Stereoselective Bio-reduction of Bulky-bulky Ketones by a Novel ADH from *Ralstonia* sp.

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Supporting Information

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1. Gene library, sequencing, cloning and expression of *Ralstonia* sp. ADH

1.1. Construction and screening of a Ralstonia *sp. gene library*

Standard molecular-biology procedures were performed according to literature.[1] Genomic bacterial DNA from *Ralstonia* sp. DSM 6428 was partially restricted with *Bsp*143I (*Sau* 3AI). DNA Fragments from 5-8 kb in size were isolated and ligated into the plasmid pBSII (SK^-). Transformation of *E. coli* Top 10F´ (Invitrogen) with the resulting library was performed by electroporation.

Cells were grown for two days at room temperature on LB plates containing 100 µg mL^{-1} ampicillin (LB-amp) and 0.2 mM IPTG. The colonies were transferred to a filter and screened for increased NADPH fluorescence[2] due to oxidation of the substrate 1-phenyl-1-propanol in the presence of $NADP^+$. 1-Phenyl-1-propanol was added to the cells as a 1% v v^{-1} solution dissolved in 5% v v^{-1} *N*,*N*-dimethylformamide in 50 mM Tris-HCl, pH 7.5. Positive clones were analyzed in a subsequent step using the 'substrate-' or the 'enzyme-coupled' approach assay.

1.2. Subcloning and expression of a Ralstonia *sp. ADH gene*

Plasmid DNA from positive clones was sequenced. The gathered sequences were analyzed by blastx for putative alcohol dehydrogenases or related genes. A putative short-chain dehydrogenase/alcohol dehydrogenase gene was amplified by PCR and cloned into the pMS470[3] based plasmid pEamTA (Figure S1).[4] The primers were ADH-f 5´-ATGTATCGACTATTAAACAAAACAGC-3´ and ADH-r 5´-TTAGACCTGGGTCAATCCACCGTCC-3´. The resulting plasmid pEam_RasADH was used to transform *E. coli* DH5α. The resulting strain was arbitrarily designated as *E. coli*/RasADH.

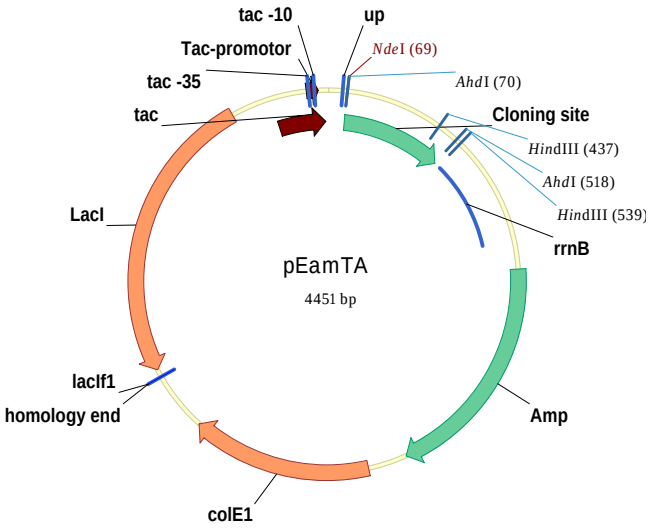

Figure S1. Plasmid containing the RasADH gene

1.3. Protein sequence

MYRLLNKTAVITGGNSGIGLATAKRFVAEGAYVFIVGRRRKELEQAAAEIGRNVTAVK ADVTKLEDLDRLYAIVREQRGSIDVLFANSGAIEQKTLEEITPEHYDRTFDVNVRGLIFTV QKALPLLRDGGSVILTSSVAGVLGLQAHDTYSAAKAAVRSLARTWTTELKGRSIRVNAV SPGAIDTPIIENQVSTQEEADELRAKFAAATPLGRVGRPEELAAAVLFLASDDSSYVAGIE LFVDGGLTQV.

1.4 Gene sequence

Accession number: EU485985

atgtatcgactattaaacaaaacagccgtcataaccggtggaaacagcggcattggcctcgccacagcgaagcgcttcgttgccgaggg tgcctatgtattcattgtcggtcgccggcggaaggaactcgagcaggcggccgcagaaatcggtcggaatgtcacggcggtcaaagccga tgtgacaaagcttgaagacctggaccgactttacgcgattgtgcgtgagcaacggggtagcatcgacgtactatttgcgaattccggcgcaat cgagcaaaagacgcttgaggagattactccggaacactatgacaggactttcgatgtcaacgttcggggattgatcttcaccgtgcagaagg

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gacggtggattgacccaggtctaa

1.5. Preparation of lyophilized cells containing recombinant catalyst E.coli/RasADH

Cultivation medium LB-amp: Luria broth (25 g L^{-1}, Sigma L-3522), ampicillin sodium salt (100 mg L^{-1}, Sigma A9518-5G), KH_2PO_4 (1.4 g L^{-1}, Fluka 60220), and K_2HPO_4 (4.4 g L^{-1}, Fluka 60355).

E. coli/RasADH was stored at –86 °C in a glycerol/LB-amp 15:85 solution. Prior to use it was plated on LB-amp, then a single colony was plated again on LB-amp (16 h, 37 °C), and finally a loop of cells were used to inoculate 250 mL of LB-amp medium in one liter baffled shake flasks. After incubation for 24 hours at 30 °C at 130 rpm an OD of ~5 was reached and the expression of the ADH was induced by the addition of IPTG (450 mg L^{-1}, 2 mM final concentration, preqlab Biotechnologie GmbH 37-2020) and again, ampicillin sodium salt (100 mg L^{-1}). The incubation was performed at 20 °C to avoid the formation of inclusion bodies for 24 hours at 130 rpm. The cells were harvested by centrifugation (8000 rpm, 3000 g, 20 min, 4 °C), the medium was decanted and the cells were resuspended in water, shock-frozen (liquid nitrogen) and lyophilized.

2. Measurement of activities

2.1. 'Enzyme-coupled' approach

Lyophilized cells of *E. coli*/RasADH (10 mg) were rehydrated in Tris-HCl buffer (600 µL, 50 mM, pH 7.5, 1 mM NADPH) for 30 min at 30 ºC and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Then, the corresponding ketone (20 g L^{-1}), glucose (5 equiv.), and GDH (1 U) were added. Reactions were shaken at 30 ºC and 120 rpm for 30 min to ensure low conversions and stopped by extraction with ethyl acetate (2 x 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried (Na_2SO_4). Conversions were determined via the standard media of three measurements using GC analysis.

2.2. 'Substrate-coupled' approach.

Lyophilized cells of *E. coli*/RasADH (10 mg) were rehydrated in Tris-HCl buffer (600 µL, 50 mM, pH 7.5, 1 mM NADPH) for 30 min at 30 ºC and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Then, 2-propanol (67 µL, 10% v v^{-1}) and the corresponding ketone (20 g L^{-1}) were added. Reactions were shaken at 30 ºC and 120 rpm for 30 min to ensure low conversions and stopped by extraction with ethyl acetate (2 x 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried (Na_2SO_4). Conversions were determined via the standard media of three measurements using GC analysis.

*2.3 Preparative reduction of ω-chloroacetophenone **12a***

Lyophilized cells of *E.coli*/*Ras*ADH (230 mg) were rehydrated in Tris-HCl buffer (6 mL, 50 mM, pH 7.5, 1 mM NADPH) for 30 min at 30°C and 150 rpm on a rotary shaker in a Falcon tube (50 mL) in horizontal position. ω-Chloroacetophenone **12a** (59.6 mg, 45 µL, 0.39 mmol), glucose

(5 equiv.) and GDH (6 U) were added. The reaction was shaken at 30°C and 150 rpm for 24 h and stopped by extraction with ethyl acetate (2 x 5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried (Na_2SO_4). After analysis of the conversion the organic solvent was evaporated under reduced pressure to yield 55 mg (91%) of optically pure (*R*)-alcohol (*e.e.* >99%).

3.**Analytics**

3.1. GC Analyses for determination of conversions

The following column was used: Chrompack Chirasil Dex (25 m x 0.32 mm x 0.25 µm, 1.0 bar H_2).

Table S1. Determination of conversions by GC.

Compound	Program[a]	Retention time (min)	
		Ketone **a**	Alcohol **b**
1	80/6.5/10/160/10	11.7	13.9
2	80/6.5/10/160/10	13.2	15.0
3	80/6.5/10/160/10	14.5	16.6
4	80/6.5/10/160/10	12.1	14.0
5	80/6.5/10/160/10	16.0	17.3
6	80/6.5/10/160/10	14.6	16.0
7	70/0/9/160/4	4.7	6.7
8	110/0/2.5/120/0/10/200/1	1.8	2.5
9	110/0/2.5/120/0/10/200/1	1.7	2.4
10	110/0/2.5/120/0/10/200/1	2.4	3.5
11	80/6.5/10/160/10	10.1	12.7
12	110/0/2.5/120/0/10/200/1	6.8	8.0
13	110/0/2.5/120/0/10/200/1	3.7	5.4

[a] Program: initial temp. (°C)/ time (min)/ slope (°C/min)/ temp. (°C)/ time (min)/ slope (°C/min)/ temp. (°C)/ time (min).

3.2. GC analyses for determination of e.e.

The following chiral GC column was used: Chrompack Chirasil Dex (25 m x 0.32 mm x 0.25 μm, 10 psi H_2).

Table S2. Determination of *e.e.* values by GC.

Compound[a]	Program[b]	Retention time (min)
4b	95/20/5/160/0/10/180/1	26.2 (*R*), 26.4 (*S*)
5b[c]	100/2/1/130/5/20/170/5	36.8 (*R*), 38.0 (*S*)
6b	80/6.5/10/160/10	15.0 (*S*), 15.2 (*R*)
7b	110/0/2.5/120/0/10/200/1	3.6 (*S*), 4.0 (*R*)
8b	110/0/2.5/120/0/10/200/1	2.4 (*S*), 2.6 (*R*)
9b	110/0/2.5/120/0/10/200/1	2.2 (*S*), 2.4 (*R*)
10b	110/0/2.5/120/0/10/200/1	3.2 (*S*), 3.4 (*R*)
11b	110/0/2.5/120/0/10/200/1	5.0 (*S*), 5.3 (*R*)
12b	110/0/2.5/120/0/10/200/1	6.9 (*R*), 7.1 (*S*)
13b	110/0/2.5/120/0/10/200/1	5.9 (*R*), 6.1 (*S*)

[a] Enantiomeric excesses determined of the corresponding acetate derivatives except otherwise stated. [b] Program: initial temp. (°C)/ time (min)/ slope (°C/min)/ temp. (°C)/ time (min)/ slope (°C/min)/ temp. (°C)/ time (min). [c] Measured as underivatized alcohol.

3.3. HPLC analyses for determination of e.e.

Column: Chiralpak OD-H (0.46 cm x 25 cm, Daicel Chemical Ind. Ltd.); isocratic eluent: *n*-heptane/*i*-propanol/ (98/2), 18 °C, flow 1.0 mL min^{-1}. Enantiomeric excesses determined of the corresponding acetate derivatives.

Retention times (min): **1b**: 4.7 (*R*), 5.2 (*S*).

2b: 4.6 (*R*), 4.9 (*S*).

3b: 4.5 (*R*), 4.7 (*S*).

4. References

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