*Article*

# Expanding the Application Range of Microbial Oxidoreductases by an Alcohol Dehydrogenase from *Comamonas testosteroni* with a Broad Substrate Spectrum and pH Profile

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## 1. Construct design

Nucleotide sequences of cloned CtADH is given as the native DNA sequence without 6xHis-tag. Length: 789 bp

ATGACTAATCTGCCTACAGCCTTGATCACCGGTGCGTCTTCCGGCATCGGTGCGACCTA
CGCTGAACGCTTCGCCCGCCGTGGCCACAACCTCGTCATGGTTGCCCGCGATAAAGTT
CGCATGGACGTCCTCGCCTCCCGCTTGCGCGAAGAGACCAAGGTCACCATCGACGTGA
TTCAAGCCGACCTTACACAACAGAAAGACCTGGCTGAGGTCGAGACGCGTCTGCGCGA
AGACACGTCGATCGGGATTTTGATCAACAACGCTGGCATGGGCCAGTCCGGGGCTTTC
GTCCAACAGAACGCGCAGAGCATTGACCGTCTGGTAATGCTCAACACTACGGCGCCCA
CGCGCCTCGCTGCAGCAGTCGCTGCACGCTTTGCTCAGGAAGGCAAAGGCTCGATCGT
CAACATCGGCTCGGTTGTTGGCTTTGCTCCCGAGCTAGGCATGACGATTTATGGTGCGA
CCAAGGCATTTGTACTGTTTCTCTCACAAGGTCTGAATTTGGAACTAGGGCCGAAAGGA
ATCTATGTGCAAGCAGTGCTGCCTGCTGCAACCCGTACTGAGATCTGGAGCCGCGCCG
GCATGGATATCAACACACTGCCAGAGGTGATGGATGTCAACGAACTGGTGGATGCCGC
ACTCATCGGCTTCGATCGTAAGGAACTGGTGACGATTCCGCCTCTGCATGTCGCAGAG
CGTTGGAATGAGCTGGACCAGGCGCGACAAGGACTGATGTCCGAAATCCGTCAAGCGC
ATGCCGCTGAGCGCTACCTGCCGCAGGCCTAA

Protein sequence of cloned CtADH included the C-terminal 6xHis-tag sequence.

Length: 272 aa.

MGMTNLPTALITGASSGIGATYAERFARRGHNLVMVARDKVRMDVLASRLREETKVTIDVIQ
ADLTQQKDLAEVETRLREDTSIGILINNAGMGQSGAFVQQNAQSIDRLVMLNTTAPTRLAAA
VAARFAQEGKGSIVNIGSVVGFAPELGMTIYGATKAFVLFLSQGLNLELGPKGIYVQAVLPAA
TRTEIWSRAGMDINTLPEVMDVNELVDAALIGFDRKELVTIPPLHVAERWNELDQARQGLMS
EIRQAHAAERYLPQALEHHHHHH

## 2. Cloning of CtADH with a 6xHis-Tag

The CtADH gene was obtained via standard PCR- techniques (1 mM dNTPs, 560 mM DMSO, 10 pmol $\mu L^{-1}$ Primer, 1x HF-Buffer (*Thermo Scientific*), 1 U Phusion polymerase) from *Comamonas testosteroni* DSM 38 wildtype cells. Primer were designed according to the sequence of unknown gene from *C. testosteroni* strain DSM 14576 (Uniprot B7X0Q9) 5´-AACCAACCATGGGAATGACTAATCTGCCTACAGCCTTGATCACC-3´and 5´-ATAATAACTCGAGTTAGGCCTGCGGCAGGTAGCGCTCAGCGGCAT-3´. The created *Nco*I-*Xho*I fragment was ligated (*Nco*I-*Xho*I) into the commercially available vector pET28a(+),which is under the control of T7-promoter. Thereby the constructed vector has a 6xhis-tag at the C-terminus and was named pET28a_CtADH(C).
The correct in-frame DNA sequence and the absence of any mutations were confirmed by sequencing.

## 3. Preparation of crude extract

A cell suspension (25% w/v) was prepared in 100 mM citrate-phosphate buffer, pH 6. Cells were disrupted by three sonication cycles of 3 min (10-20% power output) with cooling periods between cycles. The lysed cells were centrifuged at 20,000 x g for 30 min at 4 °C and the supernatant was used for determining the activity or further purification steps.

## 4. SDS-Page

Protein overexpression and purification were monitored by SDS-PAGE according to Bradford [1]. For the expected mass of approximately 30 kDa, a tris-glycine gel containing 15 % acrylamide was used. Samples were incubated for 10 min at 95 °C in loading buffer and 10 µg were loaded on the gel. The gels were stained with coomassie brilliant blue G-250. The molecular mass under denaturing conditions was determined by comparison with standard markers (Thermo Scientific, Dreieich, Germany).

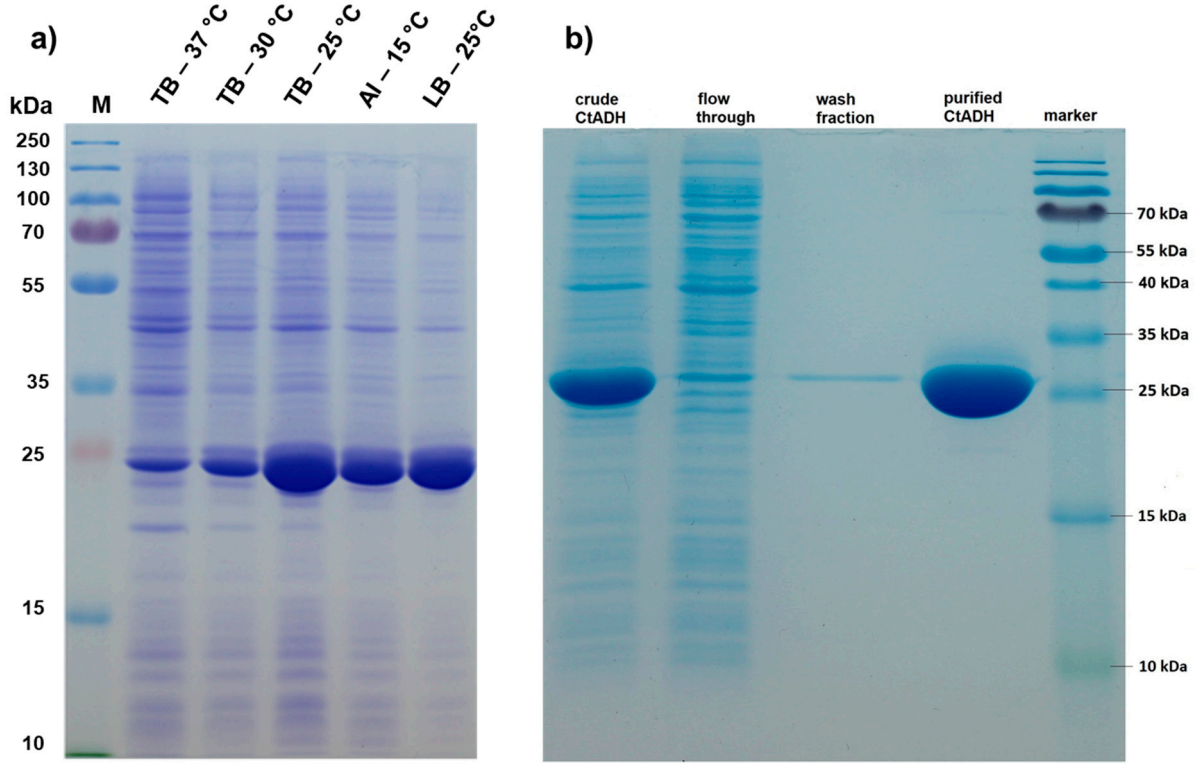


**Figure S1:** SDS-PAGE **a)** of the expression optimization of CtADH, shown are crude extracts of different expression conditions and **b)** of the Ni-NTA purification including flow through and wash fraction. 10 µg of protein were applied into each lane of part (**a**) and into the lanes “crude CtADH” and “purified CtADH” of part (**b**).

## 5. X-ray diffractometry

**Table S1:** X-ray diffraction data and structure refinement statistics.

| PDB code | | 6ZE0 | 6ZDZ |
|---|---|---|---|
| ***X-ray diffraction data collection*** | | | |
| Wavelength [Å] | | 0.97970 | 1.00000 |
| Synchrotron (beamline) | | ESRF (ID23-1) | SLS (PXIII) |
| Space group | | $P22_12_1$ | $P4_32_12$ |
| Unit cell | a, b, c [Å] | 49.929, 70.820, 210.868 | 103.481, 103.481, 78.107 |
| | α, β, γ [°] | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Monomers per asymmetric unit | | 3 | 2 |
| Resolution [Å]<br>(highest res. shell) | | 105.434 – 1.989<br>(2.126 – 1.989) [1] | 76.153 – 2.153<br>(2.361 – 2.153) [1] |
| $R_{sym}$ [%] | | 12.1 (69.8) [1] | 17.6 (288.6) [1] |
| CC1/2 | | 0.995 (0.644) [1] | 0.998 (0.680) [1] |
| Signal-to-noise ratio ($I/\sigma_I$) | | 9.5 (1.5) [1] | 17.2 (1.6) [1] |
| No. of unique refl. | | 43285 (2163) [1] | 23618 (1182)[1] |
| Completeness (spher.) [%] | | 82.4 (23.1) [1] | 70.1 (14.8) [1] |
| Completeness (ellips.) [%] | | 86.2 (25.7) [1] | 94.9 (75.7) [1] |
| Multiplicity | | 5.7 (3.2) [1] | 25.3 (24.1) [1] |
| Wilson B-factor [$Å^2$] | | 27.56 | 39.65 |
| ***Structure refinement and validation*** | | | |
| No. of reflections for $R_{work}/R_{free}$ | | 43234/1082 | 23599/1173 |
| $R_{work}/R_{free}$ [%] | | 19.91/24.04 | 19.10/22.95 |
| Number of non-H-atoms | | 6167 | 3866 |
| | Protein | 5719 | 3690 |
| | Ligands | --- | 10 |
| | Water | 448 | 485 |
| Average B-factor [$Å^2$] | | 36.89 | 48.39 |
| | Protein | 36.71 | 48.59 |
| | Ligands | --- | 41.59 |
| | water | 39.23 | 44.28 |
| RMS deviations | | | |
| | Bond lengths [Å] | 0.002 | 0.002 |
| | Bond angles [°] | 0.53 | 0.44 |
| Ramachandran plot | | | |
| | favored (%) | 97.70 | 98.32 |
| | allowed (%) | 2.03 | 1.47 |
| | outliers (%) | 0.27 | 0.21 |

[1] Data in brackets refer to the highest resolution shell according to autoPROC [2]

## 6. Enzyme assays

### 6.1 Substrate scope

The enzyme assay mixture for the standard assay and the determination of the specific activity of various substrates contained, 980 µL citrate-phosphate buffer (100 mM, pH 6.0) containing the substrate, 10 µL NADPH (20 mM in a. dest) for reductive reactions or 10 µL $NADP^+$ (20 mM) for oxidative reactions, respectively and 10 µL purified CtADH in various concentrations in a total volume of 1 mL. Reactions were started by addition of the NADPH solution and measured over 1 minute. The oxidation assay mixture contained 980 µL buffer (100 mM citrate-phosphate buffer pH 6.0), 10 µL $NADP^+$ (20 mM in a. dest.) and 10 µL enzyme solution. Reactions were started by addition of the $NADP^+$ solution and measured over 1 minute. All photometric measurements were done at least as triplicates. One unit of activity was defined as the amount of enzyme catalysing the reduction of 1 µmol NAD(P)H under standard conditions (340 nm, 22 °C, pH 6.0) using a spectrophotometer (Shimadzu).

### 6.2 pH-Optimum

The pH-optimum of Nickel-NTA purified CtADH was determined by measuring the specific activity for the test substrate propiophenone at different pH-values. The following pH-values were measured with the shown buffers.

**Table S2:** List of buffers used for the determination of the pH-optimum.

| Buffer [100 mM] | pH-value |
|---|---|
| Citrate-phosphate buffer | 4.5 |
| Citrate-phosphate buffer | 5.0 |
| Citrate-phosphate buffer | 5.5 |
| Citrate-phosphate buffer | 6.0 |
| Citrate-phosphate buffer | 5.5 |
| Citrate-phosphate buffer | 7.0 |
| Citrate-phosphate buffer | 7.5 |
| BICINE-buffer | 8.0 |
| BICINE-buffer | 8.5 |
| BICINE-buffer | 9.0 |
| BICINE-buffer | 9.5 |
| BICINE-buffer | 10.0 |
| BICINE-buffer | 10.5 |
| BICINE-buffer | 11.2 |
| BICINE-buffer | 12.2 |
| TRIS-buffer | 8.0 |
| TRIS-buffer | 8.5 |
| TRIS-buffer | 9.0 |

The specific activity for propiophenone was measured in a total volume of 1 mL. In 980 µL buffer containing 10 mM propiophenone 10 µL purified CtADH (3.3 mg $mL^{-1}$ end concentration) was added and incubated for 10 min at RT. The specific activity was determined after adding 10 µL NADPH (20 mM) through measuring the change of absorbance at 340 nm over 1 min at RT using a spectrophotometer. All photometric measurements were done at least as triplicates.

## 6.3 Determination of $K_M$-values

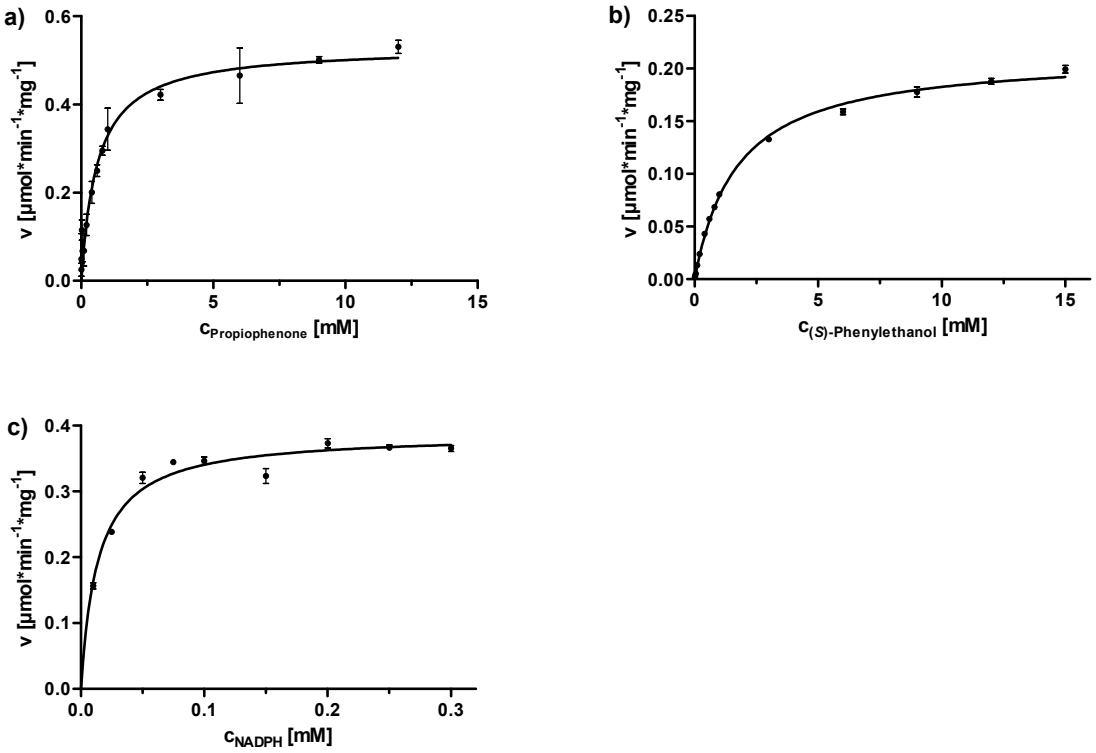


**Figure S2:** Michaelis-Menten diagrams for purified CtADH with the substrate **a)** propiophenone (10 µM – 12 mM) and fixed co-substrate NADPH (0.36 mM), **b)** (*S*)-phenylethanol (10 µM – 15 mM) and fixed co-substrate NADPH (0.36 mM) and **c)** NADPH (10 µM – 350 µM) and fixed co-substrate propiophenone (1.0 mM)

## 7. Analytical methods

Determination of the enantioselectivity was carried out by GC measurements (Shimadzu GC-2010 Plus). Measurements were conducted on a chiral BGB-174 column (30 m x 0.25 mm x 0.25 µm) or a LIPODEX-E column (25 m x 0.25 mm) with nitrogen as carrier gas. A sample amount of 1 µL was injected in all cases. The used methods and measured retention times are listed respectively in Table 3 and Table 4.

**Table S3:** List of used GC-methods.

| Method number | Split | Injector temperature /°C | Detector temperature /°C | Gradient |
|---|---|---|---|---|
| 1 | 1:100 | 200 | 210 | starting temperature 110 °C for 5 min; to 125 °C in 2 °C/min; 125 °C for 15 min; to 200 °C in 20 °C/min; 200 °C for 1 min |
| 2 | 1:100 | 180 | 210 | starting temperature 70 °C; to 100 °C in 5 °C/min; 100 °C for 20 min; to 105 °C in 1 °C/min |
| 3 | 1:10 | 250 | 275 | starting temperature 110 °C for 1 min; to 120 °C in 0.4 °C/min;120 °C for 1 min; to 200 °C in 10 °C/min; 200 °C for 1 min |
| 4 | 1:10 | 250 | 275 | starting temperature 130 °C for 1 min; to 142 °C in 0.4 °C/min; 140 °C for 5 min; to 200 °C in 12 °C/min; 200 °C for 2 min |
| 5 | 1:10 | 250 | 275 | starting temperature 100 °C for 30 min; to 200 °C in 10 °C/min; 200 °C for 1 min |
| 6 | 1:10 | 180 | 210 | starting temperature 70 °C; to 75 °C in 1 °C/min; 75 °C for 0.5 min; to 150 °C in 5 °C/min; 150 °C for 3 min |

**Table S4:** List of measured retention times.

| Substrate | Method | Retention time /min |
|---|---|---|
| 4-Chloro-1-phenyl-1-butanone | 1 | 21.52 |
| (*S*)-4-chloro-1-phenyl-1-butanone | 1 | 14.14 |
| (*R*)-4-chloro-1-phenyl-1-butanone | 1 | 14.39 |
| 2,2,2-Trifluoroacetophenone | 2 | 6.14 |
| (*S*)-2,2,2-trifluoro-1-phenylethanol | 2 | 23.50 |
| (*R*)-2,2,2-trifluoro-1-phenylethanol | 2 | 24.16 |
| Ethyl 4-chloroacetoacetate | 3 | 16.18 |
| (*S*)-ethyl 4-chloro-3-hydroxybutyrate | 3 | 21.63 |
| (*R*)-ethyl 4-chloro-3-hydroxybutyrate | 3 | 21.07 |
| Ethyl benzoylformate | 4 | 20.97 |
| (*S*)-mandelic acid ethyl ester | 4 | 21.91 |
| (*R*)-mandelic acid ethyl ester | 4 | 21.53 |
| Acetophenone | 5 | 17.27 |
| (*S*)-phenylethanol | 5 | 23.43 |
| (*R*)-phenylethanol | 5 | 24.53 |
| 4,4,4-Trifluoroacetoacetic acid ethyl ester | 6 | 5.30 |
| (*S*)-ethyl 3-hydroxy-4,4,4-trifluorobutyrate | 6 | 15.18 |
| (*R*)-ethyl 3-hydroxy-4,4,4-trifluorobutyrate | 6 | 14.90 |

## 8. Thermal shift assay

Working solutions for thermal shift assay were prepared according to Boivin [3]. In short, 2 µL of 62 x SYPRO Orange, 5 µL of 5 x buffer, 5 µL x 5 salt [optional], 2 µL protein (2.5 mg $mL^{-1}$), add to 25 µL with distilled water were added to the wells of a 96-well thin-wall PCR plate (BioRad). The plates were sealed with optical-Quality sealing tape (BioRad) and heated in a CFX96 Touch real-time PCR system (BioRad) from 20°C to 90°C in increments of 0.2°C.

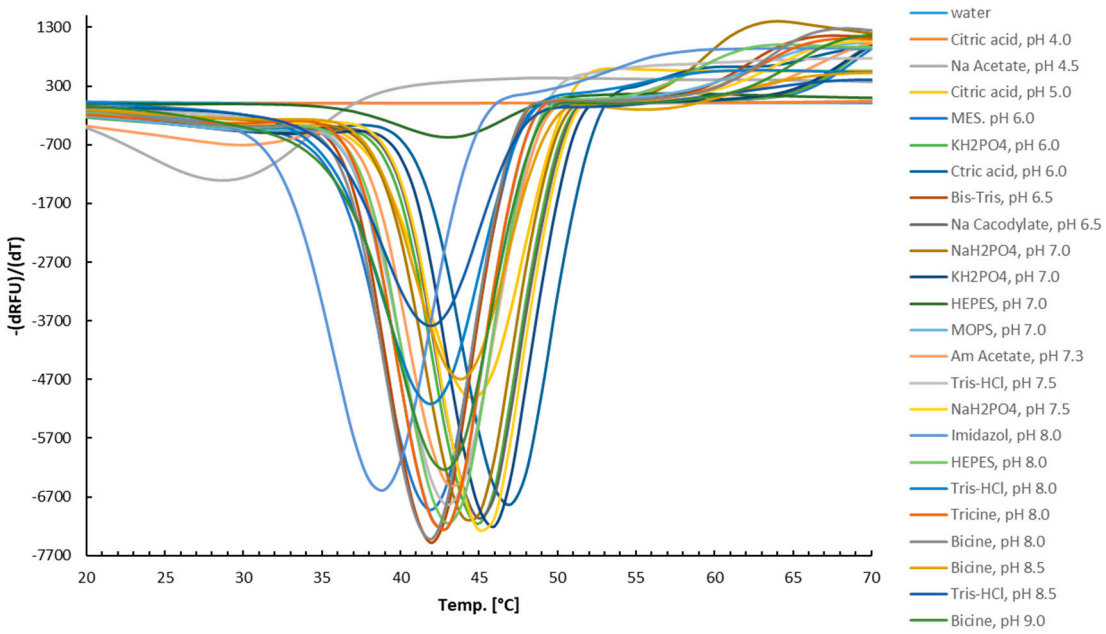


**Figure S3:** Thermal shift assay curves of CtADH obtained in the presence of various buffers (all 50 mM) and with different pH (4.0 – 9.0).

## 10. References


[1] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-54.

[2] Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T. and Bricogne, G. (2011). Data processing and analysis with the autoPROC toolbox. Acta Crystallogr D Biol Crystallogr 67, 293-302.

[3] Boivin, S., Kozak, S. and Meijers, R. (2013). Optimization of protein purification and characterization using Thermofluor screens. Protein Expr Purif 91, 192-206.