability of Mb-containing whole-cell biocatalyst

Recyclability is another attractive feature of biocatalysts used for organic synthesis [56], which is often pursued through immobilization of enzymes to solid supports [57-60]. Given the demonstrated functionality of our Mb-based catalysts in wholecell systems, we were interested in investigating the possibility of recycling Mb-containing whole cells in multiple rounds of catalysis. In particular, we envisioned that entrapment of the biocatalyst in the cell would facilitate reisolation of the biocatalyst after the reaction, while protecting it from denaturation during the product extraction process (typically performed with halogenated solvents such as CH_2Cl_2). For these experiments, *E. coli* cells expressing Mb(H64V,V68A) were applied to catalyze the cyclopropanation of *p*-methoxy-styrene (**11**) with EDA over three consecutive rounds of catalysis. After each round, the cells were pelleted, washed with buffer containing 1.5% (v/v) Tween 20 surfactant to remove residual product (and unreacted reagents), and then resuspended in buffer for use in the next round of catalysis. At each cycle, the product was isolated from the supernatant of the cell suspension and wash solutions. These experiments showed a relative activity of 80%–85% for both the second and third round of catalysis compared with the first round. In addition,

excellent levels of stereoselectivity (>99% *de* and *ee*) were observed after each rounds of catalysis, indicating that the Mb catalyst is responsible for the observed cyclopropanation activity and no erosion of stereoselectivity occurred during the biocatalyst recovery process. These results thus provide a proof-of-principle demonstration of the recyclability of the Mb carbene transferase in whole cells and its ability to maintain high levels of activity and stereoselectivity across multiple cycles of catalysis using these systems.

4. Conclusions

Our studies demonstrate that Mb-based carbene transferases show a remarkable organic solvent stability and tolerate the presence of various organic solvents such as methanol, ethanol, ACN, DMF, and DMSO, in concentrations up to 30%–50% (v/v) without significant reduction in activity and stereoselectivity. As these organic cosolvent concentrations are often required for biocatalysis at the large scale (e.g., 50% DMSO for sitagliptin manufacturing) [15], the inherent organic solvent stability of these Mb-based carbene transferases should facilitate their use in the context of these applications without requiring further protein engineering. This work also shows that these biocatalysts are able to withstand lyophilization, both as a purified protein and in whole cells, with excellent retention of catalytic activity and stereoselectivity. These lyophilized biocatalysts exhibit long shelflife stability and could be stored at room temperature for over 6 months without any loss in activity or stereoselectivity. Finally, we demonstrate the recyclability of Mb-expressing whole cells across multiple rounds of carbene transfer catalysis, and we implemented technically simple protocols for performing these biocatalytic transformations, which bypass the need for specialized instrumentation (anaerobic chamber) or techniques (cannulation) to ensure microaerobic conditions for optimal catalytic activity. Altogether, these findings demonstrate the robustness of Mb-based carbene transferases under operationally relevant conditions and should facilitate the practical exploitation of these biocatalysts for synthetic applications.

5. Acknowledgements

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