Supporting Information

Mechanistic insights in *Lactobacillus brevis* alcohol dehydrogenase: stability and active site role of Ser143 and Tyr156

E.P.J. Jongkind,a W.J. Kools,a M. Pareek,b S.C.L. Kamerlin,b,c C.E. Paul\*,a

*a Biocatalysis section, Department of Biotechnology, Delft University of Biotechnology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands; c.e.paul@tudelft.nl*

*b Department of Chemistry-BMC, Uppsala University, BMC Box 576, S-751 23 Uppsala, Sweden*

*c School of Chemistry and Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive NW, Atlanta, GA 30332, USA*

Contents

[1. General information 2](#_Toc169854538)

[2. *Lb*ADH production and purification 3](#_Toc169854539)

[2.1. Sequences 3](#_Toc169854540)

[2.2. Induced fit docking of iminium acetophenone in engineered LbADH 3](#_Toc169854541)

[2.3. FuncLib library 3](#_Toc169854542)

[2.4. Production of *Lb*ADH-wt and variants 6](#_Toc169854543)

[2.5. Affinity chromatography purification of *Lb*ADH-wt and variants 7](#_Toc169854544)

[3. Carbonyl substrates and corresponding products 11](#_Toc169854545)

[4. Activity assays 12](#_Toc169854546)

[5. Biocatalytic reactions 13](#_Toc169854547)

[5.1. Ketoreduction conditions 13](#_Toc169854548)

[5.2. Reductive amination conditions 13](#_Toc169854549)

[5.3. Imine reductase conditions 14](#_Toc169854550)

[6. GC analyses 15](#_Toc169854551)

[7. Molecular dynamics simulations 22](#_Toc169854552)

[8. References 24](#_Toc169854553)

# General information

Chemicals were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany), Janssen Chimica (Geel, Belgium), Fluka Chemica (Buchs, Switzerland), Acros organics (ThermoFisher Scientific, Ward Hill, MA, USA), Thermo Fisher Scientific (Ward Hill, MA, USA) and Enamine (Riga, Latvia) (**Table 1**). HPLC-grade ethyl acetate (EtOAc) was used as solvent for gas chromatography. HPLC-grade dimethylsulfoxide (DMSO) was used as a co-solvent. Lyophilised glucose dehydrogenase 105 (GDH-105) was purchased from Codexis (Redwood City, CA, USA). Crude lyophilised DNaseI, lyophilised lysozyme from chicken egg white and cOmplete™EDTA-free Protease I inhibitor were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany).

**Table 1**. Overview of purchased chemicals, purities and origin. **a**: Sigma-Aldrich (Merck, Darmstadt, Germany). **b**: Janssen Chimica (Geel, Belgium). **c**: Fluka Chemica (Buchs, Switzerland). **d**: Acros Organics (ThermoFisher Scientific, Ward Hill, MA, USA). **e**: Thermo Fisher Scientific (Ward Hill, MA, USA). **f**: Enamine (Riga, Latvia). **g**: Prozomix Ltd (Haltwhistle, UK). **h**: OYC EU B.V. (Rotterdam, The Netherlands).

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **CAS number** | **Purity (%)** | **Company** |
| acetophenone | 98-86-2 | 99 | a |
| phenylacetone | 103-79-7 | 99 | b |
| 4-phenylbutan-2-one | 2550-26-7 | 98 | a |
| 2-methylcyclohexanone | 583-60-8 | 99 | a |
| 3-methylcyclohexanone | 591-24-2 | 97 | a |
| 2-hexanone | 591-78-6 | n.a. | c |
| 2-heptanone | 110-43-0 | 98 | a |
| 2-octanone | 111-13-7 | n.a. | d |
| 3-octanone | 106-68-3 | 97 | c |
| hexane-2,3-dione | 3848-24-6 | 98 | a |
| benzalacetone | 1896-62-4 | 98 | c |
| phenylethanol | 98-85-1 | 98 | d |
| 1-phenyl-2-propanol | 698-97-3 | n.a. | a |
| 4-phenylbutan-2-ol | 2344-70-9 | n.a. | n.a. |
| 2-methylcyclohexanol | 583-59-5 | n.a. | c |
| 3-methylcyclohexanol | 591-23-1 | n.a. | a |
| hexanol | 111-27-3 | 98 | d |
| heptanol | 111-70-6 | 98 | a |
| 2-octanol | 123-96-6 | n.a. | c |
| 3-octanol | 589-98-0 | 97 | a |
| 1-phenylethan-1-amine | 618-36-0 | 99 | a |
| 2-methylcyclohexanamine | 7003-32-9 | 97 | e |
| heptanamine | 111-68-2 | 99 | e |
| 2-octanamine | 693-16-3 | n.a. | f |
| 3-octanamine | 24552-04-3 | n.a. | (synthesized) |
| benzaldehyde | 100-52-7 | 99.5 | a |
| pentanal | 110-62-3 | 97 | a |
| hexanal | 66-25-1 | 98 | a |
| heptanal | 111-71-7 | 92 | a |
| benzylalcohol | 100-51-6 | 99 | d |
| pentanol | 71-41-0 | 99 | d |
| hexanol | 111-27-3 | 98 | d |
| heptanol | 111-70-6 | 98 | a |
| benzylamine | 100-46-9 | 99 | d |
| pentanamine | 110-58-7 | 99 | a |
| hexanamine | 111-26-2 | 99 | a |
| heptanamine | 111-68-2 | 99 | e |
| *β*-nicotinamide adenine dinucleotide phosphate (NADP+) | 24292-60-2 | n.a. | g |
| NADPH | 2646-71-1 | n.a. | h |

The synthesis of 3-octanaminewas performed according to Mangas-Sanchez *et al.*,[1] with the following deviations. 35 mmol ammonium acetate and 7 mmol sodium cyanoborohydride were added to 3.5 mmol of ketone in 11 mL methanol. The reaction was stirred for 4 h and was quenched with 12.6 mL 1 M NaOH. The mixture was then concentrated by rotary evaporation. The product was analyzed on proton and carbon NMR (400 MHz). The spectrum of the product was in accordance with literature.[1]

# *Lb*ADH production and purification

## Sequences

***Lactobacillus brevis* alcohol dehydrogenase (*Lb*ADH)**. UniProt: Q84EX5 Genbank: AJ544275.1.

Gene sequence:

atgtctaaccgtttggatggtaaggtagcaatcattacaggtggtacgttgggtatcggtttagctatcgccacgaagttcgttgaagaaggggctaaggtcatgattaccggccggcacagcgatgttggtgaaaaagcagctaagagtgtcggcactcctgatcagattcaatttttccaacatgattcttccgatgaagacggctggacgaaattattcgatgcaacggaaaaagcctttggcccagtttctacattagttaataacgctgggatcgcggttaacaagagtgtcgaagaaaccacgactgctgaatggcgtaaattattagccgtcaaccttgatggtgtcttcttcggtacccgattagggattcaacggatgaagaacaaaggcttaggggcttccatcatcaacatgtcttcgatcgaaggctttgtgggtgatcctagcttaggggcttacaacgcatctaaaggggccgtacggattatgtccaagtcagctgccttagattgtgccctaaaggactacgatgttcgggtaaacactgttcaccctggctacatcaagacaccattggttgatgacctaccaggggccgaagaagcgatgtcacaacggaccaagacgccaatgggccatatcggtgaacctaacgatattgcctacatctgtgtttacttggcttctaacgaatctaaatttgcaacgggttctgaattcgtagttgacggtggctacactgctcaatag

Amino acid sequence:

MSNRLDGKVAIITGGTLGIGLAIATKFVEEGAKVMITGRHSDVGEKAAKSVGTPDQIQFFQHDSSDEDGWTKLFDATEKAFGPVSTLVNNAGIAVNKSVEETTTAEWRKLLAVNLDGVFFGTRLGIQRMKNKGLGASIINMSSIEGFVGDPSLGAYNASKGAVRIMSKSAALDCALKDYDVRVNTVHPGYIKTPLVDDLPGAEEAMSQRTKTPMGHIGEPNDIAYICVYLASNESKFATGSEFVVDGGYTAQ

## Induced fit docking of iminium in *Lb*ADH active site

The crystal structure of the alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*ADH, PDB ID: 1zk4) was loaded in YASARA (20.8.23). The docked acetophenone was removed from the crystal structure, and the *Lb*ADH was mutated either on position Y156 or S143 to glutamate. The energy was minimized, and the positively charged iminium ion formed from acetophenone was docked into the *Lb*ADH active site by induced fit docking (VINA). The YASARA sessions were saved as .pdb to be used as described below.

## FuncLib library

The following parameters were used for the generation of variants with the FuncLib algorithm (URL: https://funclib.weizmann.ac.il/bin/steps)[2]. Amino acid positions to diversify: 92A, 93A, 94A, 95A, 109A, 143A, 152A, 154A, 156A, 157A, 158A, 188A, 195A, 198A. Min ID: 35. Max targets: 4000. Coverage: 75. E value: 0.0001. A PDB file of *Lb*ADH-Y156E (**Table 2**) or *Lb*ADH-S143E (**Table** **3**) with the docked iminium intermediate of acetophenone as described above was used as input. Essential amino acid residue positions were: 113A, 141A, 142A, 144A, 155A, 159A, 189A, 205A. Ligands to keep during simulations were: 1270A (nicotinamide cofactor), 1682A (iminium intermediate). The single mutant and the three hits from the FuncLib algorithm with the highest predicted stability were selected for expression in the lab.

**Table 2**. List of the 50 most stable hits from Y156E-*Lb*ADH generated by FuncLib.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Rank | Label | 92A | 93A | 94A | 95A | 109A | 143A | 152A | 154A | 156A | 157A | 158A | 188A | 195A | 198A | ΔΔG (kJ/mol) |
| - | Y156E | I | A | V | N | L | I | L | A | N | A | S | G | V | L | -743.522 |
| 1 | YF1 | I | F | V | P | V | I | L | A | H | A | S | G | V | I | -759.661 |
| 2 | YF2 | I | F | V | P | L | I | L | A | H | A | S | G | L | Y | -759.462 |
| 3 | YF3 | I | Y | V | P | V | I | L | A | N | A | A | G | V | L | -759.456 |
| 4 |  | I | F | V | P | V | I | L | A | N | A | A | G | V | V | -759.404 |
| 5 |  | I | A | V | P | V | I | L | A | H | A | A | G | V | I | -759.046 |
| 6 |  | I | F | V | P | V | I | L | A | N | A | A | G | I | L | -758.942 |
| 7 |  | I | Y | V | P | I | I | L | A | H | A | S | G | V | Q | -758.734 |
| 8 |  | I | F | V | P | I | I | L | A | N | A | A | G | L | L | -758.728 |
| 9 |  | I | F | V | P | I | I | L | A | N | A | A | G | V | N | -758.599 |
| 10 |  | I | W | V | E | V | I | L | A | N | A | S | G | I | R | -758.548 |
| 11 |  | I | F | V | P | I | V | L | A | H | A | S | G | V | L | -758.397 |
| 12 |  | I | A | V | P | V | I | L | A | H | A | A | G | L | L | -758.393 |
| 13 |  | I | F | V | P | V | I | L | A | N | A | S | G | L | Q | -758.286 |
| 14 |  | M | F | V | P | L | I | L | A | N | A | S | G | L | Y | -758.272 |
| 15 |  | I | A | I | P | L | I | L | A | H | A | A | G | V | V | -758.248 |
| 16 |  | I | F | V | P | L | I | L | A | H | A | A | G | V | Y | -758.199 |
| 17 |  | I | W | V | P | V | I | L | A | H | A | S | G | L | L | -758.142 |
| 18 |  | I | F | V | P | I | I | L | A | H | A | S | G | V | Y | -758.035 |
| 19 |  | I | Y | V | N | I | I | L | A | H | A | A | G | V | Q | -758.034 |
| 20 |  | I | Y | V | P | I | I | L | A | N | A | A | G | V | V | -758.01 |
| 21 |  | I | Y | V | P | V | I | L | A | N | A | S | G | L | V | -757.948 |
| 22 |  | M | V | V | P | L | I | L | A | N | A | A | G | V | Q | -757.853 |
| 23 |  | I | F | V | N | I | I | L | A | H | A | A | G | V | I | -757.845 |
| 24 |  | I | Y | V | P | L | I | L | A | H | A | S | G | I | V | -757.789 |
| 25 |  | I | Y | V | P | L | I | L | A | H | A | A | G | V | Q | -757.729 |
| 26 |  | I | Y | V | P | I | V | L | A | N | A | S | G | L | L | -757.667 |
| 27 |  | I | Y | V | P | V | V | L | A | N | A | S | G | V | I | -757.656 |
| 28 |  | I | Y | I | P | V | I | L | A | N | A | S | G | V | N | -757.646 |
| 29 |  | I | Y | I | W | L | I | L | A | H | A | A | G | V | L | -757.605 |
| 30 |  | I | Y | I | W | L | I | L | A | N | A | A | G | L | L | -757.604 |
| 31 |  | I | T | V | E | L | I | L | A | H | A | A | G | V | R | -757.598 |
| 32 |  | I | Y | V | P | L | I | L | A | N | A | A | G | I | N | -757.549 |
| 33 |  | I | F | V | N | I | I | L | A | H | A | A | G | L | L | -757.487 |
| 34 |  | I | F | V | N | I | I | L | A | N | A | A | G | L | Q | -757.392 |
| 35 |  | I | Y | V | P | V | I | L | A | H | A | S | G | V | S | -757.371 |
| 36 |  | M | A | V | P | L | I | L | A | H | A | A | G | L | L | -757.343 |
| 37 |  | I | Y | V | P | L | V | L | A | N | A | A | G | V | N | -757.337 |
| 38 |  | I | Y | I | P | L | I | L | A | H | A | S | G | V | V | -757.321 |
| 39 |  | I | F | V | P | I | I | L | A | N | A | S | G | L | Y | -757.293 |
| 40 |  | I | Y | V | P | L | V | L | A | N | A | S | G | L | N | -757.277 |
| 41 |  | I | Y | Y | P | L | I | L | A | H | A | A | G | V | L | -757.276 |
| 42 |  | I | W | V | P | V | V | L | A | N | A | A | G | V | L | -757.271 |
| 43 |  | I | F | V | W | V | I | L | A | N | A | A | G | V | Q | -757.23 |
| 44 |  | I | W | V | P | V | I | L | A | N | A | S | G | V | Q | -757.156 |
| 45 |  | I | F | F | P | V | I | L | A | N | A | A | G | V | L | -757.153 |
| 46 |  | I | Y | V | P | V | I | L | A | N | S | S | G | V | Q | -757.148 |
| 47 |  | I | F | V | P | V | I | L | A | N | A | S | G | V | N | -757.147 |
| 48 |  | I | F | V | K | I | I | L | A | N | A | A | G | V | Q | -757.141 |
| 49 |  | I | Y | Y | P | L | I | L | A | N | A | A | G | V | Q | -757.104 |
| 50 |  | M | A | V | P | L | I | L | A | H | A | A | G | V | Y | -757.093 |

**Table 3**. List of the 50 most stable hits from S143E-*Lb*ADH generated by FuncLib.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Rank | Label | 92A | 93A | 94A | 95A | 109A | 143A | 152A | 154A | 156A | 157A | 158A | 188A | 195A | 198A | ΔΔG (kJ/mol) |
| - | S143E | I | A | V | N | L | I | L | A | N | A | S | G | V | L | -740.535 |
| 1 | SF1 | I | Y | V | P | L | I | L | A | A | A | A | G | V | I | -760.42 |
| 2 | SF2 | I | A | V | P | V | I | L | A | Q | A | A | G | V | I | -759.883 |
| 3 | SF3 | I | A | I | N | V | I | L | A | Q | A | A | G | V | I | -759.744 |
| 4 |  | I | Y | Y | N | L | I | L | A | A | A | A | G | V | I | -758.961 |
| 5 |  | I | T | I | P | L | I | L | A | Q | A | S | G | V | V | -758.929 |
| 6 |  | I | Y | V | P | L | I | L | A | C | A | A | G | V | V | -758.786 |
| 7 |  | V | T | V | P | L | I | L | A | H | A | S | G | V | I | -758.705 |
| 8 |  | I | Y | V | P | V | I | L | A | N | A | A | G | V | V | -758.703 |
| 9 |  | I | A | I | N | L | I | L | A | A | T | A | G | V | V | -758.476 |
| 10 |  | I | Y | V | L | L | I | L | A | C | A | A | G | V | I | -758.452 |
| 11 |  | V | Y | V | P | L | I | L | A | T | A | A | G | V | L | -758.421 |
| 12 |  | I | Y | V | P | V | I | L | A | T | A | S | G | V | I | -758.417 |
| 13 |  | I | T | V | P | L | I | L | A | Q | S | S | G | V | I | -758.383 |
| 14 |  | I | Y | V | P | L | I | L | A | T | A | A | G | V | M | -758.374 |
| 15 |  | I | Y | V | P | L | I | L | A | A | T | S | G | V | I | -758.339 |
| 16 |  | I | Y | V | P | L | I | L | A | N | T | A | G | V | I | -758.238 |
| 17 |  | I | T | V | L | L | I | L | A | A | A | A | G | V | I | -758.23 |
| 18 |  | I | Y | V | P | V | I | L | A | T | A | A | G | V | L | -758.189 |
| 19 |  | I | A | V | P | V | V | L | A | H | A | S | G | V | I | -758.185 |
| 20 |  | I | A | I | P | L | I | L | A | H | A | A | G | V | V | -758.163 |
| 21 |  | I | A | Y | P | V | I | L | A | Q | A | A | G | V | L | -758.129 |
| 22 |  | I | Y | V | P | L | I | L | A | N | A | A | G | I | I | -758.104 |
| 23 |  | V | Y | V | P | L | I | L | A | A | A | S | G | V | I | -758.063 |
| 24 |  | I | T | V | E | L | I | L | A | Q | A | A | G | V | V | -758.028 |
| 25 |  | I | V | I | N | L | I | L | A | Q | A | A | G | V | I | -757.988 |
| 26 |  | I | T | V | P | L | I | L | A | A | S | A | G | V | L | -757.956 |
| 27 |  | I | A | V | N | V | I | L | A | A | A | A | G | V | I | -757.914 |
| 28 |  | I | T | V | P | L | I | L | A | A | A | S | G | V | I | -757.849 |
| 29 |  | I | A | I | S | L | I | L | A | Q | A | A | G | V | I | -757.849 |
| 30 |  | I | A | V | P | L | I | L | A | H | A | A | G | I | I | -757.84 |
| 31 |  | I | A | V | P | L | I | L | A | A | T | A | G | V | M | -757.778 |
| 32 |  | I | A | V | P | V | I | L | A | H | A | A | G | V | M | -757.723 |
| 33 |  | I | Y | V | L | L | I | L | A | A | A | A | G | V | V | -757.717 |
| 34 |  | I | T | V | P | L | I | L | A | H | A | A | G | I | L | -757.673 |
| 35 |  | I | Y | V | P | L | I | L | A | T | A | A | G | I | L | -757.646 |
| 36 |  | V | Y | V | P | L | I | L | A | N | A | A | G | V | I | -757.567 |
| 37 |  | I | A | Y | N | V | I | L | A | Q | A | A | G | V | V | -757.556 |
| 38 |  | I | T | V | N | L | V | L | A | Q | A | A | G | V | I | -757.555 |
| 39 |  | I | V | V | P | L | V | L | A | H | A | S | G | V | I | -757.543 |
| 40 |  | I | A | L | P | L | I | L | A | A | A | A | G | V | I | -757.532 |
| 41 |  | I | T | V | N | L | V | L | A | H | A | A | G | V | M | -757.528 |
| 42 |  | I | Y | V | N | I | I | L | A | H | A | A | G | V | I | -757.486 |
| 43 |  | I | T | I | N | L | I | L | A | H | A | A | G | V | I | -757.461 |
| 44 |  | I | A | Y | P | I | I | L | A | Q | A | S | G | V | I | -757.372 |
| 45 |  | I | A | Y | N | L | I | L | A | T | T | A | G | V | I | -757.338 |
| 46 |  | I | T | I | P | L | I | L | A | H | A | A | G | V | L | -757.321 |
| 47 |  | V | T | I | N | L | I | L | A | Q | A | S | G | V | V | -757.296 |
| 48 |  | I | Y | V | P | L | I | L | A | H | S | A | G | V | L | -757.288 |
| 49 |  | V | A | V | P | V | I | L | A | T | A | S | G | V | I | -757.264 |
| 50 |  | V | A | I | N | L | I | L | A | Q | A | A | G | V | V | -757.257 |
| 51 |  | I | Y | V | P | L | I | L | A | H | A | S | G | V | I | -757.254 |
| 52 |  | I | A | L | P | L | I | L | A | T | A | A | G | V | V | -757.25 |

## Production of *Lb*ADH-wt and variants

The gene sequences for the *Lb*ADH-wt, the serine and tyrosine variants were ordered in a pET28a(+) vector from SynBio (Monmouth Junction, NJ, USA). The corresponding plasmids were transformed in *E. coli* C43(DE3) competent cells. The transformed cells were grown on selective LB-agar plates (50 µg/mL kanamycin) overnight at 37 °C. TB-medium (500 mL in 2L baffled flask) was inoculated with 1% overnight LB preculture of the transformed cells and incubated for 3-5 h at 37 °C, 180 rpm. After reaching an OD600 of 0.6-0.8, 0.5 mM IPTG was added, followed by overnight incubation at 22 °C. Cells were harvested (17,000 × *g*, 20 min, 4 °C) and stored at -80 °C.

For *Lb*ADH-A94D and *Lb*ADH-A94E, primers were ordered from BaseClear (Leiden, The

Netherlands). Site-directed mutagenesis was performed *via* PCR using a Q5® High-Fidelity 2X Master Mix (NEB M0492) from New England Biolabs (Ipswich, MA, USA). The produced plasmids were purified using a Monarch® Plasmid Miniprep Kit (NEB T1010) from New England Biotech and were sequenced by Macrogen (Amsterdam, The Netherlands) (Appendix C.2). The mutated genes were confirmed with an alignment with CloneManager Professional Suite (**Figure 1**).

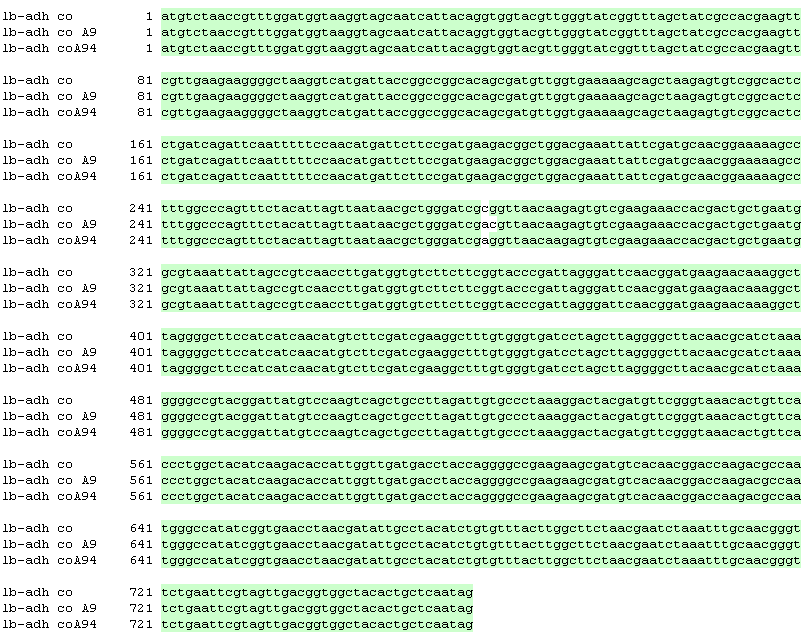
Primer sequences:

A94E forward: CGC TGG GAT CGA GGT TAA GA

A94E reverse: TTA TTA ACT AAT GTA GAA ACT GGG CC

A94D forward: CGC TGG GAT CGA CGT TAA CAA GAG

A94D reverse: TTA TTA ACT AAT GTA GAA ACT GG



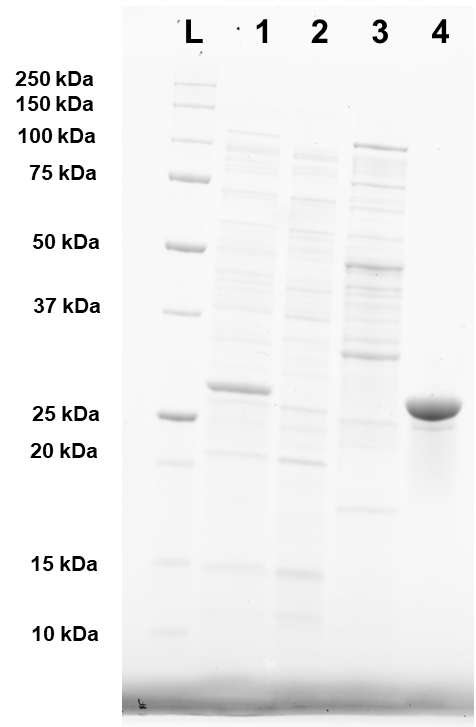
**Figure S1**. Alignment of genes encoding for *Lb*ADH-wt (top), *Lb*ADH-A94D (middle) and *Lb*ADH-A94E (bottom). Made with Clone Manager.

## Affinity chromatography purification of *Lb*ADH-wt and variants

Two buffers were prepared for each purification: a 20 mM sodium phosphate (NaPi) buffer with 0.5 M NaCl, 1 mM MgCl2, pH 7.4, titrated with 3 M NaOH (referred to as ‘binding buffer’) and a 20 mM NaPi buffer with 0.5 M NaCl, 250 mM imidazole, 1 mM MgCl2, pH 7.4, titrated with 3 M HCl (referred to as ‘elution buffer’). For preparation of the cell-free extracts the cells were resuspended in 20 mM NaPi buffer pH 7.5, 0.5 M NaCl, 1 mM MgCl2. To the buffer, EDTA-free protease inhibitor, lysozyme and DNaseI were added. After 10 min incubation, the suspension was passed through a cell disruptor at 22 kpsi with 50 mM KP*i* buffer pH 7.5 with 1 mM MgCl2 and clarified by centrifugation (32,000 × *g*, 30 min, 4 °C). After filtering the CFE (0.2 µm), IMAC was performed with 5 mL HisTrapTM FF crude column (GE Healthcare, Chicago, Illinois, U.S.). These columns were attached into a Bio-Rad NGC Chromatography system.

Protein fractions were concentrated with Amicon® Ultra-15 10 K Centrifugal Filter Devices at 4,000 rcf. Desalting was performed using PD10 desalting columns (52-1308-00 AP) from GE Healthcare (Chicago, IL, USA), equilibrated with 50 mM potassium or sodium phosphate buffer, pH 7.4, with 1 mM MgCl2. 10% glycerol and 50 mM NaCl were added to the pure protein fraction which was then stored at -80°C. When comparing storage conditions, stocks were placed either at 4 °C and -20 °C. Total protein amounts were determined by BC Assay (Uptima Protein Quantitation Kit UP40840A, Interchim, Montlucon, France).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run to show gene expression and enzyme purity levels (Figures 2 - **Figure 8**). Samples were prepared by mixing with one equivalent of Laemmli buffer and 5% v/v dithiothreitol (DTT), heated to 95 °C for 5 min, then centrifuged at 9,000 × *g* for 2 min. From these samples, 10 μL was loaded onto the gel, whereas 5 μL of protein ladder was loaded onto a Criterion TGX Stain-Free Precast Gel. Imaging was performed with a ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, California, U.S.).

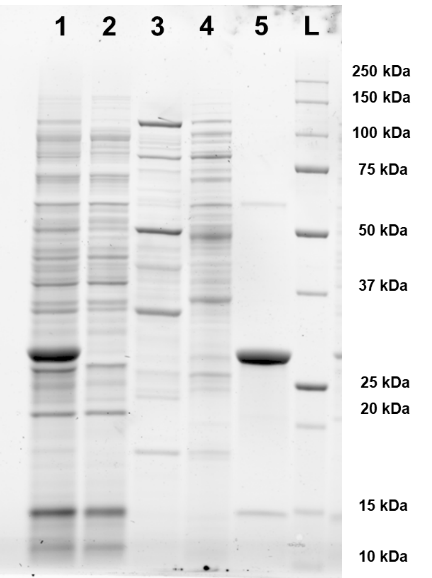


**Figure S2**. SDS-PAGE of the purification of the *Lb*ADH-wt IMAC purification. L: protein ladder. 1: cell-free extract. 2: flowthrough after loading on Histrap colum. 3: washing fraction. 4:eluted *Lb*ADH-wt.

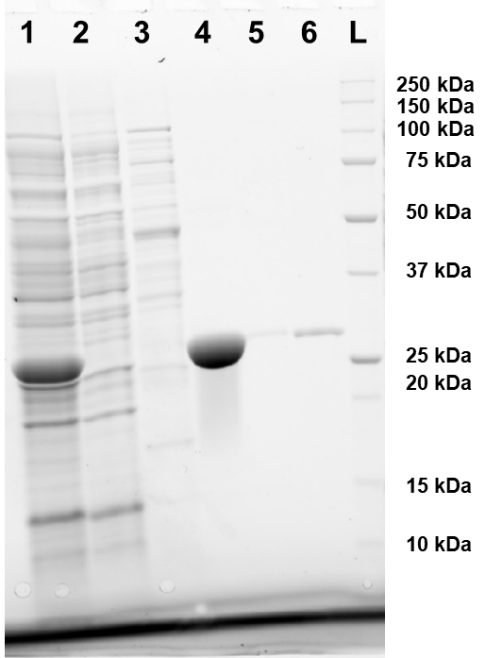
A close-up of a dna test

Description automatically generated

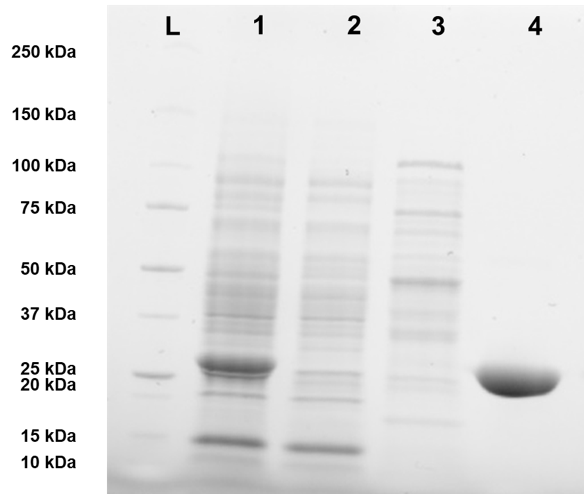
**Figure S3**. SDS-PAGE of the purification of the *Lb*ADH-Y156E IMAC purification 1: cell-free extract. 2: flowthrough after loading on Histrap column. 3-4: washing fractions. 5: eluted *Lb*ADH-Y156E.



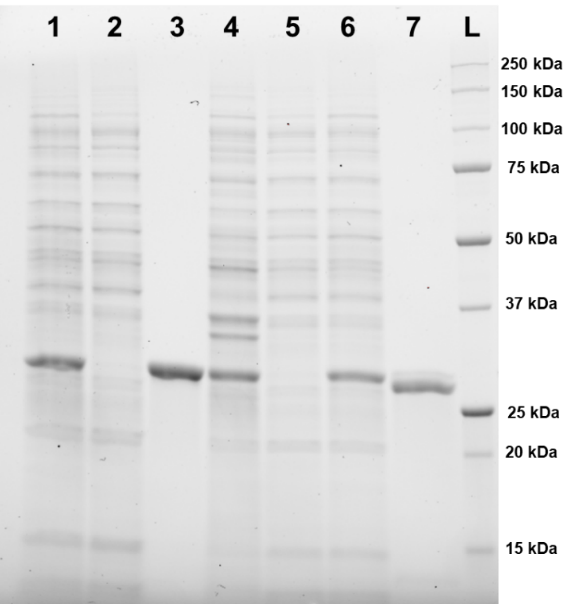
**Figure S4**. SDS-PAGE of the purification of *Lb*ADH-YF1 IMAC purification. 1: cell-free extract. 2: flowthrough after loading on Histrap column. 3-4: washing fractions. 5: eluted *Lb*ADH-YF1. L: protein ladder.



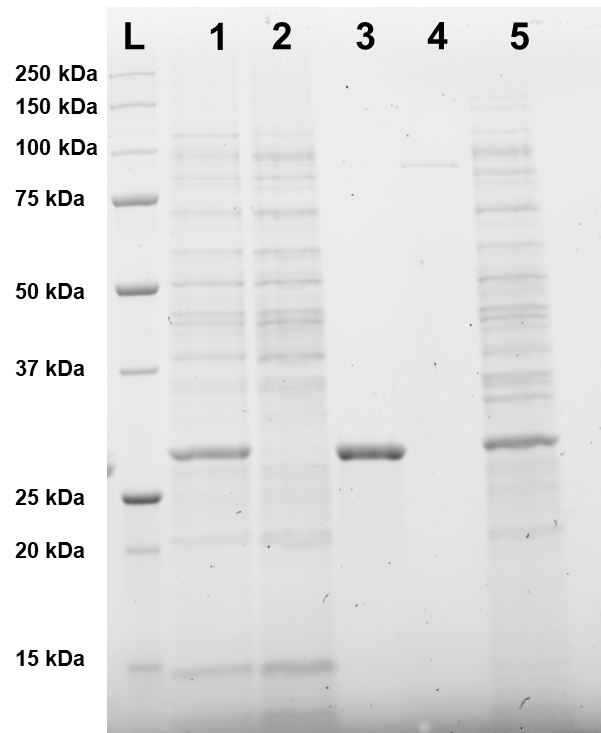
**Figure S5**. SDS-PAGE of the purification of *Lb*ADH-YF2 IMAC purification. 1: cell-free extract. 2: flowthrough after loading on Histrap column. 3: washing fractions. 4-6: eluted *Lb*ADH-YF2. L: protein ladder.



**Figure S6**. SDS-PAGE of the purification of *Lb*ADH-YF3 IMAC purification. L: protein ladder. 1: cell-free extract. 2: flowthrough after loading on Histrap column. 3: washing fractions. 4: eluted *Lb*ADH-YF3.



**Figure S7**. SDS-PAGE of the purification of *Lb*ADH-S143E (1-3) and *Lb*ADH-SF1 (4-7) IMAC purifications. 1: cell-free extract. 2: flowthrough after loading on Histrap column. 3: eluted *Lb*ADH-S143E. 4: cell-free extract. 5: flowthrough after loading on Histrap column. 6: elution fraction with *Lb*ADH-SF1. 7: pure fraction with *Lb*ADH-SF1.



**Figure S8**. SDS-PAGE of the purification of *Lb*ADH-SF2 IMAC purification. L: protein ladder. 1: cell-free extract. 2: flowthrough after loading on Histrap column. 3: eluted *Lb*ADH-SF2.

# Carbonyl substrates and corresponding products



**Figure S9**. Panel of ketone substrates used in screening of *Lb*ADH variants towards ketoreduction or reductive amination.

**Table S4**. List of carbonyl substrates, original alcohol products and targeted amine products with the corresponding labels used in this work.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Label** | **Carbonyl substrates** | **Label** | **Alcohol product** | **Label** | **Amine product** |
| **1a** | acetophenone | **2a** | phenylethanol | **3a** | phenylethylamine |
| **1b** | phenylacetone | **2b** | 1-phenyl-2-propanol | **3b** | 1-phenylpropan-2-amine |
| **1c** | 4-phenyl-2-butanone | **2c** | 4-phenyl-2-butanol | **3c** | 4-phenylbutan-2-amine |
| **1d** | 2-methylcyclohexanone | **2d** | 2-methylcyclohexanol | **3d** | 2-methylcyclohexanamine |
| **1e** | 3-methylcyclohexanone | **2e** | 3-methylcyclohexanol | **3e** | 3-methylcycolhexanamine |
| **1f** | 2-hexanone | **2f** | 2-hexanol | **3f** | 2-hexanamine |
| **1g** | 2-heptanone | **2g** | 2-heptanol | **3g** | 2-heptanamine |
| **1h** | 2-octanone | **2h** | 2-octanol | **3h** | 2-octanamine |
| **1i** | 3-octanone | **2i** | 3-octanol | **3i** | 3-octanamine |
| **1j** | hexane-2,3-dione | **2j** | hexane,2-3-diol | **3j** | 2-aminohexan-3-one |
| **1k** | hexanal | **2k** | 1-hexanol | **3k** | 1-hexanamine |
| **1l** | benzaldehyde | **2l** | benzyl alcohol | **3l** | benzylamine |

# Activity assays

Specific activity measurements were carried out with a volume of 2 mL in a 4 mL UV cuvette monitoring the consumption of NADPH at a wavelength of 340 nm on a Cary 60 UV-Vis spectrophotometer. The extinction coefficient of NAD)H was assumed to be 6220 M-1cm-1.[3] Carbonyl substrates were prepared as a 1 M stock solution in DMSO. NAD(P)H stock solution was prepared in the mentioned buffer as a 10 mM concentration (confirmed by UV spectroscopy).

**Figure S11**. Specific activity of *Lb*ADH-wt after storage in 4, -20 and -80 °C, without (light blue) and with (dark blue) 10% glycerol and 50 mM NaCl. Conditions: 11 mM acetophenone, 0.2 mM NADP+, 50 mM KP*i* buffer pH 7.5, 1 mM MgCl2, 30 °C. Averages of triplicates.

The specific activity of *Lb*ADH-wt and *Lb*ADH-YF2 was measured with the best performing substrate 2-methylcyclohexanone from the previous screening (**Table 6**). For *Lb*ADH-wt, the specific activity towards 2-methylcyclohexanone was 137 U/mg, compared with 22.6 U/mg for acetophenone in similar conditions (1% v/v DMSO as co-solvent). *Lb*ADH-YF2 gave a specific activity of 20 mU/mg.

**Table S6**. Specific activities of *Lb*ADH-wt and *Lb*ADH-YF2 with 2-methylcyclohexanone.

|  |  |
| --- | --- |
| ***Lb*ADH** | **Specific activity (U/mg)** |
| wt | 137 ± 3.3 |
| YF2 | 0.02 ± 0.00 |

Conditions: 0.2 mM NADP+, 50 mM KPi buffer pH 7.5, 1 mM MgCl2, 30 °C. Average of duplicates.

# Biocatalytic reactions

## Ketoreduction conditions

To 50 mM KP*i* buffer with 1 mM MgCl2 and pH 7.5 in a glass GC vial, glucose (20 mM), GDH-105 (6 U/mL), NADP+ (0.2 mM), *Lb*ADH (2 µM) and ketone substrate (10 mM) were added. Products were extracted with 0.5 mL EtOAc. The isolated organic layer was dried with MgSO4, centrifuged at 10,000 × *g* for 1 min, decanted to a GC vial and injected onto the GC-FID.

## Reductive amination conditions

To 1 M NH4HCOO buffer (titrated to pH 8.0 or 9.0 with 4 M NH4OH), glucose (20 mM), GDH-105 (6 U/mL), NADP+ (0.2 mM), ketone substrate (10 mM) and *Lb*ADH (10 µM) were added. In case of reactions with methylamine, 250 mM methylamine was added to 50 mM Tris-HCl buffer with 1 mM MgCl2 at pH 9.0. The reaction mixtures of 0.5 mL were incubated for 24 h at 30 °C and 500 rpm. For reductive amination conditions, 0.4 mL 10 M NaOH was added, and the reactions were extracted twice with 0.5 mL EtOAc. The isolated organic layers were combined, dried with MgSO4, centrifuged at 10,000 × *g* for 1 min, decanted to a GC vial and injected onto the GC-FID.

*trans*

*cis*

**Figure S10**. Ketoreduction of 2-methylcyclohexanone in 2-methylcyclohexanol under reductive amination conditions, and the corresponding enantiomeric excess of either the *trans*- (diamonds) or *cis*-product (triangles). *ee* was not determined for conversions under 10%. Conditions: 1 M NH4HCOO buffer, pH 8.0, 0.2 mM NADP+, 6 U/mL *Bs*GDH, 10 μM *Lb*ADH, 20 mM glucose, 24 h at 500 rpm, 30 °C. Average of duplicates.

**Table S5**. Diastereomeric excess (*de*) of the *trans*-2-methylcyclohexanol catalysed by the *Lb*ADH variants from screening in reductive amination conditions.

|  |  |
| --- | --- |
| ***Lb*ADH** | ***de* (%)** |
| wt | 8 |
| Y156E | 73 |
| YF1 | 72 |
| YF2 | 10 |
| YF3 | 91 |

## Imine reductase conditions

To 50 mM Tris-HCl buffer pH 9.0, 1 mM MgCl2 glucose (20 mM), GDH-105 (6 U/mL), NADP+ (0.2 mM), aldehyde substrate (either hexanal or benzaldehyde 10 mM), amine donor (either cyclopropylamine or allylamine, 100 mM) and *Lb*ADH (10 µM) were added. The reaction mixtures of 0.5 mL were incubated for 24 h at 30 °C and 500 rpm. For reductive amination conditions, 0.4 mL 10 M NaOH was added, and the reactions were extracted with 0.5 mL EtOAc. The isolated organic layer was dried with MgSO4, centrifuged at 10,000 × *g* for 1 min, decanted to a GC vial and injected onto the GC-FID.

# GC analyses

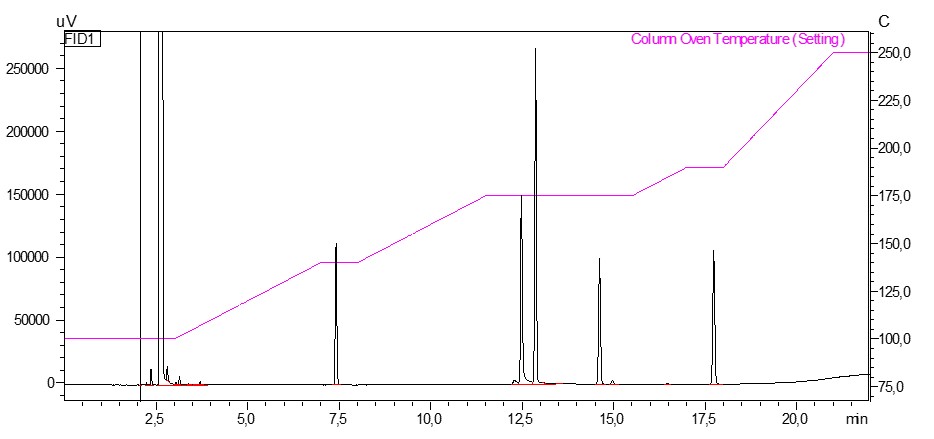
Samples were measured on Shimadzu GC-2010 gas chromatographs (Kyoto, Japan) with an AOC-20i Auto injector equipped with a flame ionization detector (FID), using nitrogen or helium as the carrier gas. Products were confirmed by reference standards. Product concentrations were obtained with a calibration curve equation using 5 mM dodecane or tridecane as an internal standard. All samples were injected with GC quality ethyl acetate (EtOAc), except where specified with diethyl ether (Et2O) or methyl *tert*-butyl ether (MTBE). Columns used in this work:

1. **CP-Wax 52 CB** (Agilent Technologies, Santa Clara, California, United States) (25 m × 0.53 mm × 2.0 µm), injection at 250 °C, split ratio 100, flow 4 mL/min, nitrogen as carrier gas.
2. **CP Chirasil-DEX** **CB** 1a, 25 m x 0.32 mm x 0.25 µm, injection at 250 °C, split ratio 100, flow 4 mL/min, helium as carrier gas.
3. **Hydrodex β-TBDM** (Macherey-Nagel, Düren, Germany), 50 m × 0.25 mm × 0.15 µm, heptakis-(2,3-di-O-methyl-6-O-*t*-butyldimethyl-silyl)-β-cyclodextrin, injection at 250 °C split ratio 50, linear velocity 38 cm/s, column flow 2.23 mL/min, helium as carrier gas.
4. **CP Sil 5 CB Split**, 25 m x 0.25 mm x 1.20 µm, injection at 250 °C, split ratio 50, flow 4 mL/min, nitrogen as carrier gas.

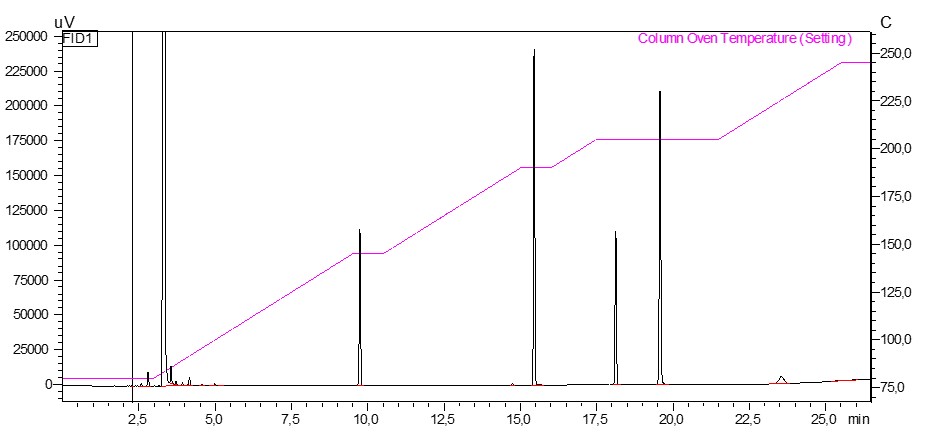
**Table S7**. Overview of GC methods and retention times for ketone substrates **1a-k** and aldehyde substrates **1l-o**.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| No. | Column | Parameters | Ramp (°C/min) | Temp. (°C) | Hold time (min) | Compound | Ret. time (min) |
| 1 | **A** | Split ratio: 50  Linear velocity: 30.0 cm/s | -  10 10 10 20 | 100 140 175 190 250 | 3 1 4 1 1 | tridecane  phenylethylamine **3a**  DMSO  acetophenone **1a**  phenylethaniminea  phenylethanol **2a** | 7.4  12.5  13.0  14.4  16.4  17.4 |
| 2 | **A** | Split ratio: 50  Linear velocity:  30.0 cm/s | - 10 10 10 10 | 80 145 190 205 245 | 3 1 1 4 1 | tridecane  DMSO  phenylacetone **1b**  1-phenyl-2-propanol **2b** | 9.8  15.6  18.2  19.7 |
| 3 | **A** | Split ratio: 50  Linear velocity: 30.0 cm/s | - 10 10 10 10 | 80 145 190 230 245 | 3 1 1 4 1 | tridecane  DMSO  4-phenyl-2-butanone **1c**  4-phenyl-2-butanol **2c** | 9.8  15.6  20.2  21.9 |
| 4 | **B** | Split ratio: 100  Linear velocity: 30.0 cm/s | - 5 5 5 10 | 70 80 90 100 220 | 2 3 3 2 1 | DMSO  2-methylcyclohexanamine **3d**  2-methylcyclohexanone **1d**  2-methylcyclohexanol **2d**  tridecane | 6.3  11.2, 13.4  11.5, 11.8  16.1, 16.2, 17.1, 17.2  20.5 |
| 5 | **B** | Split ratio: 50  Linear velocity: 38.0 cm/s | - 1.02  2  2  2  2  2  2  20 | 50 55 60 75  80  95  100  105  220 | 15.1 2 2 2  2  2  2  2  1.25 | DMSO  2-methylcyclohexanone **1e**  2-methylcyclohexanol **2e**  dodecane  tridecane | 12.4  19.1, 19.6  23.2, 23.5, 23.9  25.6  30.7 |
| 6 | **C** | Split ratio: 50  Linear velocity: 38.0 cm/s | - 5 5 20 | 80 100 120 240 | 5 10 5 1 | DMSO  3-methylcyclohexanamine **3e**  3-methylcyclohexanone **1e**  3-methylcyclohexanol **2e**  dodecane  tridecane | 12.4  13.9, 14.2, 15.8, 16.1  19.1, 19.6  23.2, 23.5, 23.9  25.6  30.7 |
| 7 | **A** | Split ratio: 50  Linear velocity: 30.0 cm/s | - 5 20 | 80 150 250 | 3 1 1 | 2-hexanamine **3f**  2-hexanone **1f**  2-heptanamine **3g**  2-heptanone **1g**  tridecane 2-heptanol **2g**  2-hexanol **2f**  DMSO | 5.6  6.8  7.8  9.4  12.1  12.8  13.9  17.6 |
| 8 | **A** | Split ratio: 50  Linear velocity: 30.0 cm/s | - 10 10 30 | 100 130 165 250 | 3 7 2 1 | 2-octanamine **3h**  tridecane  2-octanone **1h**  2-octanone **2h**  DMSO | 6.5  7.9  8.2  11.3  18.0 |
| 9 | **C** | Split ratio: 50  Linear velocity: 38.0 cm/s | - 5 5 10 20 | 80 110 125 180 220 | 3 3 3 1.5 1 | DMSO 3-octanone **1i**  3-octanamine **3i**  3-octanol **2i**  tridecane | 10.7  11.4  11.5, 11.6  15.4  22.0 |
| 10 | **C** | Split ratio: 50  Linear velocity: 38.0 cm/s | - 5 | 80 220 | 3 2 | hexane-2,3-dione **1j**  DMSO  hexane-2,3-diol **2j**  2-hydroxy-3-hexanone  tridecane | 5.8  10.6  17.8  11.6  18.9 |
| 11 | **D** | Split ratio: 100  Linear velocity: 30.0 cm/s | -  5 5 20 | 50 60 65 340 | 4 2 4 1 | hexanal **1l**  1-hexanamine **3l**  1-hexanol **2l**  benzaldehyde **1m**  DMSO  tridecane | 7.7  9.7  11.3  13.6  14.7  22.0 |
| 12 | **A** | Split ratio: 50  Linear velocity: 30.0 cm/s | -  5 20 | 80 150 250 | 3 1 1 | hexanal  dodecane  iminea  *N*-hexylcyclopropanamine  DMSO | 6.8  9.2  11.8  11.3  20.3 |

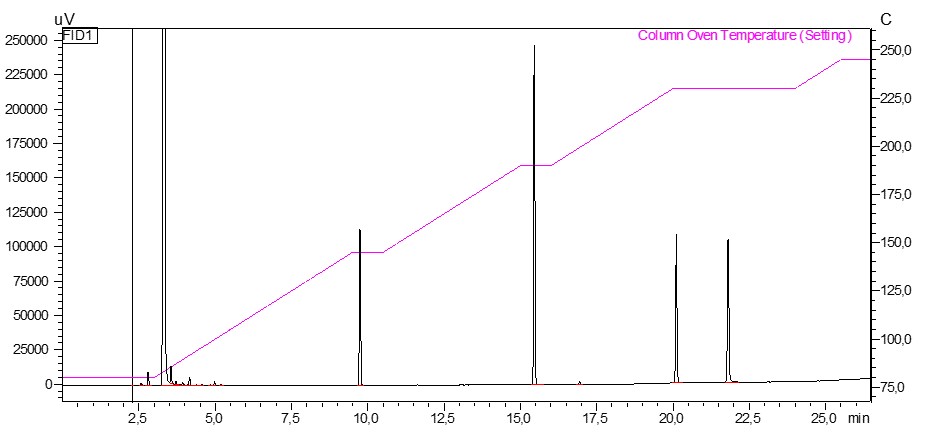
*a* hypothesized



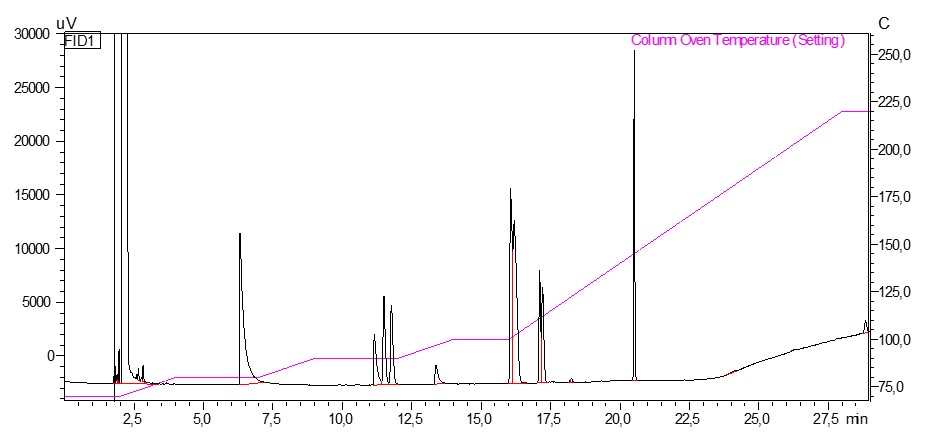
**Figure S12.** Gas chromatogram of acetophenone (14.1 min), phenylethanol (17.4) and phenylethanamine (12.5 min) (Method 1).



**Figure S13.** Gas chromatogram of phenylacetone (18.2 min) and 1-phenyl-2-propanol (19.7 min) (Method 2).



**Figure S14.** Gas chromatogram of 4-phenyl-2-butanone (20.2 min) and 4-phenyl-2-butanol (21.9 min) (Method 3).



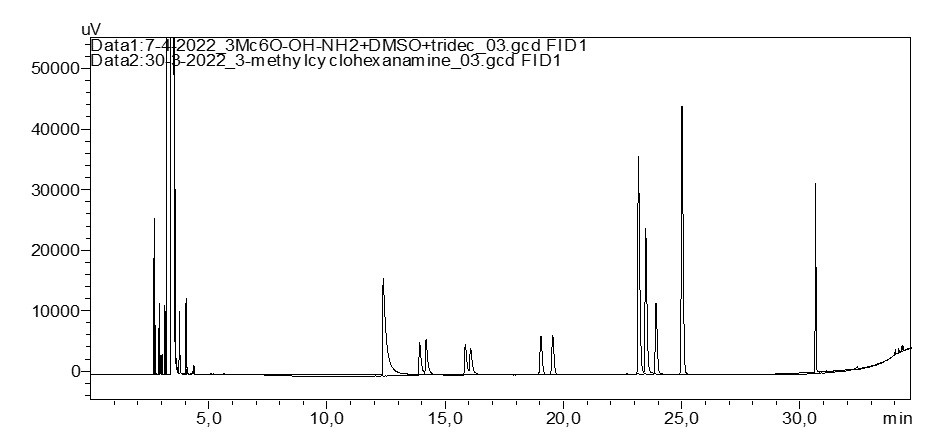
**Figure S15.** Gas chromatogram of 2-methylcyclohexanone (11.5, 11.8 min), 2-methylcyclohexanol (16.1, 16.2, 17.1, 17.2 min) and 2-methylcyclohexanamine (11.2, 13.4 min) (Method 4).



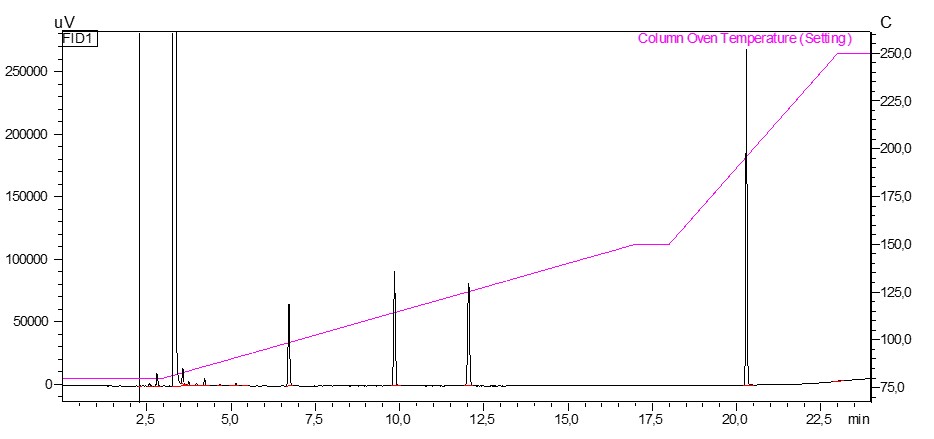




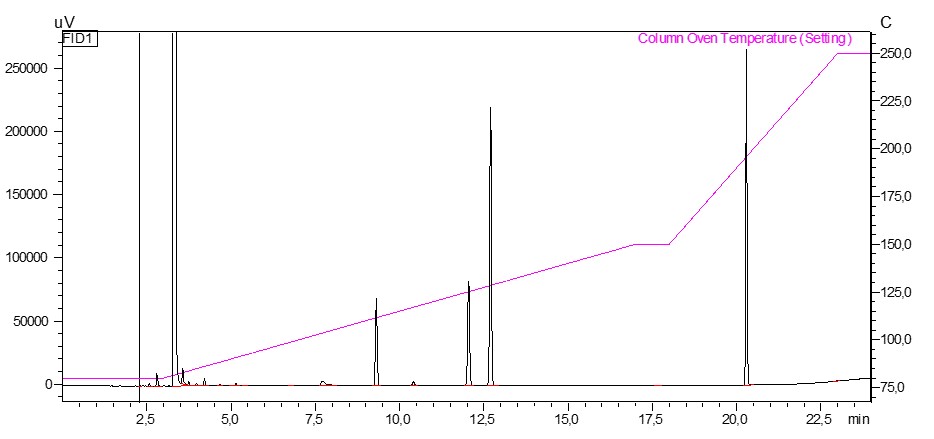
**Figure S16.** Gas chromatograms of 2-methylcyclohexanol (top), *cis*-2-methylcyclohexanol (middle) and a reaction mixture with YF2-LbADH and 2-methylcyclohexanone (bottom) (Method 5).



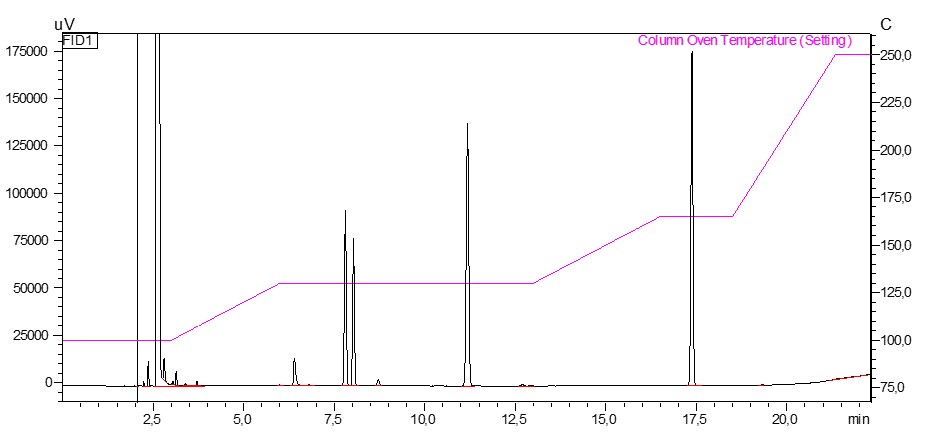
**Figure S17**.Gas chromatogram of 3-methylcyclohexanone (19.1, 19.6 min), 3-methylcyclohexanol (23.2, 23.5, 23.9 min) and 3-methylcyclohexanamine (13.9, 14.2, 15.8, 16.1 min) (Method 6).



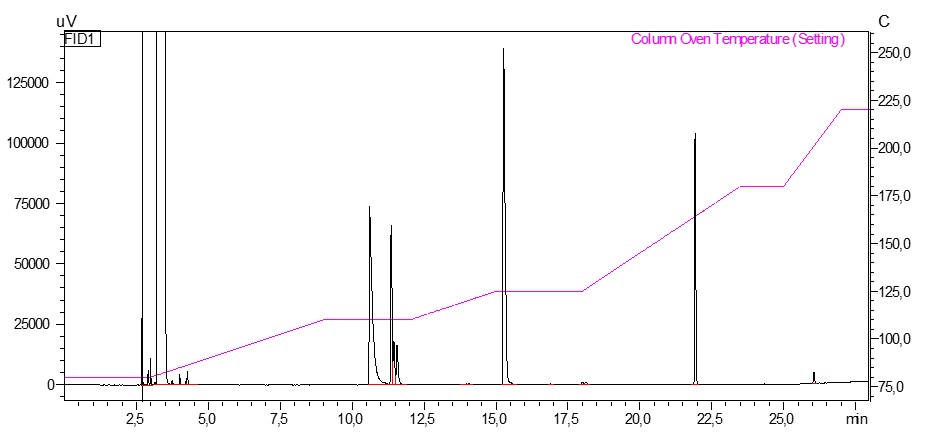
**Figure S18**. Gas chromatogram of 2-hexanone (6.8 min), 2-hexanol (13.9 min) and 2-hexanamine (5.6 min) (Method 7).



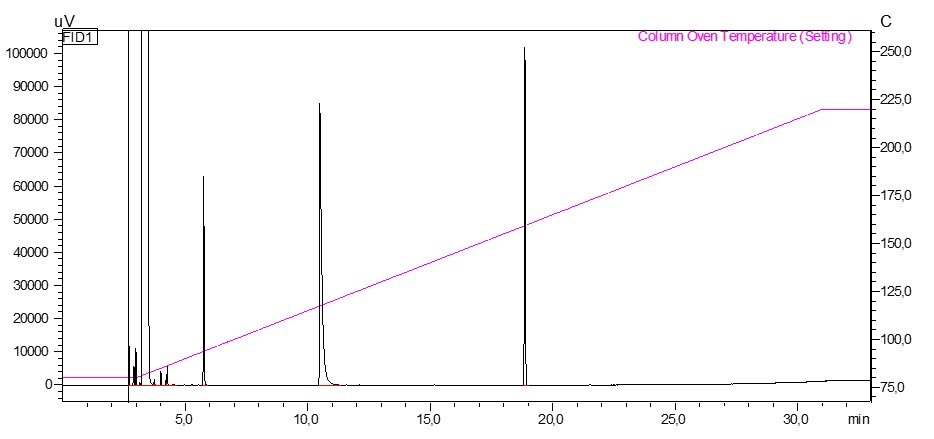
**Figure S19.** Gas chromatogram of 2-heptanone (9.4 min), 2-heptanol (12.8 min) and 2-heptanamine (7.8 min) (Method 8).



**Figure S20.** Gas chromatogram of 2-octanone (8.2 min), 2-octanol (11.3 min) and 2-octanamine (6.5 min) (Method 9).



**Figure S21.** Gas chromatogram of 3-octanone (11.4 min), 3-octanol (15.4 min) and 3-octanamine (11.5, 11.6 min) (Method 10).



**Figure S22.** Gas chromatogram of hexane-2,3-dione (5.8 min), hexane-2,3-diol (17.8 min) and 2-hydroxy-3-hexanone (11.6 min) (Method 11).



**Figure S23.** Gas chromatogram of reaction mixture with *Lb*ADH-Y156E, hexanal (6.8 min), hexanol (13.7 min) and the corresponding imine formed with cyclopropylamine (11.8 min) and amine product *N*-hexylcyclopropanamine (11.3 min) (Method 12).



**Figure S24.** Overlay of reaction mixture with *Lb*ADH-Y156E with hexanal and cyclopropylamine (pink) and a negative control of extracted hexanal and cyclopropylamine in buffer (black) (Method 12).

# Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed using the crystal structures of *Lb*ADH (PDB ID: 1ZK4)[4] from the Protein Data Bank[5] to describe the wild-type enzyme, with mutant forms of *Lb*ADH generated using the Dunbrack 2010 Rotamer library as implemented in Chimera.[6] *Lb*ADH is a homotetrameric enzyme whose quaternary structure is stabilized by the presence of two divalent magnesium ions at the dimerization interface. The structure of the homotetramer was generated in PyMOL[7] using the symmetry matrix, and protonation states of all titratable residues were assigned to their predicted states at physiological pH based on predictions using PROPKA v 3.1.[8] Histidine side-chain protonation states were assigned based on a visual inspection of their local environment to maintain optimal hydrogen bonding networks.

|  |
| --- |
| One limitation of the *Lb*ADH structure used in these simulations is that in this structure, a critical substrate binding loop (Thr192 – Lys210)[4] is found in an open conformation. This results in substrate dissociation from the active site when attempting simulations from this starting structure. In contrast, the crystal structure of a different ADH from *Lactobacillus kefir* (*Lk*ADH, PDB ID: 4RF2) presents this loop in a closed conformation, and as these two enzymes share both 70% sequence identity and high structural homology in this region (**Figure 25**), we grafted the closed loop onto the *Lb*ADH structure in our simulations, thus allowing us to retain the substrate in the active site.  A close-up of a structure  Description automatically generated |

**Figure S25.** Overlay of the structures of the ADHs from *Lactobacillus brevis* (*Lb*ADH, PDB ID: 1ZK4[4]) and *Lactobacillus kefir* (*Lk*ADH, PDB ID: 4RF2), with the active site loop in open (pink) and closed (yellow) conformations, respectively.

The substrate molecules, acetophenone (**1a**) and the corresponding iminium intermediate derived from **1a**, the (*R*) and (*S*) enantiomers of 2-methylcyclohexanone (**1d**) and the iminium intermediate formed from hexanal and cyclopropylamine for IRED activity were parametrized using Antechamber[9] and the General AMBER Force Field (GAFF)[10]. Partial charges were calculated at the HF/6-31G\* level of theory using Gaussian16 Rev.[11] and fitted using the standard RESP protocol.[12] NADPH parameters were adopted from the literature[13], and an octahedral cationic dummy model was used to describe Mg2+.[14, 15]

The simulations were performed using the GPU version of the AMBER20 simulation package[16] with the ff14SB force field[17] and TIP3P[18] water model. All systems were solvated in an octahedral box of water molecules extending to 10 Å from the closest solute atom. Sodium and chloride ions were added for overall charge neutrality.

The preparation for production MD simulations involved a series of steps, including energy minimization, system heating, and equilibration. All hydrogen atoms and solvent molecules were first minimized using 100 steps of steepest descent, followed by 900 steps of conjugate gradient minimization. A 100 kcal mol-1 Å-2 positional restraint was placed on all heavy (non-hydrogen) protein and ligand atoms during this process. The positional restraints of 25 kcal mol-1 Å-2 were applied as the system was heated from 50 to 300 K in an NVT ensemble over 50 ps of simulation time using a 2fs time step and Langevin thermostat[19] (collision frequency of 1 ps-1). After the initial steps of energy minimization, the subsequent phase involved another round of energy minimization with 5 kcal mol-1 Å-2 positional restraints specifically applied to the Cα-atoms. This process consisted of 1000 steps of steepest descent minimization. Following this, the system underwent a heating process from 25 K to 300 K over a period of 20 picoseconds (ps) in an NVT ensemble, maintaining constant volume and temperature. Following the preparatory phases, the simulations progressed into the NPT ensemble at 300 K and 1 atm pressure, employing Langevin thermostat with a collision frequency of 1 ps-1 and a Berendsen barostat[20] with a 1 ps pressure relaxation time. A simulation time step of 2 femtoseconds (fs) was utilized during the NPT simulations. During these simulations, four quasi-harmonic distance restraints of 20 kcal mol-1 Å-2 were implemented strategically. The first restraint targeted the reactive distance between the substrate and the transferrable hydride of NADPH. The remaining three restraints were crucial for maintaining specific structural features: one between the Cα-atoms of amino acid residues A94 and L195, another between N96 and L199, and the fourth between D63 and the amino group of the adenine ring of NADPH. These restraints were helpful in preventing the substrate from escaping the active site throughout the MD simulations. Gradual release of the 5 kcal mol-1 Å-2 positional restraints occurred in increments of 1 kcal mol-1 Å-2 every 10 picoseconds (ps) during 40 ps of simulation time. Subsequently, a 7 nanosecond (ns) MD simulation was conducted for further equilibration. During this phase, the distance restraints were reduced from 20 kcal mol-1 Å-2 to 5 kcal mol-1 Å-2, decreasing incrementally every 1 ns simulation time while maintaining the NPT conditions.

The final production runs consisted of 100 ns simulations (5 replicas per system) using a 2fs time step. The SHAKE[21] algorithm was employed to constrain all bonds containing hydrogen atoms. Temperature and pressure were controlled by the Langevin thermostat with a collision frequency of 1 ps-1 and the Berendsen barostat with a 1 ps coupling constant. A cutoff of 8 Å was applied to all non-bonded interactions, and long-range electrostatic interactions were evaluated using the particle mesh Ewald (PME)[22] approach. Analysis of the MD simulations was performed using CPPTRAJ.[23]

# References

[1] J. Mangas-Sanchez, M. Sharma, S. C. Cosgrove, J. I. Ramsden, J. R. Marshall, T. W. Thorpe, R. B. Palmer, G. Grogan, N. J. Turner, *Chem. Sci.* **2020**, *11*, 5052-5057.

[2] O. Khersonsky, R. Lipsh, Z. Avizemer, Y. Ashani, M. Goldsmith, H. Leader, O. Dym, S. Rogotner, D. L. Trudeau, J. Prilusky, P. Amengual-Rigo, V. Guallar, D. S. Tawfik, S. J. Fleishman, *Mol. Cell.* **2018**, *72*, 178-186 e175.

[3] J. Ziegenhorn, M. Senn, T. Bucher, *Clin. Chem.* **1976**, *22*, 151-160.

[4] N. H. Schlieben, K. Niefind, J. Muller, B. Riebel, W. Hummel, D. Schomburg, *J. Mol. Biol.* **2005**, *349*, 801-813.

[5] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235-242.

[6] M. V. Shapovalov, R. L. Dunbrack, Jr., *Structure* **2011**, *19*, 844-858.

[7] L. Schrödinger Inc., *The PyMOL Molecular Graphics System*, **n.y.**

[8] C. R. Søndergaard, M. H. Olsson, M. Rostkowski, J. H. Jensen, *J Chem Theory Comput* **2011**, *7*, 2284-2295.

[9] J. Wang, W. Wang, P. A. Kollman, D. A. Case, *J. Mol. Graph. Model.* **2006**, *25*, 247-260.

[10] J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman, D. A. Case, *J. Comput. Chem.* **2004**, *25*, 1157-1174.

[11] M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. V. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, A. F. Izmaylov, J. L. Sonnenberg, Williams, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V. G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J. A. Montgomery Jr., J. E. Peralta, F. Ogliaro, M. J. Bearpark, J. J. Heyd, E. N. Brothers, K. N. Kudin, V. N. Staroverov, T. A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. P. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, J. M. Millam, M. Klene, C. Adamo, R. Cammi, J. W. Ochterski, R. L. Martin, K. Morokuma, O. Farkas, J. B. Foresman, D. J. Fox, Wallingford, CT, **2019**.

[12] P. Cieplak, W. D. Cornell, C. Bayly, P. A. Kollman, *J. Comput. Chem.* **1995**, *16*, 1357-1377.

[13] N. Holmberg, U. Ryde, L. Bülow, *Protein Eng.* **1999**, *12*, 851-856.

[14] F. Duarte, P. Bauer, A. Barrozo, B. A. Amrein, M. Purg, J. Aqvist, S. C. Kamerlin, *J. Phys. Chem. B* **2014**, *118*, 4351-4362.

[15] Q. Liao, A. Pabis, B. Strodel, S. C. L. Kamerlin, *J. Phys. Chem. Lett.* **2017**, *8*, 5408-5414.

[16] D. A. B. Case, K.; Ben-Shalom, I. Y.; Brozell, S. R.; Cerutti, D. S.; Cheatham, T. E., III; Cruzeiro, V. W. D.; Darden, T. A.; Duke, R. E.; Giambasu, G.; Gilson, M. K.; Gohlke, H.; Goetz, A. W.; Harris, R.; Izadi, S.; Izmailov, S. A.; Kasavajhala, K.; Kovalenko, A.; Krasny, R.; Kurtzman, T.; Lee, T. S.; LeGrand, S.; Li, P.; Lin, C.; Liu, J.; Luchko, T.; Luo, R.; Man, V.; Merz, K. M.; Miao, Y.; Mikhailovskii, O.; Monard, G.; Nguyen, H.; Onufriev, A.; Pan, F.; Pantano, S.; Qi, R.; Roe, D. R.; Roitberg, A.; Sagui, C.; Schott-Verdugo, S.; Shen, J.; Simmerling, C. L.; Skyrnnikov, N. R.; Smith, J.; Swails, J.; Walker, R. C.; Wang, J.; Wilson, L.; Wolf, R. M.; Wu, X.; Xiong, Y.; Xue, Y.; York, D. M.; Kollman, P. A., *AMBER 2020*, University of California, San Francisco, **2020**.

[17] J. A. Maier, C. Martinez, K. Kasavajhala, L. Wickstrom, K. E. Hauser, C. Simmerling, *J. Chem. Theory Comput.* **2015**, *11*, 3696-3713.

[18] W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, *J. Chem. Phys.* **1983**, *79*, 926-935.

[19] R. W. Pastor, B. R. Brooks, A. Szabo, *Mol. Phys.* **1988**, *65*, 1409-1419.

[20] H. J. C. Berendsen, J. P. M. Postma, W. F. Vangunsteren, A. Dinola, J. R. Haak, *J. Chem. Phys.* **1984**, *81*, 3684-3690.

[21] J. P. Ryckaert, G. Ciccotti, H. J. C. Berendsen, *J. Comput. Phys.* **1977**, *23*, 327-341.

[22] T. Darden, D. York, L. Pedersen, *J. Chem. Phys.* **1993**, *98*, 10089-10092.

[23] D. R. Roe, T. E. Cheatham, *J. Chem. Theory Comput.* **2013**, *9*, 3084-3095.