ENZYMATIC REDUCTION OF KETONES IN 'MICRO-AQUEOUS' MEDIA CATALYZED BY ADH-'A' FROM *RHODOCOCCUS RUBER*

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Supporting Information

Contents

1. General

Ketones **1a**-**8a**, *rac*-alcohols **1b**-**4b**, **7b**, alcohol **6b**, diol **8b** and hydroxyketone **8c** are commercially available either from Sigma-Aldrich-Fluka (Vienna, Austria) or Lancaster (Frankfurt am Main, Germany). Compound **5b** was synthesized by reduction from the corresponding ketone **5a** ($NaBH_4$, MeOH, 5ºC).[1] All other reagents and the solvents employed in the biocatalyzed reductions were of the highest quality available.

TLC plates were run on silica gel Merck 60 F_{254} and visualized by UV or by spraying with a $KMnO_4$ solution.

Preparation of lyophilized cells of *E. coli* Tuner^{TM} (DE3)/pET22b+-ADH-'A' is described in reference 2.

ADH-'A' is commercially available from BioCatalytics Inc.. For the experiments described here we used the following cell-free preparation: *E. coli* Tuner^{TM} (DE3)/pET22b+-ADH-'A' was grown in LB-ampicillin medium (250 mL) with additional Zn^{2+} (100 mg L^{-1}). The medium (OD ~3) was centrifuged (Jouan KE22i, AK-500.11, 20 min, 8000 rpm, 4 °C) and the biomass was resuspended in buffer (50 mL, 50mM Tris-HCl pH 7.5). The cells were disrupted by ultrasonication (Branson, S250D CE, 200W, 5 mm spike, 50 mL tubes, 1 sec impulse, 2 sec pause, amplitude 50%, 16 min, 4 °C) and centrifuged (Jouan KE22i, AK-100.21, 20 min, 13000 rpm, 4 °C). The supernatant was transferred to an Erlenmeyer flask (250 mL) and kept at 65 °C for 25 minutes. After centrifugation (Jouan KE22i, AK-100.21, 20 min, 13000 rpm, 4 °C) the supernatant was used for the experiments. One unit of ADH-'A' will reduce 1.0 μmol of 2-octanone (**1a**) to 2-octanol (**1b**) per minute at pH 7.5 and 30ºC in presence of NADH.

Determination of absolute configurations

Absolute configurations of the alcohols **1b**-**5b**, **7b** and diol **8b** were determined by (i) comparison of elution order on GC with published data[3,4] or by (ii) co-injection with commercial available material or independent synthesized chiral reference material as described before.

2. Experimental procedures

General procedure for the biocatalytic reduction of 2-octanone employing E. coli ADH-'A' in buffer/organic solvent systems.

Unless otherwise stated, lyophilized cells of *E. coli* TunerTM (DE3)/pET22b+-ADH-'A' (5-15 mg), stored at 4ºC, were rehydrated in the corresponding volume of Tris/HCl buffer (50 mM, pH 7.5, 1mM NADH) for 30 min at 30ºC and 120 rpm on a rotatory shaker in an Eppendorf vial (1.5 mL). Then, the organic solvent (final volume of 0.6 mL), 2-propanol (97 μL, 14% v/v) and **1a** (20 μL) were added. The mixtures were shaken at 30ºC and 120 rpm for the appropriate time and stopped by extraction with ethyl acetate or CH_2Cl_2 (2 x 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried over Na_2SO_4. Conversions and enantiomeric excesses of 2-octanol were determined by GC analysis. (*S*)-**1b** was identified by co-injection with independently synthesized material or commercial reference material on two different GC columns.

General procedure for the biocatalytic reduction of ketones employing E. coli/ADH-'A' in micro-aqueous media.

Lyophilized cells of *E. coli* Tuner^{TM} (DE3)/pET22b+-ADH-'A' (5.0-15.0 mg), stored at 4ºC, were rehydrated in 6 μL of a 100 mM NADH solution (pH 7.5) for 30 min at 30ºC and 120 rpm on a rotatory shaker in an Eppendorf vial (1.5 mL). The corresponding anhydrous organic solvent (0.6 mL) saturated with buffer, 2-propanol (97 μL, 14% v/v) and substrates **1a**-**8a** (10-20 μL) were added and the reactions were shaken at 30ºC and 120 rpm for the appropriate time and stopped. Afterwards, water (60 μL) was added and the mixture was extracted with ethyl acetate or CH_2Cl_2 (2 x 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried over Na_2SO_4. Conversions and enantiomeric excesses of **1b**-**5b**, **7b** and **8b** were determined by GC analysis.

Recycling of E. coli/ADH-'A' in hexane/toluene.

To a suspension of rehydrated cells of *E. coli* /ADH-'A' (15.0 mg) in 10 μL of a 100 mM NADH solution, hexane or toluene (1.0 mL), 2-propanol (140 μL, 14% v/v) and 2-octanone (**1a**) (20 μL) were added. The reactions were shaken at 30ºC and 120 rpm for 4 hours and then stopped by extraction with ethyl acetate (3 x 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm), dried on Na_2SO_4 and analyzed by GC. The remaining cells were washed with the hexane or toluene (2 x 0.5 mL),

separated from the solvent by centrifugation, dried at 30ºC and stored at -20 ºC, in order to be repeatedly used in the subsequent enzymatic reductions, following the same procedure.

General procedure for the biocatalytic reduction of ketones employing recombinant ADH-'A' in buffer/ organic solvent.

Unless otherwise stated, to a solution of recombinant ADH-'A' (50-150 μL, 1.1-3.3 units) in Tris/HCl buffer (50 mM, pH 7.5, 1 mM NADH), the corresponding organic solvent (reaction final volume of 0.6 mL), 2-propanol (97 μL, 14% v/v) and **1a** (10-20 μL) were added. The reactions were shaken at 30ºC and 120 rpm for the appropriate time and stopped. The mixture was then extracted with ethyl acetate or CH_2Cl_2 (2 x 0.5 mL). The organic layer was dried over Na_2SO_4 and analyzed by GC in order to determine the conversions and enantiomeric excesses of the corresponding alcohols **1b-8b**.

General procedure for the biocatalytic reduction of ketones employing recombinant ADH-'A' in micro-aqueous organic solvent.

For reactions using recombinant ADH-'A' in micro-aqueous organic solvents, different volumes of ADH-'A' solution in Tris/HCl buffer (50-150 μL, 1.1 -3.3 units) were lyophilized. The enzyme was rehydrated in 6 μL of a 100 mM NADH solution (pH 7.5) for 30 min at 30ºC and 120 rpm on a rotatory shaker in an Eppendorf vial (1.5 mL). The corresponding anhydrous organic solvent saturated with buffer (0.6 mL), 2-propanol (97 μL, 14% v/v) and substrates **1a-8a** (10-20 μL) were added to the cells and the reactions were shaken at 30ºC and 120 rpm for the appropriate time and stopped. The mixture was extracted with ethyl acetate or CH_2Cl_2 (2 x 0.5 mL) and the organic layer was dried over Na_2SO_4. Conversions and enantiomeric excesses of **1b-8b** were determined by GC analysis.

3. Kinetic data for the enzymatic reductions employing aqueous buffer/ organic cosolvent.

For certain selected media, the effect of the solvent concentration was studied in more detail, as shown in Table S1. When the percentage of organic solvent increased, reaction rates became lower, probably due to a decrease of ketone concentration in the aqueous phase. Anyway, it is important to highlight that after 4 hours, moderate or good conversions were measured at high organic solvent concentrations, indicating an excellent stability of this enzyme in these solvents when using both biocatalyst preparations.

Table S1. Effect of solvent concentration on the reduction of **1a** with *E. coli*/ADH-'A' or purified ADH-'A' (t = 4 hours).[a]

	E. coli/ADH-'A'				Purified ADH-'A'		
% Solv.	hexane	toluene	dioxane	2-PrOH	hexane	toluene	2-PrOH
0	84.3	84.3	84.3	84.3	84.7	84.7	84.7
10	77.1	70.0	*n.d.*	*n.d.*	80.8	61.6	*n.d.*
30	76.8	67.2	*n.d.*	*n.d.*	74.3	50.0	*n.d.*
50	76.7	66.5	77.3	91.7	65.4	37.5	88.4
70	74.4	65.4	41.4	59.5	61.5	27.0	75.3
90	73.3	44.8	29.2	47.5	36.6	14.7	21.2
99	53.8	42.9	≤ 1.0	4.6	7.6	4.6	3.9

[a] For reaction details, see Section 2. *n.d.* not determined.

The enzymatic reductions of 2-octanone using *E. coli*/ADH-'A' or recombinant ADH-'A' in aqueous buffer (Tris/HCl 50 mM, pH 7.5) and organic solvents, performed as described in section 2, were stopped and worked up after different reaction times. The conversions measured are described in the following tables:

Table S2. Time course of the enzymatic reductions of **1a** in buffer/organic solvent (50% v v^{-1}) employing *E. coli*/ADH-'A'.[a]

time (h)	*n*-hexane	toluene	CH_2Cl_2	EtOAc	$^{i}Pr_2O$	dioxane
0.5	53.7	43.8	13.1	32.9	59.1	43.1
1	64.6	61.1	*n.d.*	45.7	74.7	57.6
2	71.2	68.2	31.8	72.3	76.4	77.4
4	76.7	69.4	51.8	76.6	78.1	77.9
6	78.8	70.3	58.9	*n.d.*	*n.d.*	*n.d.*
24	76.7	71.5	68.1	77.3	79.0	78.8

[a] Conversions determined by GC. *n.d.* not determined.

Table S3. Time course of the enzymatic reductions of **1a** in water-immiscible organic solvent (99% v v^{-1}) and water-miscible solvent (90% v v^{-1}) employing *E. coli*/ADH-'A'.[a]

time (h)	*n*-hexane	toluene	CH_2Cl_2	EtOAc	$^{i}Pr_2O$	dioxane	2-PrOH
0.5	13.3	10.5	8.7	3.8	4.9	10.4	11.1
1	22.1	19	*n.d.*	6.8	8.7	17.9	16.8
2	40.2	27.7	17.6	10.4	13.5	24.3	26.9
4	53.8	42.9	22.5	14.9	20.4	34.2	43.5
6	65.8	54.7	26.9	*n.d.*	*n.d.*	*n.d.*	*n.d.*
24	78.0	71.9	47.1	41.9	52.7	49.5	83.2

[a] Conversions determined by GC. *n.d.* not determined.

Table S4. Time course of the catalyzed reduction of 2-octanone in Tris/HCl (50 mM, pH 7.5) organic cosolvent (50% v v^{-1}) using recombinant ADH-'A'.[a]

time (h)	*n*-hexane	$^{i}Pr_2O$	1,4-dioxane
0.5	24.7	17.4	9.1
1	46.2	31.9	16.8
2	56.2	48.1	29.2
4	65.4	60.5	38.7
24	79.3	77.5	78.3

[a] Conversions determined by GC.

4. GC Analyses on achiral columns

The following columns were used for the determination of conversions: Column A: Hewlett-Packard HP 1701 (30 m x 0.25 mm x 0.25 μm, 1.0 bar N_2); column B: J&W Scientific Agilent Technologies HP-1 (30 m x 0.25 mm x 0.25 μm, 1.0 bar N_2) and column C: Chrompack Chirasil Dex (25 m x 0.25 mm x 0.25 μm, 1.0 bar N_2).

Table S5. Determination of conversion by GC.

Compound	Program[a]	Column	Retention time (min)	
			Ketone **a**	Alcohol **b**
1	80/5/10/105/30/240	A	7.0	7.3
2	100/5/12/160/10	C	2.1	5.5
3	110/0/2.5/120/0/10/200/1	C	6.8	8.3
4	55/7/5/80/0/10/160	A	6.8	7.1
5	110/0/2.5/120/0/10/200/1	C	3.9	5.4
6	120/0/20/250	B	4.6	4.4
7	33/5/20/140	C	6.7	8.1
8[b]	90/9/15/130/0/20/250	B	10.9	7.5

[a] Program: initial T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min). [b] Compound **8b** was measured as diacetate. The hydroxyketone **8c** shows a retention time of 13.6 min.

5. GC Analyses on chiral columns

For the determination of the enantiomeric excesses, alcohols **1b-8b** were acetylated to the corresponding acetate derivatives and analyzed by GC employing the following column: Chrompack Chirasil Dex (25 m x 0.25 mm x 0.25 μm, 1.0 bar N_2).

Table S6. Determination of *ee* values by chiral GC.

Compound	Program[a]	Retention times (min)
1b	110/0/2.5/120/0/10/200/1	2,5 (*S*); 2,7 (*R*)
2b	110/0/2.5/120/0/10/200/1	3.8 (*S*); 4.1 (*R*)
3b	112/0/0.5/116/0/15/170/1	7.8 (*R*); 8.6 (*S*)
4b	55/7/5/80/10/160	10.3 (*S*); 10.5 (*R*)
5b	112/0/0.5/116/0/15/170/1	5.3 (*R*); 5.5 (*S*)
6b	80/6.5/10/160/1	13.3 (*S*); 13.5 (*R*)
8b	65/5/2/150/10	26.3 (*meso*), 27.4 (2*S*,5*S*), 27.5 (2*R*,5*R*)

[a] Program: initial T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/time (min).

6. Supporting references

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