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# What to sacrifice? Fusions of cofactor regenerating enzymes with Baeyer-Villiger monoxygenases and alcohol dehydrogenases for self-sufficient redox biocatalysis

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## ABSTRACT

A collection of fusion biocatalysts has been generated that can be used for self-sufficient oxygenations or ketone reductions. These biocatalysts were created by fusing a Baeyer-Villiger monoxygenase (cyclohexanone monoxygenase from *Thermocristum municipale*: TmCHMO) or an alcohol dehydrogenase (alcohol dehydrogenase from *Lactobacillus brevis*: LbADH) with three different cofactor regeneration enzymes (formate dehydrogenase from *Burkholderia stabilis*: BsFDH; glucose dehydrogenase from *Sulfolobus tokodaii*: StGDH, and phosphite dehydrogenase from *Pseudomonas stutzeri*: PsPTDH). Their tolerance against various organic solvents, including a deep eutectic solvent, and their activity and selectivity with a variety of substrates have been studied. Excellent conversions and enantioselectivities were obtained, demonstrating that these engineered fusion enzymes can be used as biocatalysts for the synthesis of (chiral) valuable compounds.

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## 1. Introduction

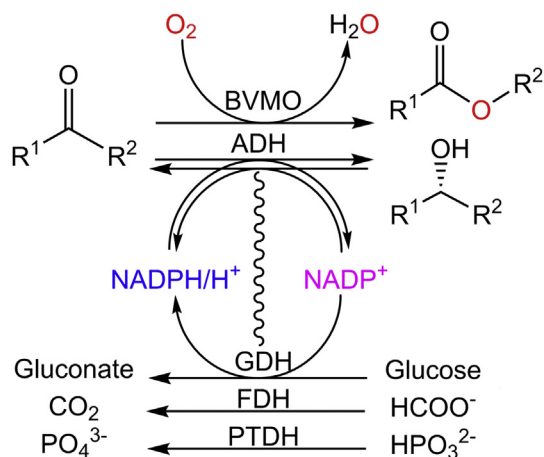
Biocatalysis can be used to synthesize chiral building blocks, such as monomers for polymer materials, and precursors for pharmaceuticals [1–4]. Enzymes are very suitable for catalyzing reactions with high enantioselectivity to obtain chiral products. For instance, alcohol dehydrogenases (ADHs, EC 1.1.1.X) – also known as carbonyl reductases or ketoreductases – depend on NAD(P)H to catalyze the asymmetric reduction of ketones to either (*R*)- or (*S*)-alcohols in excellent enantiomeric excess (*ee*) [5]. These enzymes, among others, have been applied for syntheses of pharmaceutical precursors [2,4]. Another group of redox enzymes that depend on NAD(P)H are the Baeyer-Villiger monoxygenases (BVMOs) (EC 1.14.13.X). These FAD-containing enzymes can catalyze regio- and enantioselective transformations of ketones to esters or lactones, using dioxygen and NADPH. Interest in the application of BVMOs

has grown, in particular for the transformation of substituted cyclic ketones to chiral lactones, for branched polyesters [6–9]. Specifically, the recent discovery of robust BVMOs, such as the thermostable cyclohexanone monoxygenase from *Thermocristum municipale* (TmCHMO), has led to great interest for exploring these monoxygenases for industrial applications, as most of the previously reported BVMOs were quite unstable [10].

Both ADHs and BVMOs rely on the cofactor NAD(P)H for catalysis, which is too expensive to apply in stoichiometric amounts, and therefore should be regenerated. There are a number of different approaches to recycle NAD(P)H [5,11,12]. One approach is to apply another enzyme which can use the oxidized NAD(P)<sup>+</sup> and a sacrificial cheap cosubstrate to regenerate the reduced nicotinamide cofactor: the so-called “coupled-enzyme” approach. Three commonly used coenzyme regenerating enzymes are formate dehydrogenase (FDH), glucose dehydrogenase (GDH), and phosphite dehydrogenase (PTDH), all using relatively cheap substrates [12]. Instead of producing and adding these enzymes separately, the recycling enzyme can also be covalently fused to the NADPH-dependent enzyme through enzyme engineering (Scheme 1). In

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**Scheme 1.** Representation of self-sufficient ADHs and BVMOs, with either GDH, FDH or PTDH as fusion partner to recycle the nicotinamide cofactor NADPH.

this way, a bifunctional and self-sufficient fusion biocatalyst is produced enabling conversion using merely one biocatalyst. Moreover, some studies on enzyme fusions provided evidence that tethering of two enzymes can improve the productivity of a multi-enzyme system [13]. In 2008 Torres Pazmiño et al. developed a platform for expressing BVMOs fused to PTDH for efficient cofactor regeneration [14,15]. Later, a few other studies reported enzyme fusions with FDH [16], GDH [17], or PTDH [18–20], though no study yet has compared a single biocatalyst with different regenerating enzymes.

The aim of this work is to explore different recycling enzymes as fusion partner for two oxidoreductase enzymes (BVMO and ADH), and to compare their strengths and weaknesses for biocatalytic applications. We fused three different regenerating enzymes to a BVMO (*TmCHMO*, from *Thermocristum municipale*) [10] and an (*R*)-selective alcohol dehydrogenase (*LbADH*, from *Lactobacillus brevis*) [21–23], to generate a panel of self-sufficient biocatalysts. The three recycling enzymes are: FDH from *Burkholderia stabilis* (*BsFDH*) [24], GDH from *Sulfolobus tokodaii* (*StGDH*) [25], and an engineered PTDH from *Pseudomonas stutzeri* (*PsPTDH*) [26,27]. The fused enzymes were produced and purified, and applied for biotransformations. Both enzymes act on ketone substrates to form interesting products, though many of such organic substrates have low solubility in water. Therefore, the enzyme fusions were tested with a wide range of solvents. In particular, the tolerance of these biocatalysts to deep-eutectic solvents (DES) was evaluated, which at present has not been studied to a great extent.

## 2. Results and discussion

### 2.1. Cloning, expression and purification

The cloning approach was similar to that of previous fusion cloning work [14,15]. As linker region a variation of the short-linker from previous work was used, which was found to be optimal for fusions with BVMOs [15]. For ease of denoting, *TmCHMO* is named B (BVMO) while the *LbADH* is named A (ADH), see Table 1. The cofactor regeneration enzymes are also denoted in single letter codes: F for FDH, G for GDH and P for PTDH. After cloning of the six constructs (Table 1), the plasmids were used to transform competent *E. coli* NEB 10b cells. These cells were then used for recombinant expression and subsequent enzyme purification. Only one of the six fusion constructs had an inadequate level of soluble expression: G-A. Although it was used for some of the initial

**Table 1**  
Fusion constructs.

Enzyme Fusion	N-terminal	Linker	C-terminal	Mw (kDa)
F-B	<i>BsFDH</i>	SGSAAG	<i>TmCHMO</i>	104
G-B	<i>StGDH</i>	SGSAAG	<i>TmCHMO</i>	102
P-B	<i>PsPTDH</i>	SRSAAG	<i>TmCHMO</i>	99
F-A	<i>BsFDH</i>	SGSAAG	<i>LbADH</i>	71
G-A	<i>StGDH</i>	SGSAAG	<i>LbADH</i>	69
P-A	<i>PsPTDH</i>	SGSAAG	<i>LbADH</i>	65

experiments, in later experiments this fusion enzyme was omitted. The other fusion enzymes could be produced in high quantities (>50 mg/L culture). After lysis of the cells and protein purification through affinity column chromatography, the enzyme fusions were used for further testing and biotransformations.

### 2.2. Reaction optimization

The first step was to establish optimal reaction conditions. The reactions were set up using cyclohexanone (5 mM) as model substrate for the *TmCHMO* constructs, and acetophenone (5 mM) for the *LbADH* constructs (see Supporting Information, Section 2.1). The optimal reaction conditions for each biocatalyst system were similar. All fusions were able to fully convert the substrate under mild reaction conditions: phosphate buffer 100 mM pH 8.5 for *TmCHMO* fusions, and phosphate buffer 100 mM pH 7.5 for *LbADH* constructs, at 30 °C in most cases. Previous studies have shown that PTDH is not inhibited by phosphate buffer [28]. The only exception was G-B that afforded a better selectivity towards the formation of caprolactone at 24 °C, since at 30 °C a significant amount of cyclohexanol was observed due to the undesired activity of *StGDH* with cyclohexanone.

The biotransformations were monitored over time (Fig. 1). The reactions catalyzed by *BsFDH* and *PsPTDH* constructs were mostly completed after 16 h, while the *StGDH* fusions needed 24 h to achieve full conversions. The conversion with the *TmCHMO* fusions resulted in a linear increase of product in time, whereas the *LbADH* fusions showed a higher initial rate up to 70% conversion, and then it decreased until full conversion. This was probably due to the relatively high  $K_M$  value for acetophenone (2.8 mM) of *LbADH* and/or product inhibition.

Assuming that the *TmCHMO* and *LbADH* enzymes had roughly the same catalytic properties (activity and  $K_M$ ) for the three different fusions, the only difference concerning the kinetic performance of the fusion enzymes is due to the recycling enzyme. The amount of sacrificial substrate (10 mM glucose, 50 mM sodium formate or 20 mM sodium phosphite) was decided based on the reported  $K_M$  values of each enzyme, ensuring maximal activity ( $V_{max}$ ) throughout the whole reaction. The  $k_{cat}$  values for the three recycling enzymes PTDH, FDH and GDH with  $NADP^+$  are, respectively:  $6.5\text{ s}^{-1}$  (at 25 °C) [15],  $4.75\text{ s}^{-1}$  (at 30 °C) [24], and  $\sim 3.8\text{ s}^{-1}$  (at 35 °C, derived from reported activity and temperature optimum) [25]. This was in agreement with the time course of the biotransformations (Fig. 1): PTDH fusions were the fastest, while FDH fusions were the second. From this initial experiment it was clear that GDH constructs had the weakest performance. The respective biotransformations were the slowest, maybe due to the fact that the reaction with G-B worked better at 24 °C while the *StGDH* has a relatively high temperature optimum. Furthermore, G-A showed also a poor expression.

### 2.3. Enzymatic preparations

The need for NADPH is a cost factor when considering the

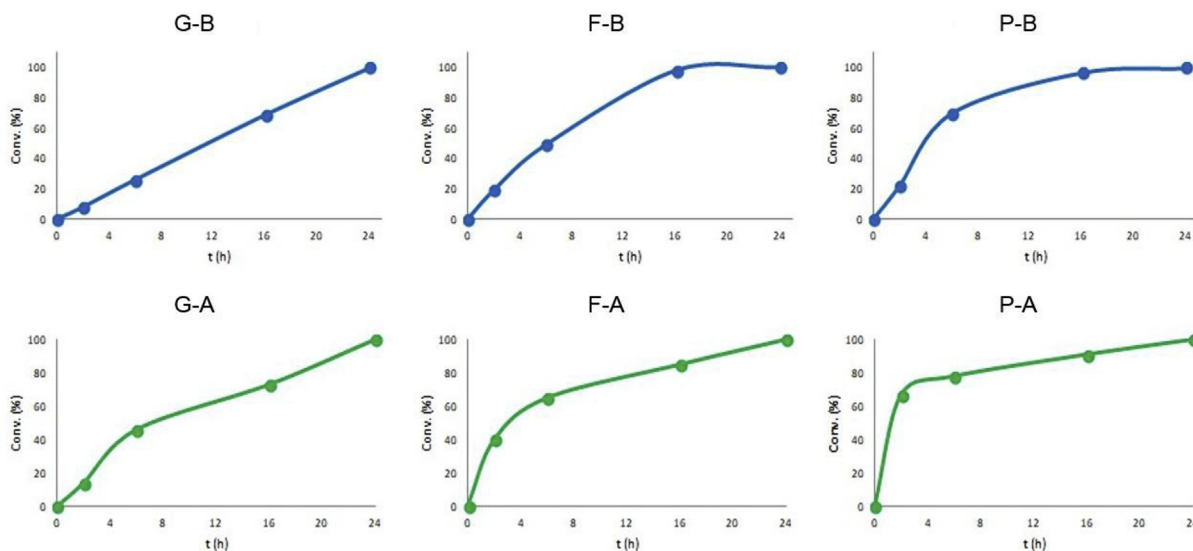


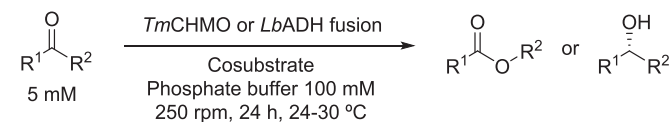
Fig. 1. Monitoring of the conversions in the self-sufficient *TmCHMO* and *LbADH*-catalyzed enzymatic biotransformations.

utilization of biocatalysts at larger scale. In this context, Torres Pazmiño et al. published interesting results using cell-free extract preparations of different self-sufficient BVMOs without external addition of the nicotinamide cofactor [14]. This approach was also tested for our fusions (Table 2, entries 1, 4, 7, 10, 13 and 16), but resulted in good results only for G-B and P-B fusions. F-B and *LbADH* fusions had a significantly lower activity. Apart from that, and still in order to make the process economically feasible, each biotransformation using the purified enzyme was set up adding

just 0.5 mM NADPH (Table 2, entries 3, 6, 9, 15 and 18). High conversions and excellent *ee* values were found, though the reactions with *LbADH* (entries 15 and 18) could not reach full conversion. Although the  $K_M$  value for NADPH is 40  $\mu$ M for the native *LbADH*, possibly the  $K_M$  of the respective fusion enzymes is a bit higher, as was observed for other PTDH-BVMO fusions [14]. This could slow down the reaction, in particular in combination with the aforementioned slower reaction rates after 70% conversion, due to the high  $K_M$  for acetophenone (Fig. 1).

Table 2

Biotransformations mediated by self-sufficient *TmCHMO*s and *LbADH*s using different enzymatic preparations and different amounts of cofactor.



Entry	Fusion	Protein preparation	[NADPH] (mM)	Conv. (%) <sup>b</sup>	<i>ee</i> (%) <sup>b,c</sup>
1	G-B	Cell-free extract <sup>a</sup>	—	92	—
2	G-B	Purified	1	>99	—
3	G-B	Purified	0.5	96	—
4	F-B	Cell-free extract <sup>a</sup>	—	10	—
5	F-B	Purified	1	>99	—
6	F-B	Purified	0.5	99	—
7	P-B	Cell-free extract <sup>a</sup>	—	>99	—
8	P-B	Purified	1	>99	—
9	P-B	Purified	0.5	96	—
10	G-A	Cell-free extract <sup>a</sup>	—	13	81 (R)
11	G-A	Purified	1	>99	>99 (R)
12	G-A	Purified	0.5	n.d.	n.d.
13	F-A	Cell-free extract <sup>a</sup>	—	68	96 (R)
14	F-A	Purified	1	>99	>99 (R)
15	F-A	Purified	0.5	74	>99 (R)
16	P-A	Cell-free extract <sup>a</sup>	—	42	>99 (R)
17	P-A	Purified	1	>99	>99 (R)
18	P-A	Purified	0.5	86	>99 (R)

<sup>a</sup> Cell-free extract preparations were prepared as described in the Experimental Section.

<sup>b</sup> Measured by GC analysis.

<sup>c</sup> Caprolactone is not an optically active molecule so no *ee* value is associated to *TmCHMO* constructs results. For the reactions with the purified enzyme, 2  $\mu$ M of *TmCHMO* fusion or 0.2 mg/mL (approx. 2.5  $\mu$ M) of *LbADH* fusion were used.

#### 2.4. Cosolvent screening

At this point, the model reaction for each fusion was fully studied. However, in order to perform reactions with high substrate concentrations, the low water solubility of many ketone substrates must be addressed. One of the common strategies is the employment of an organic cosolvent. For this reason, the enzyme fusions were first tested with a wide range of organic solvents (1% v/v, Fig. 2), including *tert*-butyl methyl ether (MTBE), methanol (MeOH), ethanol (EtOH), 1,4-dioxane and acetonitrile (MeCN). First of all, it must be pointed out that not a single cosolvent affected the selectivity of *LbADH* fusion enzymes (data not shown), thus providing the enantiopure (*R*)-alcohol. MTBE and acetonitrile revealed to be the best cosolvents for all enzyme fusions, as they led to high conversions (>90%) in all cases. Methanol and ethanol showed very good results with most of the constructs, with the exception of P-B using MeOH and G-A using EtOH; in these cases the conversion values dropped down to 51% and 69%, respectively. Finally, 1,4-dioxane caused a huge drop of activity (90% decrease) with G-B and F-B fusions, and a 40% decrease for G-A.

These results are surprising, as it seems that particular combinations of enzymes can be sensitive to a cosolvent, even though other combinations containing one of those enzymes are tolerant. For instance, with 1,4-dioxane and F-B, it is not simply that *TmCHMO* was intolerant, since P-B did give full conversion. The same was true for *BsFDH*, as F-A was able to afford 90% conversion. Based on those two results, one would expect F-B to give high conversion as well, yet it was only 10%. In other words, it was observed that particular combinations of enzymes can be more tolerant to organic solvents than other combinations with the same enzyme. It could be that certain enzymes can transmit tolerance for

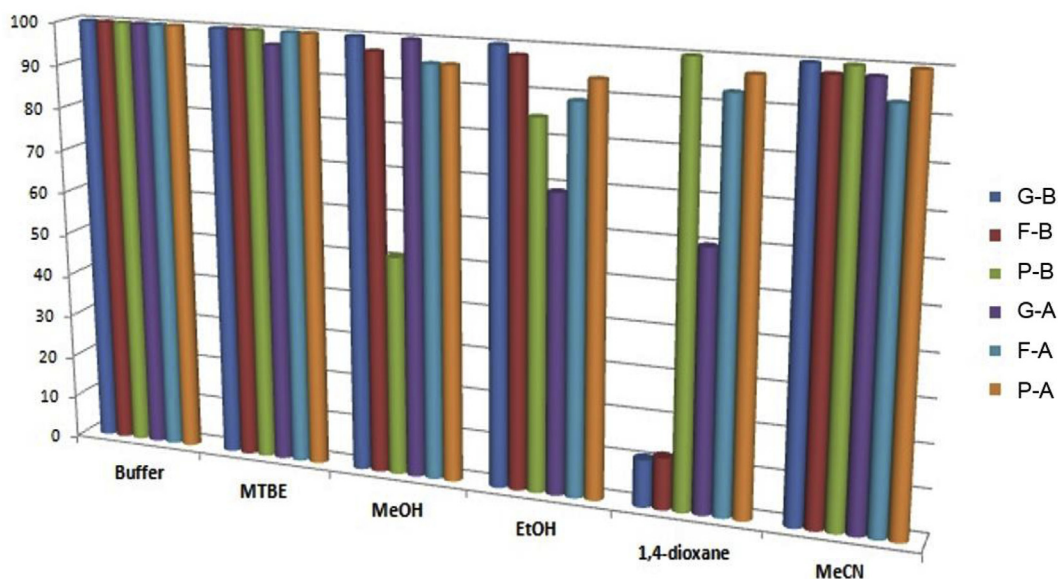


Fig. 2. Biotransformation of model substrates using different organic cosolvents (1% v/v).

a particular cosolvent when fused to its partner enzyme, e.g., PTDH in P-B for 1,4-dioxane.

Taking all these data into account, we decided to test the fusions behavior in a biphasic system using larger amounts of MTBE, as it was the best cosolvent among the tested ones (see Supporting information, Section 2.2). The *Tm*CHMO fusions were able to catalyze the Baeyer-Villiger oxidation of cyclohexanone with full conversion using up to 10% v/v of MTBE. An amount of 15% v/v of MTBE led to full conversion using *Bs*FDH and *Ps*PTDH constructs, but caused a drop in the *St*GDH fusion activity, indicating again that this fusion was less stable. On the other hand, F-A was capable to convert acetophenone into (*R*)-1-phenylethanol using up to 40% v/v of MTBE, while P-A showed a small drop of activity at 20% v/v of MTBE. Overall, it was clear that for this cosolvent *Bs*FDH was the most tolerant NADPH-regenerating enzyme.

Deep eutectic solvents (DES) represent a promising new generation of environmentally-friendly solvents [29]. We evaluated the use of DES as cosolvents for the biotransformations catalyzed by the engineered fusion biocatalysts. Concentrations of 10%–30% v/v of DES derived from choline chloride and glycerol (ChCl:Gly 1:2 mol/mol) were employed (see Supporting information, Section 2.3). G-B and F-B appeared as the most robust enzymes as they could achieve full conversion to caprolactone using up to 20% v/v of DES. Besides, F-A showed better results than P-A. As with the results with MTBE, *Bs*FDH was the most tolerant cofactor regenerating enzyme in non-conventional media. Furthermore, a glucose-based DES (ChCl:glucose at 1.5:1 mol/mol) was tested both as cosolvent and cosubstrate for producing caprolactone by G-B (see Supporting information, Section 2.4). The corresponding results are very interesting as the biotransformation (without adding external glucose) led to full conversions.

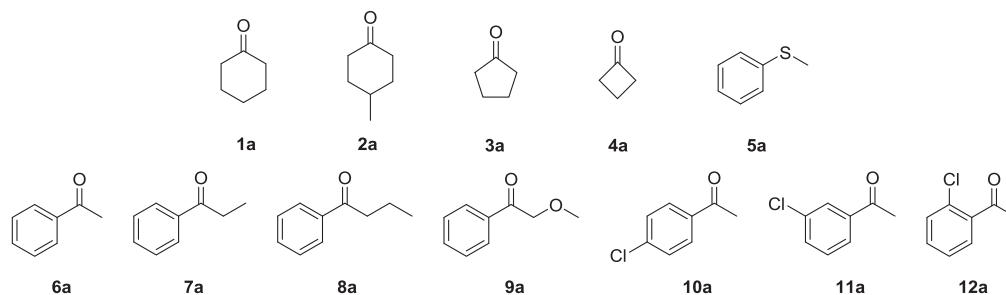
### 2.5. Substrate screening

In order to check the synthetic versatility of the engineered fusion biocatalysts, other substrates were tested (see Fig. 3). *Tm*CHMO constructs were employed to catalyze the Baeyer-Villiger oxidation of substrates differing on the ring substitution and the ring size (substrates 2–4a, Table 3). In this manner, 4-methylcyclohexanone (2a) was converted into (*S*)-4-methyl-

$\epsilon$ -caprolactone (*S*-2b) in full conversion and excellent enantioselectivity (Table 3, entries 4–6). Cyclopentanone (3a) and cyclobutanone (4a) were also tested as substrates, but unfortunately they were not converted at all (Table 3, entries 7–12). This was quite surprising, as the native *Tm*CHMO has been reported to be active with both substrates [10]. The only reported difference compared to substrate 1a was that 3a and 4a showed some substrate inhibition. Perhaps the fusion gives an even stronger substrate inhibition, or somehow the active site is slightly altered through having a fusion partner, disabling *Tm*CHMO to accept 3a or 4a.

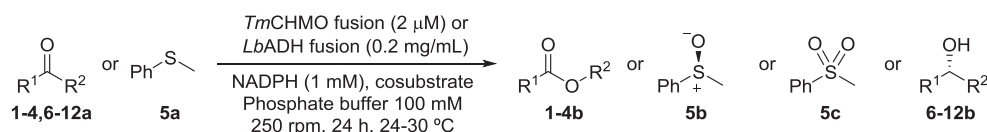
Looking for a further exploitation of these enzyme fusions, thioanisole (5a) was tested as substrate (Table 3, entries 13–15). Mixtures of enantiopure methyl phenyl sulfoxide (5b) and the corresponding sulfone (5c) were obtained, as was observed in a recent study using another robust BVMO [30]. A time-point study performed with the G-B confirmed the early formation of 5b and its subsequent oxidation to 5c (see Supporting Information, Section 2.5).

Keeping in mind previous studies on ketone reductions with ADHs [23], some acetophenone derivatives were chosen as alternative substrates for the *Lb*ADH fusions (substrates 7–12a, Table 3). The lengthening of chain  $R^2$  from a methyl group (acetophenone, 6a; Table 3, entries 16 and 17) to an ethyl group (propiophenone, 7a; Table 3, entries 18 and 19) caused a loss of activity, although the stereoselectivity remained perfect. As expected [23], when bioreductions were set up using butyrophenone (8a; Table 3, entries 20 and 21) or 2-methoxy-1-phenylethanol (9a; Table 3, entries 22 and 23) as substrates, a dramatic loss of activity was observed. At this point, we decided to evaluate the influence of the aromatic pattern substitution at different positions. For this purpose, we studied various chlorine-substituted acetophenone derivatives (10–12a). *Bs*FDH fusion behaved reasonably well with *p*-chloroacetophenone (10a; Table 3, entry 24) and *m*-chloroacetophenone (11a; Table 3, entry 26) as substrates. On the contrary, although *Ps*PTDH construct maintained excellent selectivity, it afforded low conversions for the same substrates (Table 3, entries 25 and 27). Finally, the biotransformation of *o*-chloroacetophenone (12a) catalyzed by the *Bs*FDH fusion was set up leading to low conversions (Table 3, entry 28).



**Fig. 3.** Panel of substrates studied in this work. Substrate **5a** for *Tm*CHMO can be oxidized to methyl phenyl sulfoxide (**5b**) or to (methylsulfonyl)benzene (**5c**).

**Table 3**  
Substrate scope of the engineered self-sufficient *Tm*CHMO and *Lb*ADH fusion biocatalysts.



Entry	Fusion	Substrate	Conv. (%) <sup>a</sup>	ee (%) <sup>a,b</sup>
1	G-B	<b>1a</b>	>99	–
2	F-B	<b>1a</b>	>99	–
3	P-B	<b>1a</b>	>99	–
4	G-B	<b>2a</b>	>99	>99 (S)
5	F-B	<b>2a</b>	>99	>99 (S)
6	P-B	<b>2a</b>	>99	>99 (S)
7	G-B	<b>3a</b>	<1	–
8	F-B	<b>3a</b>	<1	–
9	P-B	<b>3a</b>	<1	–
10	G-B	<b>4a</b>	<1	–
11	F-B	<b>4a</b>	<1	–
12	P-B	<b>4a</b>	<1	–
13	G-B	<b>5a</b>	75% <b>5b</b> + 21% <b>5c</b>	>99 [(R)- <b>5b</b> ]
14	F-B	<b>5a</b>	26% <b>5b</b> + 74% <b>5c</b>	>99 [(R)- <b>5b</b> ]
15	P-B	<b>5a</b>	51% <b>5b</b> + 47% <b>5c</b>	>99 [(R)- <b>5b</b> ]
16	F-A	<b>6a</b>	>99	>99 (R)
17	P-A	<b>6a</b>	>99	>99 (R)
18	F-A	<b>7a</b>	85	>99 (R)
19	P-A	<b>7a</b>	55	>99 (R)
20	F-A	<b>8a</b>	<1	n.d.
21	P-A	<b>8a</b>	<1	n.d.
22	F-A	<b>9a</b>	10	>99 (S) <sup>c</sup>
23	P-A	<b>9a</b>	2	n.d.
24	F-A	<b>10a</b>	90	>99 (R)
25	P-A	<b>10a</b>	10	>99 (R)
26	F-A	<b>11a</b>	88	>99 (R)
27	P-A	<b>11a</b>	15	>99 (R)
28	F-A	<b>12a</b>	16	>99 (R)

<sup>a</sup> Determined by GC analysis.

<sup>b</sup> Enantiomeric excess values of product **5b** were determined by chiral HPLC analysis.

<sup>c</sup> Change in Cahn-Ingold-Prelog priority. n.d. not determined.

## 2.6. Scale-up biotransformations

At this point, we aimed to show the applicability of the enzyme fusions by scaling-up some selected biotransformations. Before that, the effect of the substrate concentration and the amount of biocatalyst were studied (Supporting information, Section 2.6) in order to identify the best conditions for each preparation. Once optimized, several semi-preparative biotransformations were set up using the best conditions for each fusion without observing the formation of any by-product. Hence, for P-B 25 mg of cyclohexanone (10 mM) were transformed into caprolactone at pH 8.5 with full conversion but in moderate isolated yield (51%) after

purification by extraction and centrifugation of the combined organic phases. Suspecting that part of the caprolactone had been hydrolyzed, the biotransformation was set up using a phosphate buffer pH 7.5. In this case, it was possible to obtain the caprolactone in high isolated yield (90%). Afterwards, the same Baeyer-Villiger oxidation were performed using F-B and G-B. Again, full conversions and high isolated yields (89% and 80%, respectively) were attained for caprolactone after an extraction protocol. The *Lb*ADH constructs were also tested in semi-preparative biotransformations. However, F-A revealed worse results than those obtained at small scale. Using 15 mg of acetophenone (20 mM), it was transformed into (*R*)-1-phenylethanol in 65% conversion. After

separation of the remaining ketone, the desired enantiopure alcohol was obtained in 51% isolated yield. On the other hand, the result obtained in the same bioreduction catalyzed by P-A was as good as that obtained at small scale, and the corresponding alcohol was produced in full conversion, with excellent enantioselectivity and high isolated yield (85%).

### 3. Conclusions

In this study we have developed and investigated three NADPH-regenerating fusion partners with two different enzyme systems: a BVMO and an ADH. With the exception of one fusion construct (StGDH with *LbADH*), all fusion enzymes resulted in good soluble expression as well as fully functional as self-sufficient biocatalysts. The fusion biocatalysts displayed tolerance to various organic cosolvents, including DES. From the different fusion partners, FDH was the most tolerant to organic solvents, fully converting cyclohexanone for the BVMO fusion at 15% v/v MTBE, and acetophenone for the ADH fusion even at 40% v/v MTBE. We also demonstrate that the fusion enzymes can be used as enantioselective biocatalysts for a large variety of reactions by retaining almost all catalytic characteristics of the native non-fused enzymes. We demonstrated the applicability of three different NADPH regenerating fusions for these two enzyme systems, which broadens the choice of regenerating enzymes for future applications of these systems.

### 4. Experimental section

#### 4.1. General materials and methods

Oligonucleotide primers were ordered from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). T4 ligase and restriction enzyme *BsaI* were ordered from New England Biolabs. The PfuUltra Hotstart PCR master mix was purchased from Agilent Technologies. *Escherichia coli* NEB<sup>®</sup> 10-beta (New England Biolabs) chemically competent cells were used as host for cloning of the recombinant plasmids, and as host for protein expression.

All acetophenones, cyclohexanones, cyclopentanone, cyclobutanone and thioanisole were purchased from Sigma-Aldrich-Fluka. NADPH as enzyme cofactor and all the other chemical reagents were obtained with the highest quality available from Sigma-Aldrich-Fluka (Steinheim, Germany).

NMR spectra were recorded on a Bruker AV300 MHz spectrometer (Bruker Co., Faellanden, Switzerland). All chemical shifts ( $\delta$ ) are given in parts per million (ppm) and referenced to the residual solvent signal as internal standard.

Gas chromatography (GC) and high performance liquid chromatography (HPLC) analyses were performed for conversion and enantiomeric excess measurements. GC analyses were performed on an Agilent HP6820 GC chromatograph equipped with a FID detector. HPLC analyses were carried out in a Hewlett Packard 1100 chromatograph UV detector at 210 nm. For more details, see Section 1 in the Supporting Information. Thin-layer chromatography (TLC) was conducted with Merck Silica Gel 60 F254 precoated plates and visualized with UV and potassium permanganate stain. Column chromatography was performed using Merck Silica Gel 60 (230–400 mesh).

#### 4.2. Cloning and purification

Cloning of fusion constructs: Three fusion constructs with the *lbadh* gene were cloned into a pBAD vector through the Golden Gate method. The pBAD vector contains a region coding for an N-terminal His-tag (6xHis) upstream from the first *BsaI* restriction site, an AraC promoter, and an ampicillin-resistance gene (*bla*).

Primers were designed to have a non-overlapping region coding for the *BsaI* restriction site (GGTCTCNNNNNN), such that the vector and PCR product have a four base-pair overlap after digestion with *BsaI*. These primers were used for PCR to amplify the *lbadh* gene (accession: Q84EX5) from an in-house plasmid, *fdh* (accession: EU825923.1) and *gdh* (accession: Q96ZY7) synthetic codon-optimized genes were ordered from Genscript (NJ, USA), and *ptdh* x12 mutant was also cloned from an in-house plasmid. Primers were designed such that the *lbadh* would be the second gene of the fusion; at the C-terminal side of the fusion enzyme. In addition to the *BsaI* sites, an additional 'linker' region was added to the reverse primers of the first gene and the forward primer of the second gene of a construct. These introduced 'linker' regions together code for a short peptide linker (SGSAAG), after ligation of the PCR products.

The fusion constructs were produced by incubating together: two PCR products (*lbadh* gene and either *fdh*, *gdh* or *ptdh*), pBAD vector containing *BsaI* sites, *BsaI* restriction enzyme, T4 ligase, ligation buffer, and sterile miliQ water (total volume of 20  $\mu$ L). The incubation temperature alternated between 16 °C (for 5 min) and 37 °C (for 10 min) for 30 cycles, then was set to 55 °C for 10 min, and finally to 80 °C for 20 min to inactivate the enzymes. To transform host cells with the fusion constructs, 3  $\mu$ L of the Golden Gate reaction was added to chemically competent *E. coli* cells, and a heat shock was applied at 42 °C for 30 s. After overnight growth on a lysogeny broth (LB) agar plate with ampicillin, colonies were picked, grown in liquid LB with appropriate antibiotic, and the plasmids were isolated and sent for sequencing (GATC, Germany) to confirm correct ligation of the genes. For the three constructs with *TmCHMO*, traditional restriction-digestion cloning was used.

All enzymes were expressed using NEB 10- $\beta$  *Escherichia coli* cells. The freshly-prepared strains were cultivated in TB medium supplemented with 50  $\mu$ g/mL ampicillin at 37 °C. Protein expression was induced by adding L-arabinose (0.02% w/v) when A600 reached 0.5. G-B and F-B were incubated overnight at 30 °C, P-A and P-B were incubated at 24 °C for 48 h and G-A and F-A were incubated at 17 °C for 72 h. After this time, the cells were harvested by centrifugation.

The cells were resuspended in 20 mM phosphate buffer pH 7.5 (*TmCHMO* fusions were implemented with FAD) and lysed in an iced bath by ultrasonication (cycles of 20s on/20s off for 7 min). After centrifugation, the supernatant was used either for protein purification using Ni-sepharose material or as cell-free extract preparations for biotransformations.

For lyophilization purposes, the cells obtained after expression were resuspended in the minimum amount of 20 mM phosphate buffer pH 7.5 and lyophilized using an Alpha 2–4 DLplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

#### 4.3. Conversion of cyclohexanone and derivatives, cyclopentanone, cyclobutanone and thioanisole using self-sufficient *TmCHMOs*

Purified self-sufficient *TmCHMOs* (2  $\mu$ M or 4  $\mu$ M) were employed for biotransformation of cyclohexanone (5–25 mM), 4-methylcyclohexanone (5 mM), cyclopentanone (5 mM), cyclobutanone (5 mM) and thioanisole (5 mM) at 30 °C (F-B and P-B) or 24 °C (G-B) and 250 rpm. All reactions contained 1 or 0.5 mmol NADPH, the co-substrate (10 mM glucose for G-B, 50 mM sodium formate for F-B and 20 mM sodium phosphite for P-B) and 100 mM phosphate buffer pH 8.5. Additionally, G-B fusion needs the external addition of magnesium chloride (1 mM). In some cases, a cosolvent (1% v/v 1,4-dioxane, 1% v/v acetonitrile, 1% v/v ethanol, 1% v/v methanol, 1–50% v/v *tert*-butyl methyl ether) or ChCl:Gly (1:2 mol/mol) was added. In any case, the final volume of the reaction was adjusted up to 0.5 mL in a 2 mL eppendorf tube.

For cell-free extract biotransformations, 5 mM cyclohexanone

was used as substrate and the reaction was implemented with the corresponding co-substrate (10 mM glucose for G-B, 50 mM sodium formate for F-B and 20 mM sodium phosphite for P-B) and 1 mM of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  when G-B was employed. Cell-free extract preparations were used at 8 mg/mL concentration and the final volume was adjusted with 100 mM phosphate buffer pH 8.5 up to a 0.5 mL in a 2 mL eppendorf tube. The reactions were incubated at 30 °C (F-B and P-B) or 24 °C (G-B) and 250 rpm for 24 h.

When using lyophilized preparations (10 mg), cyclohexanone (5 mM) was used as substrate and reactions were implemented with NADPH (1 mM), the corresponding co-substrate (10 mM glucose for G-B, 50 mM sodium formate for F-B and 20 mM sodium phosphite for P-B) and 1 mM of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  when G-B was employed. The final volume was adjusted up to 0.5 mL with 100 mM phosphate buffer pH 8.5 in a 2 mL eppendorf tube and the reactions were incubated at 30 °C (F-B and P-B) or 24 °C (G-B) and 250 rpm for 24 h.

After incubation, all reactions were extracted with ethyl acetate ( $2 \times 0.2$  mL) and the organic layers were combined and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The results were analyzed using GC and/or HPLC.

#### 4.4. Preparative biotransformation of cyclohexanone into caprolactone using TmCHMO fusions

G-B (2  $\mu\text{M}$ ) was employed to convert 30 mg of cyclohexanone (5 mM) into caprolactone. The reaction media was composed of 100 mM phosphate buffer pH 8.5 implemented with NADPH (1 mM), glucose (10 mM) and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 mM). The reaction was set up in a 50 mL Falcon tube and incubated at 24 °C and 250 rpm for 24 h. After this time, the mixture was extracted with ethyl acetate ( $3 \times 20$  mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Conversion was determined by GC. Caprolactone was obtained in 80% isolated yield (>99% conv.).

F-B (4  $\mu\text{M}$ ) was used to convert 30 mg of cyclohexanone (15 mM) into caprolactone in 100 mM phosphate buffer pH 8.5 in a 50 mL Falcon tube. The reaction was implemented with NADPH (1 mM) and sodium formate (50 mM) and incubated at 30 °C for 24 h. Afterwards, the mixture was extracted with ethyl acetate ( $3 \times 20$  mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Conversion was determined by GC. Caprolactone was obtained in 89% isolated yield (>99% conv.).

P-B (2  $\mu\text{M}$ ) was employed to convert 30 mg of cyclohexanone (10 mM) into caprolactone. The reaction media (100 mM phosphate buffer pH 7.5) was implemented with NADPH (1 mM) and sodium phosphite (20 mM) and the reaction was incubated at 30 °C for 24 h. After this time, the mixture was extracted with ethyl acetate ( $3 \times 20$  mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Conversion was determined by GC. Caprolactone was obtained in 90% isolated yield (>99% conv.).

#### 4.5. General procedures for conversion of acetophenone and derivatives using self-sufficient LbADHs

Purified self-sufficient LbADHs (0.2 mg/mL or 4 mg/mL) were employed for biotransformation of acetophenone (5–25 mM) and derivatives (5 mM) at 30 °C and 250 rpm. All reactions contained 1 or 0.5 mmol NADPH, the co-substrate (10 mM glucose for G-A, 50 mM sodium formate for F-A and 20 mM sodium phosphite for P-A),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 mM) and 100 mM phosphate buffer pH 7.5. In some cases, a cosolvent (1% v/v 1,4-dioxane, 1% v/v acetonitrile, 1% v/v ethanol, 1% v/v methanol, 1–50% v/v *tert*-butyl methyl ether),

ChCl:Gly (1:2 mol/mol) or ChCl:Glu (1.5:1 mol/mol) was added. In any case, the final volume of the reaction was adjusted up to 0.5 mL in a 1.5 mL eppendorf tube.

For cell-free extract biotransformations, 5 mM acetophenone was used as substrate and the reaction was implemented with the corresponding co-substrate (10 mM glucose for G-A, 50 mM sodium formate for F-A and 20 mM sodium phosphite for P-A) and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 mM). Cell-free extract preparations were used at 8 mg/mL concentration and the final volume was adjusted with 100 mM phosphate buffer pH 7.5 up to a 0.5 mL in a 1.5 mL eppendorf tube. The reactions were incubated at 30 °C and 250 rpm for 24 h.

When using lyophilized preparations (10 mg), acetophenone (5 mM) was used as substrate and reactions were implemented with NADPH (1 mM), the corresponding co-substrate (10 mM glucose for G-A, 50 mM sodium formate for F-A and 20 mM sodium phosphite for P-A) and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 mM). The final volume was adjusted up to 0.5 mL with 100 mM phosphate buffer pH 7.5 in a 1.5 mL eppendorf tube and the reactions were incubated at 30 °C and 250 rpm for 24 h.

After incubation, all reactions were extracted with ethyl acetate ( $2 \times 0.2$  mL) and the organic layers were combined and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The results were analyzed using GC and/or HPLC.

#### 4.6. Preparative biotransformation of acetophenone into (*R*)-1-phenylethanol using LbADH fusions

F-A (0.4 mg/mL) was used to convert 30 mg of acetophenone (20 mM) into (*R*)-1-phenylethanol. The reaction media contained NADPH (1 mM), sodium formate (50 mM),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 mM) and 100 mM phosphate buffer pH 7.5. This mixture was incubated at 30 °C for 24 h and, afterwards, it was extracted with ethyl acetate ( $3 \times 20$  mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Conversion and enantiomeric excess were determined by GC. (*R*)-1-phenylethanol was obtained in 51% isolated yield (65% conv.) after purification by column chromatography (Hex: EtOAc 3:1).

P-A (0.2 mg/mL) was employed to convert 30 mg of acetophenone (10 mM) into (*R*)-1-phenylethanol. The reaction media (100 mM phosphate buffer pH 8.5) was implemented with NADPH (1 mM), sodium phosphite (20 mM) and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 mM) and the reaction was incubated at 30 °C for 24 h. After this time, the mixture was extracted with ethyl acetate ( $3 \times 20$  mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Conversion and enantiomeric excess were determined by GC. (*R*)-1-phenylethanol was obtained in 85% isolated yield (>99% conv.).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tet.2019.02.015>.

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