Supporting Information

More efficient redox biocatalysis by utilising 1,4-butanediol as 'smart cosubstrate'

Selin Kara,[a] Dominik Spickermann,[b] Joerg H. Schrittwieser,[a] Christan Leggewie,[b] Willem J. H. van Berkel,[c] Isabel W. C. E. Arends,[a] and Frank Hollmann*[a]

a Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628BL Delft (The Netherlands)

b evocatal GmbH Merowingerplatz 1A, 40225 Düsseldorf (Germany)

c Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen (The Netherlands)

CONTENTS

1 Materials

HLADH, isoform E recombinantly expressed in *Escherichia coli* is commercially available from evocatal GmbH (Duesseldorf, Germany). *Ts*ER was produced according to literature procedure (Opperman et al., 2008) by recombinant expression in *E. coli* followed by heat-purification. 3HB6H was produced by recombinant expression in *E. coli* following a literature procedure (Montersino and van Berkel, 2012). All chemicals were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands) in the highest purity available and used as received.

2 Experimental details and supporting information

2.1 Estimation of the the conversion achievable as a function of molar cosubstrate surplus

The reversible MPV-reduction can be described by the law of mass action (Equation 1). Using isopropanol as cosubstrate for the reduction of ketones, it is justified to assume an equilibrium constant of 1.

$$\boldsymbol{K = \frac{c(Coproduct)\times c(Product)}{c(Cosubstrate)\times c(Substrate)} = 1}$$ (Equation 1, equilibrium concentrations)

From this Eq.2 can be derived, which relates the conversion to the initial concentrations of substrate and cosubstrate.

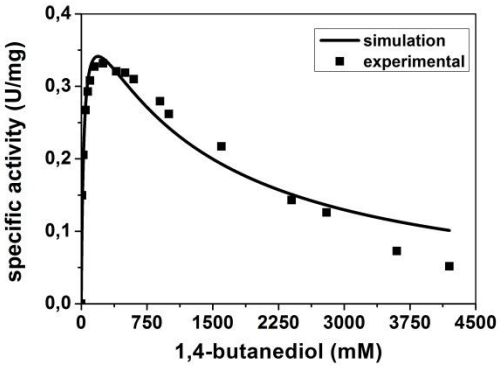
$$\boldsymbol{conversion = \frac{[Cosubstrate]0}{[Cosubstrate)0+[Substrate]0}}$$ (Equation 2)

All calculations shown in Scheme 1 were done according to these eqations.

2.2 Kinetic Analysis of HLADH-Catalysed Oxidation of 1,4-Butanediol

Stock solutions of 1,4-butanediol (1,4-BD, 5 M), NAD^+ (25 mM) and HLADH (12 gL^{-1}) were freshly prepared in 50 mM Tris-HCl buffer at pH 7. Reaction mixture containing reaction buffer (V µL), 1,4-butanediol stock (970 µL – V µL) and NAD^+ stock (20 µL) was incubated at 30 °C for 3 min. Reactions were started by the addition of the HLADH stock (10 µL). Formation of NADH was followed by UV/Vis spectroscopy and quantified using an absorption coefficient of 6.22 mM^{-1} cm^{-1}. The starting concentrations of NAD^+ and HLADH were 0.5 mM and 0.12 gL^{-1}, respectively. The following graph (Fig. S1) shows the kinetic behavior of HLADH for 1,4-butanediol oxidation. The simulation was performed using MATLAB® 2010 based on Michaelis-Menten double substrate kinetics. In case of excess substrate inhibition the following equation was used:

$$V = \frac{V_{\max} \times c(1,4-\text{butanediol}) \times c(\text{NAD}^+)}{\left((K_\text{M}1,4-\text{butanediol} + c(1,4-\text{butanediol}) \times \left(1 + \frac{c(1,4-\text{butanediol})}{K_i 1,4-\text{butanediol}}\right)\right) \times (K_\text{M}\text{NAD}^+ + c(\text{NAD}^+))}$$



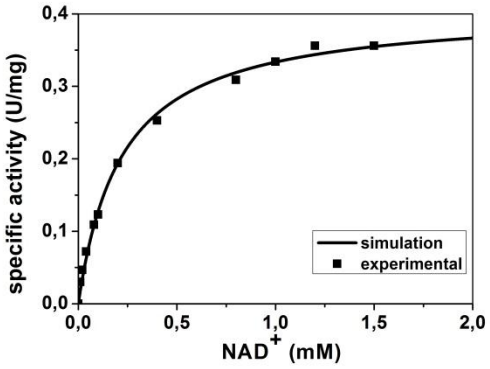


Fig. S1 Kinetic analysis of oxidation of 1,4-butanediol catalysed by HLADH. Left: Reaction conditions: 0–4200 mM 1,4-butanediol, 0.5 mM NAD^+, 0.12 gL^{-1} enzyme, 50 mM Tris-HCl at pH 7 and 30 °C. $K_{M,1,4\text{-BD}}$= 23.3 ± 8.8 mM, V_{max} = 0.34 ± 0.01 Umg^{-1}, K_i/K_M = 57. Right: Reaction conditions: 0–1.5 mM NAD^+, 150 mM 1,4-BD, 0.12 gL^{-1} enzyme, 50 mM Tris-HCl at pH 7 and 30 °C. $K_{M,NAD+}$ = 0.16 ± 0.04 mM, V_{max} = 0.35 ± 0.03 Umg^{-1}.

Similarly the kinetic properties for ethanol (EtOH) and isopropanol (^{i}PrOH) were determined (Table S1).

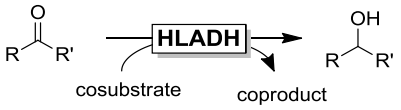
Table S1 Summary of the kinetic parameters of some selected cosubstrates with HLADH.

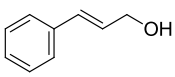
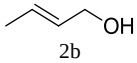
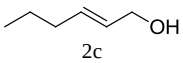
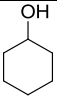
Cosubstrate	V_{max} [Umg^{-1}]	K_M [mM]	K_i/K_M [-]
1,4-BD	0.34 ± 0.001	23.3 ± 8.8	57
EtOH	0.26 ± 0.03	5.8 ± 0.5	n.d.
^{i}PrOH	0.03 ± 0.001	261.1 ± 51.0	n.d.

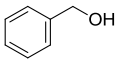
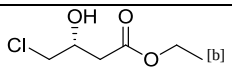
2.3 HLADH-Catalysed Reductions with Cofactor Regeneration

Due to the poor solubility of some of the applied substrates (1c, 1e and 1f), stock solutions (0.5 M) were prepared in acetonitrile (MeCN). The final MeCN concentration was kept at maximum 1% (v/v). There was no negative effect on the enzyme activity with this amount of MeCN (data not shown). Cosubstrate stocks (30 mM each), NAD^+ stock (15 mM) and HLADH stock (5 gL^{-1}) were freshly prepared in 50 mM Tris-HCl buffer at pH 7. The mixture of substrate stock (15 µL), cosubstrate stock (125 µL), NAD^+ stock (10 µL), buffer (1200 µL) was incubated at 30 °C for 3 min. Biotransformations were initiated with the HLADH solution (150 µL) and the reaction mixtures (1.5 mL) were efficiently shaken (700 rpm) in screw-capped glass vials at 30 °C. Aliquots (50 µL) were removed at intervals and mixed with 200 µL of ethyl acetate (EtOAc). The mixture was vortexed for 20 sec, followed by centrifugation (16,100 × g, 0.5 min). The clear organic phase was removed and dried over anhydrous $MgSO_4$. Dodecane (5 mM) was used as an external standard in EtOAc and the product formation was assessed using GC (see 3. Analytics). For all substrates we ran negative control experiments whereby no HLADH was added. No formation of reduction product or γ-butyrolactone (GBL) was detected in the negative controls. Following table summarises the maximum conversions achieved using either isopropanol (^{i}PrOH), ethanol (EtOH) or 1,4-butanediol (1,4-BD) as cosubstrate.

Table S2 Evaluation of isopropanol (^{i}PrOH), ethanol (EtOH) and 1,4-butanediol (1,4-BD) as cosubstrates for the HLADH-catalysed reductions.



Entry	Product	Cosubstrate	Conversion [%]
1	 2a	^{i}PrOH	17.8 ± 0.5
		^{i}PrOH[a]	38.6 ± 1.6
		EtOH	24.4 ± 1.9
		1,4-BD	58.6 ± 2.8
2	 2b	^{i}PrOH	0
		EtOH	5.3 ± 0.5
		1,4-BD	44.2 ± 2.6
3	 2c	^{i}PrOH	3.5 ± 0.2
		EtOH	6.0 ± 0.3
		BDO	48.8 ± 2.3
4		^{i}PrOH	30.1 ± 2.1
		EtOH	44.6 ± 1.7

	2d	1,4-BD	73.0 ± 2.3
5	 2e	^{i}PrOH	25.9 ± 2.2
		EtOH	47.5 ± 0.2
		1,4-BD	97.7 ± 1.7
6	 2f	EtOH	12.9 ± 1.1
		EtOH[c]	17.0 ± 0.8
		1,4-BD	44.1 ± 0.6

Conditions: *c*(substrate) = 5 mM, *c*(cosubstrate) = 2.5 mM, *c*(NAD^+) = 0.1 mM, *c*(HLADH) = 0.5 gL^{-1}, buffer: Tris-HCl (50 mM, pH 7.0), *T* = 30°C, reaction time 96 h. In case of poorly water-soluble substrates 1% (v/v) acetonitrile was added. Entry 1 *c*(HLADH) = 1 gL^{-1}. [a] 5 eq of isopropanol, [b] *ee*>98% (*R*), [c] 1 eq of ethanol.

2.4 Analysis of Product Inhibition for the Reduction of *Trans*-Cinnamaldehyde

Since in most of cases full conversion to the reduction product could not be observed, we examined a potential product inhibition. Stock solutions of *trans*-cinnamaldehyde (0.5 M) and *trans*-cinnamyl alcohol (0.5 M) were prepared in DMSO. Stocks of NADH (5 mM) and HLADH (12 gL^{-1}) were freshly prepared in 50 mM Tris-HCl buffer at pH 7. The mixture containing reaction buffer (960-V µL), *trans*-cinnamaldehyde stock (10 µL), NADH stock (20 µL) and *trans*-cinnamyl alcohol (V µL) was incubated at 30 °C for 3 min. Reactions were started by the addition of the HLADH stock (10 µL). Depletion of NADH was followed by UV/Vis spectroscopy and quantified using an absorption coefficient of 6.22 mM^{-1} cm^{-1}. Maximum 2% (v/v) of DMSO was applied whereby no negative effect of DMSO at this concentration was detected (data not shown). The following graph (Fig. S2) shows decrease in the reduction activity of HLADH towards *trans*-cinnamaldehyde in the presence of *trans*-cinnamyl alcohol. Our experiments showed a severe product inhibition: loss of 75% of activity in the presence in the presence of 0.2 eq alcohol (approx. corresponding to 20% conversion).

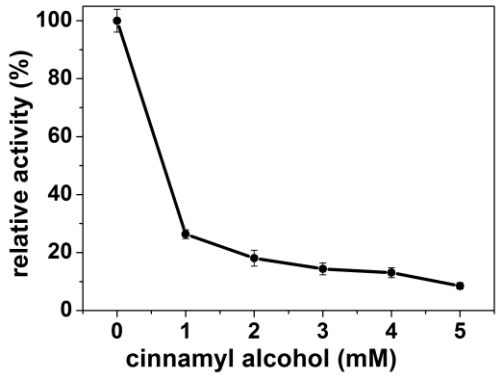


Fig. S2 Reduction of *trans*-cinnamaldehyde in the presence of *trans*-cinnamyl alcohol (0–5 mM). Reaction conditions: 5 mM *trans*-cinnamaldehyde, 0.1 mM NADH, 0.12 gL^{-1} enyzme, 50 mM Tris-HCl at pH 7 and 30 °C.

2.5 HLADH-Catalysed Reduction of 2-Phenyl-1-Propanal

2-Phenyl-1-propanal stock (0.5 M) was prepared in MeCN. Cosubstrate stocks (30 mM each), NAD^+ stock (15 mM) and HLADH stock (0.1 gL^{-1}) were freshly prepared in 50 mM Tris-HCl buffer at pH 7. The mixture of 2-phenyl-1-propanal stock (15 µL), cosubstrate stock (125 µL), NAD^+ stock (10 µL), reaction buffer (1200 µL) was incubated at 30°C for 3 min. Reactions were started by the addition of HLADH solution (150 µL, 0.1 gL^{-1}) and were incubated at 30 °C in screw-capped glass vials by effectively shaking (700 rpm). Aliquot samples (150 µL) were removed at intervals and mixed with 50 µL of TFA (10%) and 400 µL of *n*-heptane. The mixture was vortexed for 45 sec, followed by centrifugation (16,100 × g, 1 min). The clear organic phase was separated and dried over anhydrous $MgSO_4$. Conversion of 2-phenyl-1-propanal and the formation of (*R*/*S*)-2-phenyl-1-propanol were monitored by HPLC. Formation of GBL was followed by GC analysis (see 3. Analytics, Table S2).

2.6 *Ts*ER-Catalysed Reduction of Ketoisophorone

A ketoisophorone stock (KIP, 1M) stock was prepared in MeCN, whereas NAD^+ stock (15 mM), HLADH stock (10 gL^{-1}) and cosubstrate stocks (EtOH or 1,4-BD, 50 mM) were freshly prepared in 50 mM MOPS buffer, 5 mM $CaCl_2$, at pH 7. The mixture of KIP stock (15 µL), cosubstrate stock (150 µL), NAD^+ stock (100 µL) and buffer (710 µL) was incubated at 30 °C for 3 min. Reactions were started by the addition of *Ts*ER (375 µL, 1 gL^{-1}) and HLADH solution (150 µL, 10 gL^{-1}) and were incubated at 30°C in screw-capped glass vials by shaking (800 rpm). The reaction conditions were: 10 mM KIP, 5 mM cosubstrate, 1 mM NAD^+, 0.25 gL^{-1} *Ts*ER and 1 gL^{-1} HLADH in 1.5 mL. Aliquot samples (50 µL) were removed at intervals and mixed with 200 µL of EtOAc containing 5 mM dodecane. The mixture was vortexed for 20 sec, followed by centrifugation (16,100 × g, 1 min). The clear organic phase was separated and dried over anhydrous $MgSO_4$. The consumption of KIP and the formation of (*R*/*S*)-levodione were monitored by GC (see 3. Analytics, Table S3).

2.7 3HB6H-Catalysed Hydroxylation of 3-Hydroxybenzoate

The stocks 3-hydroxybenzoate (3HB, 10 mM), cosubstrates (EtOH, ^{i}PrOH or 1,4-BD, 30 mM), NAD^+ (15 mM), HLADH (10 gL^{-1}) were prepared in 20 mM Tris-SO_4 buffer at pH 8.0. The mixture containing 3HB stock (750 µL), cosubstrate stock (125 µL), NAD^+ stock (100 µL) and the reaction buffer (350 µL) were incubated at 30 °C for 3 min. The reactions were started with the addition of 3HB6H (25 µL) and HLADH (150 µL) and incubated at 30 °C and 800 rpm in screw-capped vials. The reaction conditions were: 5 mM 3HB, 2.5 mM cosubstrate, 1 mM NAD^+, 0.25 gL^{-1} 3HB6H and 1 gL^{-1} HLADH in 1.5 mL. Aliquot samples (100 µL) were removed at intervals and mixed with 100 µL of MeCN. The mixture was vortexed for 30 sec, followed by centrifugation (16,100 × g, 1 min). The consumption of 3HB and the formation of 2,5-dihydroxybenzoate (2,5-DHB) were monitored by HPLC (see 3. Analytics, Table S3).

2.8 Hydroxylation of 3-Hydroxybenzoate at 50 mM-Scale

The stocks of 1,4-BD (250 mM), NAD^+ (20 mM) and HLADH (12.5 gL^{-1}) were freshly prepared in 100 mM Tris-SO_4 at pH 8. First, 550 µL of reaction buffer (100 mM Tris-SO_4, pH 8) was given to 3HB (6.9 mg, 50 µmol) and pH was readjusted to 8 using 4M of NaOH (~10 µL was needed). After that, 100 µL of NAD^+ was added and reaction mixture was incubated at 30 °C for 3 min. The reactions were started with the addition of 3HB6H (50 µL, 15 gL^{-1}) and HLADH (200 µL, 12.5 gL^{-1}) and incubated at 30 °C and 800 rpm in screw-capped vials. The reaction conditions were: 50 mM 3HB, 25 mM 1,4-BD, 2 mM NAD^+, 0.75 gL^{-1} 3HB6H and 2.5 gL^{-1} HLADH in 1.0 mL. Aliquot samples (10 µL) were removed at intervals and mixed with 190 µL of MeCN. The mixture was vortexed for 30 sec, followed by centrifugation (16,100 × g, 3 min). The consumption of 3HB and the formation of 2,5-DHB were monitored by HPLC (see 3. Analytics, Table S4). The formation of 2,5-DHB is shown in the following graph.

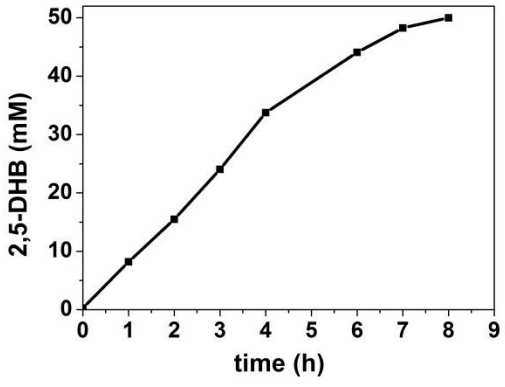
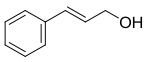
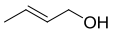
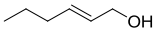
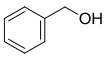
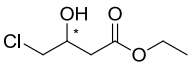
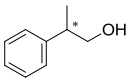


Fig. S3 Hydroxylation of 3-hydroxybenzoate (3HB) to 2,5-dihydroxybenzoate (2,5-DHB). Reaction conditions: *c*(3-hydroxybenzoate) = 50 mM, *c*(1,4-BD) = 25 mM, *c*(NAD^+) = 2 mM, *c*(3HB6H) = 0.75 gL^{-1}, *c*(HLADH) = 2.5 gL^{-1}, buffer: Tris-SO_4 (100 mM, pH 8), T = 30°C.

3 Analytics

The reaction progress and the optical purity of the products were determined using GC or HPLC.

Table S3 Details for the analytics used in ADH-catalysed reductions.

Entry	Product	Analysis – Column	Temperature Profile			t_R [min]
1	 2a	GC[a] CP-Wax 52 CB, 50 m × 0.53 mm × 2 µm	R [°C/min]	T [°C]	H [min]	Substrate = 9.9 Product = 11.1 γ-Butyrolactone = 6.1 1,4-Butanediol = 8.4
			-	60	1.0	
			50	150	2.2	
			50	160	1.5	
			50	190	2.0	
			50	250	1.0	
2	 2b	GC[a] CP-Wax 52 CB, 50 m × 0.53 mm × 2 µm	R [°C/min]	T [°C]	H [min]	Substrate = 5.6 Product = 8.6 γ-Butyrolactone = 12.0 1,4-Butanediol = 12.8
			-	50	5.0	
			40	85	3.0	
			40	250	1.0	
3	 2c	GC[a] CP-Wax 52 CB, 50 m × 0.53 mm × 2 µm	R [°C/min]	T [°C]	H [min]	Substrate = 4.3 Product = 6.3 γ-Butyrolactone = 11.2 1,4-Butanediol = 12.8
			-	80	3.0	
			40	120	6.0	
			40	250	1.0	
4	 2d	GC[a] CP-Wax 52 CB, 50 m × 0.53 mm × 2 µm	R [°C/min]	T [°C]	H [min]	Substrate = 5.1 Product = 6.3 γ-Butyrolactone = 9.7 1,4-Butanediol = 10.9
			-	80	3.0	
			40	120	4.0	
			40	250	1.0	
5	 2e	GC[b] CP-Wax 52 CB, 50 m × 0.53 mm × 2 µm	R [°C/min]	T [°C]	H [min]	Substrate = 15.3 Product = 18.5 γ-Butyrolactone = 16.3 1,4-Butanediol = 18.8
			-	55	3.0	
			25	60	7.0	
			20	160	2.0	
			25	250	3.0	
6	 2f	GC[c] CP-Chiralsil dex CB, 25 m × 0.32 mm × 0.25 µm	R [°C/min]	T [°C]	H [min]	Substrate = 11.2 (*R*)-product = 15.1 (*S*)-product = 15.4 γ-Butyrolactone = 7.1 1,4-Butanediol = 12.9
			-	100	5.0	
			20	130	3.0	
			20	140	9.0	
			50	220	4.0	
		Derivatisation of the chiral hydroxy-ester product (2f): Reaction sample was extracted with EtOAc (700 µL) and incubated with 4-dimethylaminopyridine (3 gL^{-1} in EtOAc, 300 µL) and acetic anhydride (50 µL) for 90 min. After incubation, 300 µL NaOH (4 M) was added and vigorously mixed. The organic layer was then removed and 300 µL HCl (6 M) was given to this seperated phase. The mixture was vortexed; the organic phase was separated and dried over anhydrous $MgSO_4$ for GC analysis.				
7	 2g	HPLC[d] Chiralpak AD-H, 0.46 cm × 25 cm	R [°C/min]	T [°C]	H [min]	Substrate = 6.3 (*S*)-product = 19.5 (*R*)-product = 22.2
			-	40	25	
		GC[e] CB-Sil 5 CB, 50 m × 0.53 mm × 1 µm	R [°C/min]	T [°C]	H [min]	Substrate = 5.5 Product = 7.0 γ-Butyrolactone = 2.6 1,4-Butanediol = 3.2
			-	80	3.0	
			40	105	3.0	
			40	160	3.0	
			40	335	1.0	

[a] Entry 1 to 4: Total flow = 18 $mLmin^{-1}$, N_2 as carrier gas.
[b] Entry 5: Total flow = 20 $mLmin^{-1}$, N_2 as carrier.
[c] Entry 6: Pressure = 39.7 kPa, total flow = 24.4 $mLmin^{-1}$, column flow = 1.02 $mLmin^{-1}$, linear velocity = 22 cms^{-1}, split = 20, He as carrier gas.
[d] Entry 7: (HPLC analysis) Column flow = 1.0 $mLmin^{-1}$, detection at 210 nm. Eluent = heptane:^{i}PrOH:TFA (99:1:0.1), TFA: trifluoroacetic acid.
[e] Entry 7: (GC Analysis) Total flow = 20 $mLmin^{-1}$, N_2 as carrier.
H: hold time (min), R: temperature ramp (°C/min).
Entry 1 to 5: GC equipment: Shimadzu GC-2014
Entry 6: GC equipment: Shimadzu GC-2010 Plus
Entry 7: HPLC equipment: Waters 515 Pump, Waters 717 Plus Autosampler, Shimadzu SPD-10A VP Detector, GC Equipment: Shimadzu GC-2014

Following chromatograms (Fig. S4) show the conversion of substrate *trans*-cinnamaldehyde and the synthesis of product cinnamyl alcohol.

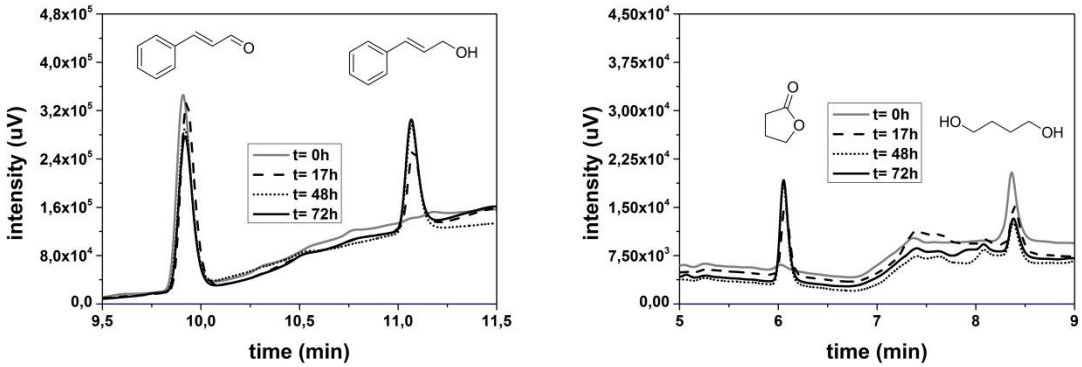
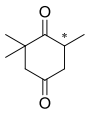
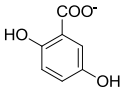


Fig. S4 Left: The GC chromatograms of the reduction of *trans*-cinnamaldehyde (t_R = 9.9 min) and the formation of *trans*-cinnamyl alcohol (t_R = 11.1 min) catalysed by HLADH using 1,4-butanediol as cosubstrate. Right: The GC chromatograms of the oxidation of 1,4-butanediol (t_R = 8.4 min) to γ-butyrolactone (t_R = 6.1 min) catalysed by HLADH.

Table S4 Details for the analytics used in ER- and 3HB6H-catalysed reactions, respectively.

Entry	Product	Analysis – Column	Temperature Profile			t_R [min]
1	 2h	GC[a] Chrialdex GTA, 50 m × 0.25 mm × 0.12 µm	R [°C/min]	T [°C]	H [min]	Substrate = 10.7 (*S*)-Product = 13.9 (*R*)-Product = 14.4 γ-Butyrolactone = 6.4 1,4-Butanediol = 6.8
			-	110	25.0	
			40	170	3.0	
2	 2i	HPLC[b] SpeedROD RP-18E, 4.6 mm × 50 mm	R [°C/min]	T [°C]	H [min]	Substrate = 5.6 Product = 4.6
			-	35	7.5	

[a] Entry 1: Pressure = 128 kPa, total flow = 27.9 mLmin^{-1}, column flow = 0.8 mLmin^{-1}, linear velocity = 20.5 cms^{-1}, split = 30, He as carrier gas.

[b] Entry 2: (HPLC analysis) Column flow = 0.8 mLmin^{-1}, detection at 240 nm. Eluent = MilliQ:MeCN:TFA (95:5:0.1), TFA: trifluoroacetic acid.

H: hold time (min), R: temperature ramp (°C/min).

Entry 1: GC equipment: Shimadzu GC 2010

Entry 2: HPLC Equipment: Shimadzu LC-20AT with SDP-M20A IVDD Detector

The following chromatograms (Fig. S5) show the conversion of ketoisophorone and the formation of (*S*/*R*)-levodione.

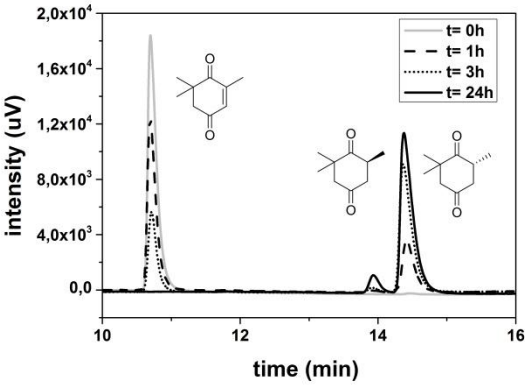


Fig. S5 The GC chromatogram from the course of reaction at time 0, 1h, 3h and 24 h. The hydrogenation of KIP (t_R = 10.7 min) catalyzed by *Ts*ER using 1,4-BD in HLADH-catalyzed NADH regeneration.

Similarly following chromatograms (Fig. S6) represent the conversion of 3HB to 2,5-DHB catalysed by 3HB6H followed by HPLC.

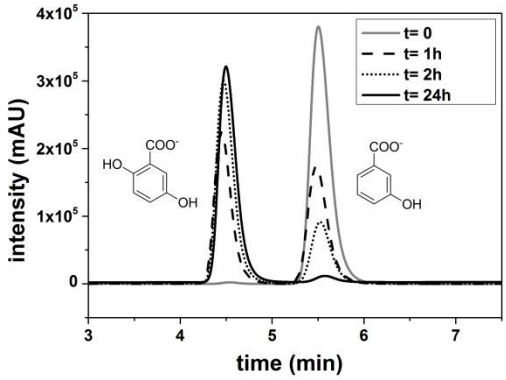


Fig. S6 The HPLC chromatogram from the course of reaction at time 0, 1h, 2h and 24 h. The hydroxylation of 3HB (t_R = 5.6 min) to 2,5-DHB (t_R = 4.6 min) catalyzed by 3HB6H using 1,4-BD in HLADH-catalyzed NADH regeneration.