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1 **Unique alcohol dehydrogenases involved in algal sugar utilization by marine**
2 **bacteria**

3

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28

29 **Abstract**

30 Marine algae produce complex polysaccharides, which can be degraded by marine
31 heterotrophic bacteria utilizing carbohydrate-active enzymes. The red algal polysaccharide
32 porphyran contains the methoxy sugar 6-O-methyl-D-galactose (G6Me). In the degradation of
33 porphyran, an oxidative demethylation of this monosaccharide towards D-galactose and
34 formaldehyde occurs, which is catalyzed by a cytochrome P450 monooxygenase and its redox
35 partners. In direct proximity to the genes encoding for the key enzymes of this oxidative
36 demethylation, genes encoding for zinc-dependent alcohol dehydrogenases (ADHs) were
37 identified, which seem to be conserved in porphyran utilizing marine *Flavobacteriia*.
38 Considering the fact that dehydrogenases could play an auxiliary role in carbohydrate
39 degradation we aimed to elucidate the physiological role of these marine ADHs. Although, our
40 results reveal that the ADHs are not involved in formaldehyde detoxification, a knockout of the
41 ADH gene causes a dramatic growth defect of *Zobellia galactanivorans* with G6Me as
42 substrate. This indicates that the ADH is required for G6Me utilization. Complete biochemical
43 characterizations of the ADHs from *F. agariphila* KMM 3901^T (FoADH) and *Z. galactanivorans*
44 Dsij^T (ZoADH) were performed and the substrate screening revealed that these enzymes
45 preferentially convert aromatic aldehydes. Additionally, we elucidated the crystal structures of
46 FoADH and ZoADH in complex with NAD⁺ and show that the strict substrate specificity of these
47 new auxiliary enzymes is based on a narrow active site.

48 **Key points**

- 49 ▪ Knockout of the ADH encoding gene revealed its role in 6-O-methyl-D-galactose utilization,
50 suggesting a new auxiliary activity in marine carbohydrate degradation
- 51 ▪ Complete enzyme characterization indicated no function in a subsequent reaction of the
52 oxidative demethylation such as formaldehyde detoxification
- 53 ▪ These marine ADHs preferentially convert aromatic compounds and their strict substrate
54 specificity is based on a narrow active site

55

56 **Keywords**

57 Alcohol dehydrogenase · Porphyran · CAZyme · Bacteroidetes · *Zobellia galactanivorans*
58 · Auxiliary activity

59 Introduction

60 Marine algae represent one of the most crucial primary producers within the marine carbon
61 cycle and contribute to approximately 50% of the total global primary production (Field 1998).
62 For instance, macroalgae sequester approximately 173 Tg of carbon dioxide per year (Krause-
63 Jensen and Duarte 2016) and accumulate the excess carbon in form of carbohydrates, which
64 they utilize as cell wall constituents or energy storage (Arnosti et al. 2021). Degradation of
65 these marine polysaccharides can be extremely complicated due their complexity and the
66 occurrence of side chain modifications like sulfatations, methylations or acetylations (Bäumgen
67 et al. 2021a). It was shown that complex enzymatic cascades are required for the breakdown
68 of a single algal polysaccharide (Reisky et al. 2019; Sichert et al. 2020). Members of the
69 bacterial phylum *Bacteroidetes* are considered specialists in the pivotal degradation of marine
70 polysaccharides (Thomas et al. 2011a) and are observed as first responders after micro- and
71 macroalgal blooms (Teeling et al. 2012; Brunet et al. 2021). They contain specific gene clusters
72 referred to as polysaccharide utilization loci (PULs) (Grondin et al. 2017), which encode for
73 carbohydrate-active enzymes (CAZymes) that catalyze the breakdown of the carbohydrates
74 (Lapébie et al. 2019) as well as proteins essential for the binding and uptake of smaller sugar
75 molecules (Bauer et al. 2006; Martens et al. 2009). Characterizing individual CAZymes helps
76 elucidating complete degradation pathways of marine carbohydrates and provides a deeper
77 understanding of the global carbon cycle. Which has been successfully performed for instance
78 for ulvan from green algae (Reisky et al. 2019; Bäumgen et al. 2021b), fucoidan from brown
79 algae (Sichert et al. 2020) and carrageenan from red algae (Ficko-Blean et al. 2017).

80 Recently, we have demonstrated that in the degradation process of the red algal galactan
81 porphyran (Fig. 1a) by marine bacteria, an oxidative demethylation of the methoxy sugar
82 6-O-methyl-D-galactose (G6Me) occurs (Reisky et al. 2018). This reaction which is catalyzed
83 by a cytochrome P450 monooxygenase (CYP) and its respective redox partners consisting of
84 ferredoxin reductase and ferredoxin leads to the formation of equimolar amounts of
85 D-galactose and formaldehyde (Fig. 1b) (Reisky et al. 2018). It was hypothesized that this
86 reaction is crucial in terms of G6Me utilization, as it removes the highly stable methyl ether,
87 consequently generating an easily metabolizable compound (Reisky et al. 2018). The crystal
88 structure of the CYP from *Zobellia galactanivorans* Dsij^T provided additional information on the
89 binding of G6Me as well as other mechanistic insights (Robb et al. 2018). In addition to the key
90 enzymes for the oxidative demethylation of G6Me, glycoside hydrolases (GH2 and GH16), an
91 esterase and a putative zinc-dependent alcohol dehydrogenase (ADH) were also observed in
92 the genomic context of the marine *Flavobacteriia Formosa agariphila* KMM 3901^T (Fig. 1c)
93 (Reisky et al. 2018). A similar genomic context was also found in *Zobellia galactanivorans* Dsij^T
94 (Fig. 1d).

95 Considering the fact that dehydrogenases play only a minor auxiliary role in the carbohydrate
96 degradation and are poorly represented in the Carbohydrate-Active enZymes (CAZy)
97 database, with some exceptions in the AA3, AA6, AA7 and AA12 families (Takeda et al. 2015;
98 Kracher and Ludwig 2016; Sützl et al. 2018), it remains unclear which biological function this
99 ADH provides for the organism. ADHs belong to the enzyme class of oxidoreductases and
100 catalyze the reversible oxidation of an alcohol to the corresponding aldehyde or ketone
101 employing the nicotinamide adenine dinucleotide (NAD⁺) or nicotinamide adenine dinucleotide
102 phosphate (NADP⁺) cofactor. Depending on the size of the substrate-binding domain, it is
103 possible for ADHs to possess a broad substrate scope; while some exhibit only activities for
104 small aliphatic alcohols, others can convert sterically challenging cyclic components (Persson
105 et al. 2008; Sirota et al. 2021). A major family of ADHs includes the group of zinc-dependent
106 ADHs, which exhibit a typical Rossmann fold (Rao and Rossmann 1973) and contain a
107 catalytic zinc in the active site as well as an additional non-catalytic zinc supporting the stability
108 of an external loop structure (Hambidge et al. 2000). Various biological functions are observed
109 within this family (Sirota et al. 2021) including polyol dehydrogenases catalyzing the
110 conversion between sugar and sugar alcohol (Lu et al. 2019), cinnamyl alcohol
111 dehydrogenases (Larroy et al. 2002; Pick et al. 2013) and glutathione-dependent
112 formaldehyde dehydrogenases (Gutheil et al. 1992; Sanghani et al. 2000; Achkor et al. 2003)
113 which play an important part in the detoxification of formaldehyde (Vorholt 2002). Additionally,
114 ADHs provide numerous advantageous properties for organic synthesis including high
115 enantioselectivity and applicability under mild reaction conditions (Koesoema et al. 2020).
116 Consequently, they are now employed in numerous biotechnological applications such as the
117 preparation of chiral alcohols (Zhang et al. 2015), rare sugars (Lu et al. 2019), fine chemicals
118 as well as the synthesis of building blocks for various essential pharmaceuticals (Hall and
119 Bommarius 2011; Zheng et al. 2017). Discovering and characterizing additional ADHs with
120 unique biochemical properties, is thus also desirable for potential industrial applications.

121 In this study, we aimed to elucidated the putative function of these ADHs, which are
122 consistently located in close proximity to genes that are essential for the oxidative
123 demethylation of G6Me of polysaccharide utilizing marine *Flavobacteriia*. We provide a
124 detailed biochemical characterization as well as the crystal structures for the ADHs from
125 *Formosa agariphila* KMM 3901^T (FoADH) and *Zobellia galactanivorans* Dsij^T (ZoADH). We
126 propose the putative biological functions of these ADHs and demonstrate their importance for
127 the utilization of G6Me via growth studies with a *Z. galactanivorans* knockout strain.

128

129

130 **Materials and Methods**

131 Materials, strains and plasmids

132 All chemicals and reagents used, unless otherwise specified, were purchased from Sigma-
133 Aldrich (St. Louis, MO, USA), Thermo Fisher Scientific (Waltham, MA, USA), Th. Geyer (Berlin,
134 Germany), ABCR GmbH (Karlsruhe, Germany), Honeywell Fluka™ (Morristown, NJ, USA),
135 Carl Roth GmbH (Karlsruhe, Germany), chemPUR GmbH (Karlsruhe, Germany), TCI
136 Deutschland GmbH (Eschborn, Germany) and Cayman Chemical Company (Ann Arbor, MI,
137 USA). Porphyrin and G6Me were obtained from Biosynth Carbosynth (Staad, Switzerland).
138 Primers were obtained from Invitrogen (Waltham, MA, USA). Phage-resistant *Escherichia coli*
139 (*E. coli*) BL21 (genotype: *fhuA2* [*lon*] *ompT gal* (λ *DE3*) [*dcm*] Δ *hsdS* [λ *DE3* = λ *sBamHI*]
140 Δ *EcoRI-B* int::(*lacI::PlacUV5::T7 gene1*) *i21* Δ *nin5*) was obtained from New England Biolabs
141 (Ipswich, MA, USA). The conjugative strain *E. coli* S17-1 λ *pir* (genotype λ *pir hsdR pro thi*;
142 chromosomal integrated RP4-2 Tc::Mu Km::Tn7) (de Lorenzo and Timmis 1994) was grown
143 from in-house glycerol stocks. A construct for the expression of the FoADH (GenBank
144 accession number: OP548117) from *F. agariphila* KMM 3901^T was prepared using the
145 FastCloning strategy (Li et al. 2011) with genomic DNA as template for the amplification of the
146 insert. *F. agariphila* KMM 3901^T (collection number DSM-15362) was obtained from the DSMZ
147 (Braunschweig, Germany). The pET28a vector was amplified with the 5-GCG GCC GCA CTC
148 GAG CA-3' and 5-CAT ATG GCT GCC GCG C-3' oligonucleotides while the insert was
149 amplified with the 5'-CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC CAT ATG TCC
150 ATA ATT TCA AAA TGC GCT ATT G-3' and 5'-CAG TGG TGG TGG TGG TGG TGC TCG
151 AGT GCG GCC GCT TAA AAA ATA ATT ACA CCC TTT GCA TTC-3' oligonucleotides. A
152 synthetic gene, codon optimized for expression in *E. coli*, encoding the ZoADH (GenBank
153 accession number: OP548118) from *Z. galactanivorans* Dsij^T, was synthesized and cloned into
154 a pET28a vector by BioCat GmbH (Heidelberg, Germany). The constructs encoded the
155 recombinant protein as fusion to a N-terminal Strep-tag for affinity purification.

156 Computational analysis for FoADH and ZoADH

157 Sequences of FoADH (Uniprot ID: T2KM87) and ZoADH (Uniprot ID: G0L712) were blasted
158 against the MarDB and MarRef database using the Marine Metagenomic Portal (Klemetsen et
159 al. 2018; Priyam et al. 2019) with the -e value of $1e^{-5}$ and maximal target sequences of 1000.
160 The automated fasta hit table of both blasts were fused and used for the generation of a
161 sequence similarity network (Zallot et al. 2019). An alignment score of 150 was chosen for the
162 refinement and generation of a genome neighborhood analysis of ten genes down and

163 upstream of the ADHs genes (Zallot et al. 2019). Resulting diagrams were visualized via
164 Cytoscape (Paul Shannon et al. 2003) and genome neighborhood diagrams were generated
165 from the online server. Only shared sequences of the MarDB/MarRef database with the
166 UniProtKB databases could be incorporated in the genome neighborhood analysis.

167 ADH knockout in *Z. galactanivorans* and growth studies

168 The deletion mutant of the ADH gene *zgal_4674* in *Z. galactanivorans* Dsij^T (collection number
169 DSM-12802) was constructed using a *sacB* system (Zhu et al. 2017) as previously described
170 for the deletion variant of the CYP gene (Brott et al. 2022). Briefly, to delete *zgal_4674*, a
171 2,448 bp fragment including the last 43 bp of *zgal_4674* and 2,405 bp of downstream sequence
172 was amplified using primers OFT0041 and OFT0043 on genomic DNA from
173 *Z. galactanivorans* Dsij^T. The fragment was digested with BamHI and XbaI and ligated into
174 pYT313 that had been digested with the same enzymes, to generate pFT12. A 2,077 bp
175 fragment including the first 29 bp of *zgal_4674* and 2,048 bp of upstream sequence was
176 amplified using primers OFT0040 and OFT0042. The fragment was cloned into XbaI and Sall
177 sites of pFT12 to generate the *zgal_4674* deletion construct pFT13. Conjugative transfer of
178 pFT13 from *E. coli* S17-1 into the wild-type *Z. galactanivorans* Dsij^T and second recombination
179 steps were carried out as described previously (Zhu et al. 2017). Deletions were confirmed by
180 PCR and sequencing on isolated colonies using primer pairs OFT0044- OFT0045 to identify
181 the *zgal_4674* deletion mutant (mZG_0080). Primers employed are displayed in Table S1 in
182 the Supplementary Information (SI). For growth studies, precultures of three
183 *Z. galactanivorans* strains (wild type, knockout ADH and knockout CYP) were prepared in
184 Zobell 2216E medium (Zobell 1941). The 3-day precultures were then rinsed twice with sterile
185 saline solution. Marine minimal medium (Thomas et al. 2011a) amended with D-galactose or
186 G6Me (4 g L⁻¹) was then inoculated so that an initial optical density (OD₆₀₀) of 0.05 was
187 achieved. Appropriate cultures were incubated for 3 days at room temperature.

188 Enzyme production and purification

189 Chemically competent *E. coli* BL21 (DE3) cells were transformed with the plasmids harboring
190 FoADH or ZoADH and were spread on lysogeny broth (LB) agar plates containing 50 µg mL⁻¹
191 kanamycin. The agar plates were incubated overnight at 37 °C. One colony was picked and
192 used to inoculate 5 mL LB medium which contained 50 µg mL⁻¹ kanamycin and was then
193 incubated at 37 °C and 180 rpm overnight. For overexpression the cultivation was performed
194 in terrific broth (TB) medium containing 50 µg mL⁻¹ kanamycin. The TB medium was inoculated
195 with the overnight culture so that a starting OD₆₀₀ of 0.05 was obtained. Cells were then
196 incubated at 37 °C and 180 rpm until an OD₆₀₀ of 0.8 was reached. Expression of target
197 enzymes was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

198 The cultivation was performed at 25 °C and 180 rpm overnight. Cells were harvested by
199 centrifugation at 10,000 x g and 4 °C for 1 h, washed with 50 mM sodium phosphate buffer
200 (NaPi) pH 7.5, and subsequently stored at -20 °C until cell disruption. The purification
201 procedures of FoADH and ZoADH for crystallization and enzyme assays are identical. Cells
202 were resuspended in 50 mM Tris-HCl buffer pH 8.0 containing 200 mM NaCl. Following cell
203 lysis by ultra-sonication (2 x 3 min, 50% power, 50% cycle), cell debris was removed by
204 centrifugation at 10,000 x g, at 4 °C for 20 min. The clarified supernatant was loaded on a
205 gravity flow column containing Strep-Tactin XT Sepharose® 50% suspension (IBA-
206 Lifesciences GmbH, Göttingen, Germany) as column material. The column was washed with
207 100 mM Tris-HCl buffer pH 8.0 containing 150 mM NaCl in order to remove unbound and
208 undesirable proteins. The target enzymes were then eluted with same buffer containing
209 additionally 50 mM biotin. Elution fractions were pooled and concentrated using a Vivaspin 6
210 centrifugal concentrator with a 10 kDa molecular weight cut-off (Sartorius AG, Göttingen,
211 Germany). Size exclusion chromatography was subsequently performed via the Äkta™ pure
212 chromatography system (Cytiva Europe GmbH, Germany). The concentrated enzyme solution
213 was applied to a HiPrep™ 16/60 Sephacryl® S-200 HR column (Cytiva Europe GmbH, Freiburg,
214 Germany) that was previously equilibrated with 10 mM Tris-HCl buffer pH 8.0 containing
215 200 mM NaCl. Elution fractions were collected and the purity was verified by sodium dodecyl
216 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pure fractions were combined and
217 concentrated like mentioned above. The enzyme solution was stored at 4 °C for crystallization.
218 For application in enzyme assays, a PD-10 desalting column (Cytiva Europe GmbH, Freiburg,
219 Germany) was employed to desalt the protein sample and exchange the buffer.

220 SDS-PAGE and determination of protein content

221 SDS-PAGE was performed to verify the purity of the target enzymes. 20 µL protein sample
222 was mixed with 5 µL of a 5-fold stock of SDS sample buffer (100 mM Tris-HCl buffer pH 6.8
223 containing 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 25 mM
224 ethylenediaminetetraacetic acid (EDTA) and 0.04% (w/v) bromophenol blue) and denatured at
225 99 °C for 15 min. For the SDS-PAGE a 12.5% acrylamide gel (separating gel) and a 4.0%
226 loading gel were used. Electrophoresis was carried out at 200 V. Proteins were stained with
227 Coomassie Blue (PhastGel® Blue R). As reference the Pierce™ Unstained protein molecular
228 weight marker (Thermo Fisher Scientific, Waltham, MA, USA) was used. Protein
229 concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher
230 Scientific, Waltham, MA, USA) with bovine serum albumin as protein standard.

231 Crystallization

232 Purified FoADH (25 mg mL⁻¹) and ZoADH (25 mg mL⁻¹) were incubated with 20 mM NAD⁺
233 overnight. Initial crystallization screen was performed using sitting drop vapor-diffusion method
234 at 22 °C. The droplets contained 0.2 µL of protein and 0.2 µL of reservoir solution. Microcrystals
235 of FoADH were obtained from reservoir solution containing 0.1 M Tris-HCl pH 7.5, 0.2 M KCl
236 and 22% (w/v) polyethylene glycol 3350. Microcrystals of ZoADH were obtained from reservoir
237 solution containing 0.1 M Tris-HCl pH 7.5, 0.2 M KCl and 20% (w/v) polyethylene glycol 3350.
238 Further crystal optimization was performed by scale-up of the droplets containing 2 µL of
239 protein and 2 µL of reservoir solution, using the hanging drop vapor-diffusion method at 22 °C.
240 Suitable FoADH and ZoADH crystals for X-ray diffraction were obtained from 0.1 M Tris-HCl,
241 pH 7.5, 0.2 M KCl and 20-22% (w/v) polyethylene glycol 3350 within one day.

242 Data collection

243 X-ray diffraction data were collected at beamline 11C at Pohang Light Source II (PLS-II,
244 Pohang, South Korea) with a Pilatus 6M detector (Dectris, Swiss). The FoADH crystals were
245 equilibrated in a cryoprotectant buffer containing reservoir buffer plus 20% (v/v) ethylene glycol.
246 ZoADH crystals were equilibrated in a cryoprotectant buffer containing reservoir buffer plus
247 20% (v/v) glycerol. The crystal was mounted on the goniometer and cooled under a nitrogen
248 gas stream at 100 K. The diffraction data were indexed, integrated, and scaled using HKL2000
249 program (Otwinowski and Minor 1997). A data collection statistic is given in Table S2.

250 Structure determination

251 The electron density maps of FoADH and ZoADH were obtained via the molecular replacement
252 method using the MOLREP program (Vagin and Teplyakov 2010). The crystal structure of an
253 ADH from *Artemisia annua* (PDB code: 6LJH, unpublished) was used as search model for both
254 FoADH and ZoADH. Model building and refinement were performed with the COOT program
255 (Emsley and Cowtan 2004) and phenix.refinement in PHENIX (Liebschner et al. 2019),
256 respectively. The geometry of final models was evaluated with MolProbity (Williams et al. 2018).
257 Structural figures were generated with PyMOL (www.pymol.org). Structure-based sequence
258 alignments were generated using Clustal-Omega (Sievers et al. 2011) and ESPript (Gouet et
259 al. 1999). Tetrameric interfaces of ADHs were analyzed by PDBePISA (Krissinel and Henrick
260 2007). The interaction between ADHs and ligands were analyzed using PLIP (Salentin et al.
261 2015). The structure factor and coordinates are deposited in the Protein Data Bank under PDB
262 codes 8H2A (FoADH-NAD) and 8H2B (ZoADH-NAD).

263 Enzyme activity determination and substrate screening

264 For determining the enzyme activity of the ADHs, the absorbance maximum of NADH at
265 340 nm was utilized. The absorbance at 340 nm was measured every minute over a 10 min

266 period using a microplate spectrophotometer (BioTek Synergy H1, Agilent Technologies,
267 Santa Clara, CA, USA), and the slope over time was used to determine activities or relative
268 activities. One unit of activity is defined as oxidation or formation of 1 μmol of NADH per minute.
269 For calculation of activity, the molar absorption coefficient of NADH was determined via a
270 standard curve that covered the range of 0 to 0.5 mM. For the initial substrate screening,
271 several alcohols/aldehydes/ketones were employed at a final concentration of 10 mM. For
272 increased substrate solubility, these reactions contained 3.5% (v/v) dimethyl sulfoxide (DMSO).
273 The total volume for all reactions was 0.2 mL. The oxidation and reduction were both
274 conducted at an incubation temperature of 70 °C. Reduction of aldehydes was performed in
275 the presence of a 50 mM succinate buffer pH 6.5, while oxidation reactions were assayed in
276 the presence of a 50 mM NaPi buffer pH 8.5. The final enzyme concentrations used to provide
277 a linear absorbance increase or decrease ranged from 20-100 $\mu\text{g mL}^{-1}$ for the oxidation
278 reactions and from 0.25-2.5 $\mu\text{g mL}^{-1}$ for the reduction reactions. The reaction was initialized by
279 the addition of 0.5 mM NAD⁺ or NADH. For the measurement with sugar substrates, a reduced
280 reaction temperature of 40 °C and an increased measuring time of 30 min was chosen. Various
281 sugars were used at a final substrate concentration of 30 mM. A concentration of 0.2% (w/v)
282 was used for porphyran. Oxidation and reduction reactions were performed in the identical
283 buffers as used for substrate screening, the final enzyme concentration was 0.1 mg mL⁻¹. The
284 reaction was initialized by the addition of 0.5 mM NAD⁺ or NADH. For the determination of
285 cofactor utilization, the oxidation of 10 mM benzyl alcohol was performed in the presence of
286 different NAD⁺ or NADP⁺ concentrations ranging from 0 to 5 mM in 50 mM HEPES buffer
287 pH 8.5 at 25 °C and a final enzyme concentration of 0.1 mg mL⁻¹. For the determination of the
288 kinetic parameters, a final protein content of 0.1 mg mL⁻¹ (corresponding to a protein
289 concentration of 2.44 μM) was used for the oxidation reactions. When determining K_m and V_{max}
290 values for NAD⁺, 15 mM benzyl alcohol was used as the final substrate concentration, while a
291 final cofactor concentration of 5 mM NAD⁺ was used for the measurement for benzyl alcohol.
292 The oxidation reactions were carried out in 50 mM NaPi buffer pH 8.5 and at a reaction
293 temperature of 70 °C. A final protein content of 5 $\mu\text{g mL}^{-1}$ was used in the reduction reaction
294 (corresponding to a protein concentration of 0.012 μM). For the determination of the kinetic
295 parameters for NADH, 2.5 mM pyridine-3-carbaldehyde was used as the final substrate
296 concentration, while a final cofactor concentration of 0.5 mM NADH was used for the
297 determination of the kinetic parameters for pyridine-3-carbaldehyde. The reduction reactions
298 were carried out in 50 mM succinate buffer pH 6.5 and at 70 °C. In order to test for thiol-
299 dependent formaldehyde detoxification, different thiols were evaluated as potential cofactors.
300 For this reaction, the thiol cofactor and formaldehyde were used in a 1:1 ratio at a final
301 concentration of 0.5 mM. The measurement was performed in the 50 mM NaPi buffer pH 8.5
302 at 70 °C with a final enzyme concentration of 0.2 mg mL⁻¹. The reaction was started by the

303 addition of 0.5 mM NAD⁺. The ADH catalyzed disproportionation of formaldehyde into
304 methanol and formate was monitored by a pH change utilizing the phenol red assay (Martínez-
305 Martínez et al. 2018). This measurement was performed in a microtiter plate and the reaction
306 volume was 0.2 mL. 5 mM formaldehyde was used as substrate, 0.5 mM NAD⁺ as cosubstrate
307 and 0.1 mg mL⁻¹ as final enzyme concentration. The pH indicator phenol red was used at a
308 final concentration of 91 μM. The reaction was performed in a 5 mM HEPES buffer pH 8.5 at
309 40 °C. Absorbance at 560 nm was measured every minute for 20 min.

310 Influence of pH and buffer components

311 To determine the pH optimum of the enzymes, the oxidation and reduction reactions were both
312 investigated in the presence of varying pH values. All buffers had a concentration of 50 mM. A
313 citrate buffer was used in the pH range of 5 to 6, a NaPi buffer in the range of 6 to 8.5, a CHES
314 buffer in the range of 8.5 to 10 and a CAPS buffer in the range of 10 to 12.5. The assay
315 conditions for the oxidation reaction were as follows: the reaction volume was 200 μL, 10 mM
316 benzyl alcohol and 0.5 mM NAD⁺ was used as substrate. The reaction was started by the
317 addition of 0.1 mg mL⁻¹ ADH. For the reduction reaction, instead of benzyl alcohol and NAD⁺,
318 10 mM benzaldehyde and 0.5 mM NADH were used. Since benzaldehyde was less soluble in
319 the buffer than benzyl alcohol, both reactions contained 3.5% (v/v) DMSO, in order to achieve
320 better comparability. The reaction was carried out at 25 °C in the respective buffers. To
321 examine the influence of buffer components on enzyme activity, different buffers with a
322 concentration of 50 mM were used. The buffers had a pH of 6.5 for the reduction reaction,
323 whereas it was 8.5 for the oxidation reaction. The reaction was carried out under the same
324 conditions as those for the pH optimum. Relative activities were determined as described
325 above.

326 Influence of temperature and thermostability

327 The temperature optimum was determined by conducting the oxidation reaction at different
328 temperatures in the range between 20 and 90 °C. For this, the reaction mixture without enzyme
329 was preheated to the desired temperature in a reaction tube by using a heating block
330 (Eppendorf ThermoMixer®C, Eppendorf SE, Hamburg, Germany) for at least 45 min. The
331 reaction mixture had a volume of 200 μL. 30 mM benzyl alcohol and 0.5 mM NAD⁺ were
332 employed as substrates, and the reaction was carried out at different temperatures ranging
333 from 20 to 90 °C in a 50 mM NaPi buffer pH 7.5. The reaction was initiated by the addition of
334 enzyme with a final concentration of 0.1 mg mL⁻¹. For the thermostability determination, the
335 purified ADH (1 mg mL⁻¹) was incubated in 50 mM NaPi buffer pH 7.5 for 1 or 4 h in a gradient
336 thermal cycler (FlexCycler², Analytik Jena, Jena, Germany) at various temperatures ranging
337 from 20 to 80 °C. Residual activity was then determined as described above and compared

338 with a control that was incubated on ice. The assay conditions were as follows: the reaction
339 volume was 200 μL , the final enzyme concentration was 0.1 mg mL^{-1} , 10 mM benzyl alcohol
340 was used as substrate, the reaction was performed at 40 °C in 50 mM NaPi buffer pH 7.5. The
341 reaction was initiated by the addition of 0.5 mM NAD^+ .

342 Influence of sodium chloride

343 Determination of NaCl influence on enzyme activity was performed by carrying out the
344 oxidation reaction in the presence of different NaCl concentrations varying from 0 to 800 mM.
345 The relative activities were determined as described above and were compared with a control
346 where no additional NaCl was present. Assay conditions were as follows: the reaction volume
347 was 200 μL , 10 mM benzyl alcohol was used as substrate, the final enzyme concentration was
348 0.1 mg mL^{-1} and the NaCl concentration was between 0 and 800 mM. The reaction was carried
349 out at 25 °C in a 50 mM NaPi buffer pH 8.5 or in a 50 mM Tricine buffer pH 8.5 and started by
350 the addition of 0.5 mM NAD^+ .

351 Influence of metal ions and other small molecules

352 For the determination of the influence of various metal ions on enzyme activity, the ADHs with
353 a concentration of 1 mg mL^{-1} were incubated with either 1 or 10 mM metal ion at RT for 1 h
354 before activity measurement. A sample without additional metal ion served as a control. For
355 the activity measurement, the standard assay was used with the following conditions: the
356 reaction mixture had a total volume of 200 μL , 10 mM benzyl alcohol was used as substrate,
357 a final enzyme concentration of 0.1 mg mL^{-1} was employed and the reaction was performed in
358 50 mM HEPES buffer pH 8.5 at 25 °C. The reaction was initiated by the addition of 0.5 mM
359 NAD^+ . In order to determine the effect of EDTA, dithiothreitol (DTT) and 2-mercaptoethanol
360 (2-ME) on enzyme activity, the ADHs were incubated at a protein concentration of 1 mg mL^{-1}
361 with these components at concentrations of 1, 10 or 25 mM for 1h at RT before activity
362 determination. Higher concentrations up to 100 mM were additionally tested for EDTA. The
363 untreated enzyme served as a control. The activity measurement was performed as described
364 for the influence of metal ions.

365 Influence of solvents and formaldehyde

366 To evaluate the influence of selected water-miscible solvents on the activity of both ADHs, the
367 oxidation reaction was conducted in the presence of 5, 10, and 20% (v/v) solvent and
368 compared with a control containing no additional solvent. The relative activity was determined
369 as described above. The total reaction volume was 0.2 mL and 0.1 mg mL^{-1} of enzyme was
370 used as final enzyme concentration. The reactions were performed in 50 mM NaPi buffer at
371 25 °C. 10 mM benzyl alcohol was employed as substrate and the reactions were started by

372 adding 0.5 mM NAD⁺. The enzymes were incubated at a concentration of 1 mg mL⁻¹ with
373 different concentrations of formaldehyde varying from 0 to 50 mM for 1 h at RT prior to activity
374 measurement to evaluate the effect of formaldehyde on enzyme activity. Relative activity was
375 determined as described above. For the activity measurement, the same conditions were used
376 as for the influence of solvent.

377 **Results**

378 Distribution and gene neighborhood analysis

379 In order to obtain an overview regarding the distribution and function of these ADHs in marine
380 bacteria, we queried the MarDB and MarRef databases for ADHs with similar sequences to
381 FoADH and ZoADH and constructed a sequence similarity network based on an alignment
382 score of 150 and a sequence identity of 63.14%. This analysis revealed six main clusters,
383 which we define here as clusters containing at least 34 sequences, with FoADH and ZoADH
384 included in main cluster 2 (Fig. S1). This main cluster primarily contained sequences that were
385 annotated as zinc-dependent ADHs, histidine kinases, ADH GroES-like domains and some
386 glutathione dependent formaldehyde dehydrogenases/ADHs. However, glutathione-
387 dependent and mycothiol-dependent formaldehyde dehydrogenases were identified
388 predominantly in clusters 1 and 4, respectively. Based on main cluster 2, we performed a
389 genome neighborhood analysis to obtain a general sense of which genes are located in close
390 proximity to the ADH gene. Similar genomic arrangements consisting of CYP, redox partners,
391 an esterase and the ADH can be identified in several marine bacteria that are capable of
392 degrading marine polysaccharides (Fig. S2), including members of the genera *Polaribacter*,
393 *Maribacter* and *Arenibacter*. Minor differences in gene arrangement can be observed among
394 some organisms such as *F. agariphila* or *Algibacter lectus*, where genes encoding for
395 CAZymes (GH2 and GH16) are located between the ADH and the esterase gene. Additionally,
396 some genes encoding for sulfatases and SusC/SusD homologs, which are responsible for the
397 binding and transport of sugar molecules (Martens et al. 2009), are located up- and
398 downstream of the ADH gene. Considering that the ADH gene consistently appears in the
399 proximity of the genes, which encode for CAZymes and key enzymes for the oxidative
400 demethylation of G6Me, it is conceivable that the ADH possesses a specific function in
401 carbohydrate utilization or a subsequent reaction.

402 Knockout of the ADH encoding gene in *Z. galactanivorans* and growth studies

403 As an attempt to elucidate the biological relevance of the ADHs for the organisms, a knock-out
404 of the gene, which encodes for the ADH in *Z. galactanivorans* was performed followed by
405 growth experiments. The controls employed for these growth studies were the wild type (WT)
406 and an additional knock-out strain of *Z. galactanivorans* in which the CYP gene was deleted.

407 When G6Me was employed as the sole carbon source, impaired growth was observed for the
408 ADH and CYP knock-out strains, while the WT exhibited normal growth (Fig. 2). In contrast,
409 regular growth was observable for all three strains in a control, which contained D-galactose
410 as sole carbon source. Consequently, the ADH possessed an impact on the G6Me utilization
411 of *Z. galactanivorans*.

412 Functional overexpression and purification of the ADHs

413 Since we could demonstrate a biological significance of the ADH for the utilization of G6Me by
414 the gene knockout in *Z. galactanivorans*, our next aim was to identify the enzyme function. We
415 therefore cloned the gene encoding for the ADH from *F. agariphila* into a pET28a vector. For
416 the ADH from *Z. galactanivorans* a synthetic gene was ordered in the pET28a vector. Both
417 enzymes were successfully overexpressed and purified (Fig. S3), which established the basis
418 to elucidate putative biological functions of these ADHs by performing biochemical and
419 structural biological characterizations.

420 Substrate spectrum of the ADHs

421 In order to obtain a preliminary understanding of the substrate spectrum of these ADHs, their
422 ability for the alcohol oxidation as well as the reduction of various aldehydes and ketones were
423 examined. Both enzymes converted predominantly aromatic substrates (Tables 1 and 2). The
424 highest specific activity of 64.1 U mg⁻¹ for FoADH and 54.9 U mg⁻¹ for ZoADH was observed
425 for the reduction of pyridine-3-carbaldehyde. In addition to compounds containing a benzene
426 ring, substrates harboring a furan or thiophene ring, such as furfural and thiophene-3-
427 carbaldhyde, were also preferentially converted. Positions of additional substituents at the
428 benzene ring influenced the activity. A difference in the specific activities for the constitutional
429 isomers of terephthalaldehyde and tolualdehyde were observed for both enzymes. In particular,
430 substrates that possessed an additional substituent in *ortho*-position were converted
431 significantly less efficiently. In addition, the length of the aldehyde substituent at the benzene
432 ring also affected the activity. For instance, hydrocinnamaldehyde was converted by both
433 enzymes, whereas for phenylacetaldehyde no activity was observable. In contrast to
434 benzaldehyde, the structurally similar acetophenone could not be oxidized. Thus, both ADHs
435 were unable to convert ketones to secondary alcohols. In comparison to the reduction reaction,
436 significant reduced specific activities were noticed for the oxidation reactions (Table 2).
437 Simultaneously, lower K_m values in the range of 0.6 to 0.8 mM could be determined for pyridine-
438 3-carbaldehyde compared to the K_m values of 3.6 and 5.3 mM for benzyl alcohol (Fig. S4). The
439 highest specific activity of 490 mU mg⁻¹ for FoADH and 290 mU mg⁻¹ for ZoADH was observed
440 for 2,5-bis(hydroxymethyl)furan. Both ADHs lacked any activity for smaller aliphatic alcohols
441 such as methanol and ethanol. Since the ADHs exhibited predominantly activities for

442 substrates containing a ring structure, several sugars were also considered as possible
443 substrates. However, no activity was observed for the oxidation or reduction of galactose,
444 G6Me and additional monosaccharides and disaccharides (Table S3). Additionally, the marine
445 carbohydrate porphyran was also evaluated as a potential substrate, however, no activity was
446 detected either. As mentioned earlier in the introduction, ADHs require either NAD^+ or NADP^+
447 as cofactor for their enzymatic activity. In order to identify the preferred cofactor for both ADHs,
448 the oxidation of benzyl alcohol was conducted in the presence of varying NAD^+ and NADP^+
449 concentrations. Both ADHs utilize NAD^+ as cofactor, whereas in the presence of up to 5 mM
450 NADP^+ no activity for the oxidation reaction was observed.

451 Testing for formaldehyde detoxification activity

452 Since activity was neither observed for galactose nor for G6Me, we hypothesized that the
453 ADHs may participate in formaldehyde detoxification, considering that formaldehyde is formed
454 as a by-product in the oxidative demethylation reaction. Members of the zinc-dependent ADHs
455 may catalyze the glutathione-dependent formaldehyde detoxification, therefore various thiols
456 were considered as potential cofactors. Thiol-dependent detoxification of formaldehyde
457 proceeds via a spontaneous reaction between the sulfhydryl group of the thiol cofactor and the
458 carbon atom of formaldehyde, resulting in the formation of an alcohol (Fig. 3a) (Chen et al.
459 2016). Subsequently, this alcohol can be oxidized by the ADH to a thioester, which is then
460 converted by an esterase to formate and the starting thiol cofactor (Gonzalez et al. 2006).
461 Based on the results of our genome neighborhood analysis, where we have also demonstrated
462 that a gene encoding for an esterase is located in the vicinity of the ADH gene, it is quite
463 possible that a thiol-dependent detoxification of formaldehyde can proceed via both enzymes.
464 In addition to glutathione, mainly mycothiol (Misset-Smits et al. 1997; Newton and Fahey 2002)
465 and bacillithiol (Newton et al. 2009; Chandrangu et al. 2018) are well known cofactors in
466 formaldehyde detoxification (Fig. 3b). However, no activity was detected for these thiols.
467 Furthermore, common thiols abundant in nature such as cysteine, coenzyme A and
468 L-ergothioneine (Hand and Honek 2005) were also investigated as cofactors. Nevertheless, no
469 activity was observed for these substrates in combination with formaldehyde either.
470 Considering that the ADHs mainly exhibited activity for aromatic substrates, aromatic thiols
471 such as 2-mercaptoimidazole or 4-mercaptophenol were considered as possible substrates as
472 well. However, even with these compounds, no oxidation reaction was detected. Furthermore,
473 neither enzyme exhibited activity for the oxidation or reduction of formaldehyde in the presence
474 of only NAD^+ or NADH as cofactors. In addition, a disproportionation reaction of formaldehyde
475 into methanol and formate catalyzed by the ADH was also checked. However, no activity could
476 be detected. Consequently, the ADHs possessed no activities for the substrate nor for the

477 products of the oxidative demethylation of G6Me. To provide additional insights into these
478 ADHs, we performed further biochemical characterizations of both enzymes.

479

480 Influence of pH and buffer components on enzyme activity

481 In order to determine the optimal pH for the enzymatic reaction, several buffers were
482 investigated in the pH range from 5.5 to 12.5. A similar pH optimum was observed for both
483 enzymes (Fig. 4). The reduction reaction was most efficiently catalyzed at pH 6.5, while
484 oxidation was found to be most efficient at pH 8.5 (Fig.4 a-b and d-e). At pH 5 and at 12.5, no
485 activity was detected for either enzymes; precipitation was noticed at pH 5 while employing
486 higher protein concentrations. Since a considerable difference in activity was observed
487 between NaPi and CHES buffer at pH 8.5, other buffers were also evaluated at pH 6.5 (Fig. 4 c)
488 and 8.5 (Fig. 4f) to investigate the influence of buffer components on the activity. For the
489 oxidation reaction at pH 8.5, it was shown that by employing a Tris-HCl buffer, an
490 approximately 60 to 80% increased activity was obtained compared to the activity in the NaPi
491 buffer. In contrast, a significant activity decrease of 95% was observed for both enzymes in
492 the presence of a borate-NaOH buffer. For the reduction reaction at pH 6.5, a slight increase
493 in activity of ~8 to 16% could be detected using citrate and succinate buffer compared to the
494 NaPi buffer, with the highest activity found for the succinate buffer.

495 Influence of temperature and enzyme thermostability

496 In addition to the pH value, the temperature influence is essential for enzymatic activity. At the
497 same time, elevated temperatures promote substrate solubility and thus the application of
498 higher concentrations which also may shift the reaction equilibrium towards product formation
499 (Unsworth et al. 2007). Therefore, the impact of temperature in the range between 20 and
500 90 °C was investigated for both enzymes. The ADHs possessed a similar temperature profile,
501 where activity increased with rising temperature, reaching an optimum between 65 to 75 °C
502 (Fig. 5a). However, at higher temperatures the activity decreased rapidly, whereas at room
503 temperature only a relative activity of about 18% for FoADH and 10% for ZoADH was observed.
504 The measurement for the temperature optimum was performed for 10 min to ensure that any
505 influence of thermostability would not affect the results. Thermostability of enzymes is an
506 important parameter for biocatalysis, since many industrial processes operate at higher
507 temperatures for longer time periods, leading to increased product yields. The thermostability
508 of the ADHs was therefore evaluated next by incubating the enzymes for 1 or 4 h at various
509 temperatures ranging from 20 to 80 °C followed by determination of residual activity. After 1 h
510 incubation at 59 °C as well as lower temperatures, no decrease in activity was detected for
511 FoADH compared to a control incubated on ice (Fig. 5b). Residual activity only diminished at

512 higher incubation temperatures and a residual activity of roughly 20% was still observed for
513 80 °C. In contrast, after 4 h incubation, almost no residual activity was observed at this
514 temperature. Nevertheless, even after this extended incubation period, a high remaining
515 activity of approximately $\leq 85\%$ was detected for the temperature range of 20 to 59 °C. ZoADH
516 exhibited a similar behavior in thermostability as FoADH, however an initial activity decrease
517 of 20% was observed for the 1 h incubation already at 57 °C (Fig. 5c). A severe activity loss of
518 almost 95 to 100% was observed for ZoADH when incubated for 4 h at temperatures >73 °C.

519 Influence of sodium chloride

520 Enzymes originating from marine organisms may possess habitat-related characteristics such
521 as an increased salt tolerance (Trincone 2011). Considering that both enzymes originate from
522 marine bacteria, the influence of NaCl on the enzyme activity was tested. For this purpose, the
523 relative activities for the oxidation reaction were determined in the presence of different NaCl
524 concentrations ranging from 0 to 800 mM in the NaPi and Tricine buffer, respectively. Both
525 ADHs displayed a similar behavior in the presence of rising NaCl concentrations (Fig. S5). An
526 increase in relative activity of approximately 10% was observed in the range from 0 to 150 mM
527 NaCl for FoADH using the Tricine buffer. In contrast, only a minor increase in activity was
528 observed for the NaCl concentration of 100 mM in the NaPi buffer. A difference between the
529 NaCl influence depending on the selected buffer was also noticed for ZoADH, with a higher
530 effect in the Tricine buffer. For ZoADH, an increase in relative activity of 20% was also detected
531 in the range of 0 to 200 mM NaCl. At NaCl concentrations ≥ 400 mM, a diminished relative
532 activity was observed for both enzymes.

533 Influence of metal ions and other small molecules

534 Both enzymes are annotated as zinc-dependent ADHs, which contain a catalytic zinc ion in
535 the active site. An influence of various metal ions on the enzyme activity is thus possible and
536 was therefore investigated next. For this purpose, the enzymes were incubated with different
537 metal ions at concentrations of 1 or 10 mM for 1 h prior to activity measurement and the relative
538 activities were determined. A high dependence on metal ions was observed for both ADHs,
539 with nearly all ions assayed exhibiting a beneficial effect on enzyme activity (Table 3 and
540 Fig. S6). Particularly higher concentrations of Ni^{2+} , Co^{2+} and Mn^{2+} led to a 10 to 14-fold increase
541 in relative activity for both enzymes compared to the control which contained no additional
542 metal ion. In contrast, complete inhibition for both enzymes was only observed for Cu^{2+} , Zn^{2+}
543 as well as 10 mM Fe^{3+} . Additionally, we analyzed whether the chelating agent EDTA, which is
544 capable of complexing bivalent metal ions, affects the enzymatic activity. After 1 h incubation
545 in the presence of 25 mM EDTA, a reduction in the relative activity for both enzymes was found,
546 while an almost complete inhibition was observable at an EDTA concentration of 100 mM

547 (Table 3 and Fig. S7). The influence of DTT and 2-mercaptoethanol (2-ME) on activity was
548 also investigated, since these compounds can affect enzyme stability. DTT had a lesser impact
549 on both enzymes than 2-ME. A major decline in relative activity of over 70% was observed for
550 both enzymes after 1 h incubation with 10 mM 2-ME (Table 3 and Fig. S7). When compared
551 to ZoADH, the effect of the reducing agents was more pronounced for the activity of FoADH.

552 Influence of solvents and formaldehyde

553 The influence of water-miscible solvents on the enzyme activity of both ADHs was also
554 investigated. Increasing the amount of solvent in the reaction led to a decrease in the relative
555 activity for all tested solvents (Fig. S8). Compared to the other solvents, methanol and DMSO
556 had the weakest negative effects on the enzyme activity, leading to a relative activity of still
557 50% in the presence of 10% (v/v) solvent. In addition, the presence of formaldehyde on the
558 enzyme activity was examined, since formaldehyde is released during the oxidative
559 demethylation of G6Me and the ADHs are most likely involved in this reaction. Therefore, the
560 ADHs were incubated with a variety of formaldehyde concentrations in the range between
561 0 and 50 mM for 1 h at RT and the relative activities was determined. In the presence of 0 to
562 1 mM formaldehyde, no reduction in activity was observed. An initial decrease in relative
563 activity of approximately 10-20% could be perceived in the presence of 2.5 mM formaldehyde
564 (Fig. S9). At higher formaldehyde concentrations, a more severe activity decrease was found,
565 while no activity was observed for both enzymes in the presence of 50 mM formaldehyde.

566 Overall structures of FoADH and ZoADH

567 In order to gain a deeper understanding of the molecular function, we performed X-ray
568 crystallography studies of FoADH and ZoADH. For the determination of the functional states
569 of both ADHs, the essential NAD⁺ cofactor was added to purified FoADH and ZoADH proteins
570 before crystallization. The crystal structures of FoADH and ZoADH in complex with NAD⁺ were
571 determined at a resolution of 2.5 and 2.1 Å, respectively (Table S2). FoADH and ZoADH
572 crystals belong to space group monoclinic P2₁ and orthorhombic P2₁2₁2₁, respectively and
573 contain four and eight molecules in asymmetric unit, respectively (Fig. S10). The electron
574 density map of FoADH and ZoADH clearly showed the almost entire polypeptide chain, except
575 for a partially disordered fragment of the loop between the β5 and β6-strands (Gly111-His115
576 in both enzymes), which is involved in substrate binding and specificity. The monomer
577 structures of FoADH and ZoADH comprise the catalytic domain (residues 1–149 and residues
578 283–326 for both enzymes) and the cofactor-binding domain (residues 150–282 for both
579 enzymes) (Fig. 6a), which are separated by a cleft containing a deep pocket, which
580 accommodates the substrate and the NAD⁺ cofactor. The catalytic domain contains two zinc-
581 binding sites, Zn1 and Zn2, which are responsible for catalytic activity and structural stability,

582 respectively. The cofactor binding domain adopts a typical Rossmann fold with the conserved
583 sequence "GXGXXG". FoADH and ZoADH had a 76.0% similarity in amino acid sequence
584 (Fig. S11), and their monomer structures showed a similarity with a root-mean-square
585 deviation (r.m.s.d.) of 0.350-0.772 Å (Table S4).

586 In FoADH, molecules A/B/C/D and E/F/G/H form a tetrameric formation (Fig. S10). In
587 superimposition of monomeric FoADH molecules, the A, B, C, E and G molecules showed
588 structural similarity (denoted as closed form) with r.m.s.d. of 0.256-0.353 Å, whereas
589 molecules D and H (denoted as open form) showed the relatively high r.m.s.d. value of 0.457-
590 0.626 Å when superimposed with molecule A, B, C, E and G (Fig. 6b and Table S5). On the
591 other hand, molecule F maintains the intermediate conformation between the closed and open
592 conformations. When the cofactor binding domains of molecules A and H of FoADH were
593 superimposed, the catalytic binding of molecule H was shifted by approximately 2.0-3.3 Å in
594 the opposite direction of the substrate-binding cleft compared to molecule A (Fig. 6b).

595 In ZoADH, superimposition of molecules A, B and C exhibited a similar conformation (denoted
596 as closed form) with r.m.s.d. of 0.198-0.226 Å, whereas molecule D (denoted as open form)
597 showed a relatively high r.m.s.d. value of 0.314-0.471 Å when superimposed with molecules
598 A, B and C (Fig. 6b and Table S6). Superposition of the cofactor binding domains of molecules
599 A and D clearly revealed the conformational difference between the catalytic domains. The
600 catalytic domain of molecule D is shifted about 2.2-3.3 Å to the outside of the substrate binding
601 cleft of ZoADH compared to molecule A. Accordingly, in the structure of NAD⁺-bound FoADH,
602 molecules A/B/C and D represent closed and open conformations of the substrate binding site,
603 respectively. Collectively, the crystal structures of NAD⁺-bound ZoADH and FoADH contain
604 the open and closed conformations between catalytic and cofactor-binding domains (see
605 below).

606 The crystal structures of FoADH and ZoADH showed the tetrameric formation via the
607 arrangement of a dimer of dimers (Fig. 6c). In both ADHs, the β17 and β18-strands of the
608 cofactor binding domains are stabilized by forming an antiparallel β-sheet with the β17* and
609 β18*strands (asterisk indicates the second monomer), respectively (Figs. S12 and S13). For
610 FoADH, the dimeric interface is stabilized by the main chain interactions of Ile297-Ile299*
611 (* denoting the partner molecule) and Ile299-Ile297* between the β17 strands and Tyr310-
612 Tyr310* between β18 strands (Fig. S12). In addition, numerous hydrogen and salt bridges
613 were observed in the dimer interface with a buried surface area of 1654 Å² (Table S7). The
614 dimer of dimers was stabilized by hydrogen interaction and the buried interface of dimers of
615 dimers is 1193 Å² (Table S7). For ZoADH, the dimeric interface is stabilized by the main chain
616 interactions of Ile298-Ile300* and Ile300-Ile298* between the β17 strand and Tyr311-Tyr311*
617 between β18 strand (Fig. S13). Moreover, numerous hydrogen and salt bridges were observed

618 at the dimer interface with a buried surface area of 1640 Å² (Table S8). The dimer of dimers
619 was stabilized by hydrogen interactions and salt bridges and the buried interface of dimers of
620 dimers is ~1205 Å² (Table S8). All active sites of the tetrameric ADH in the crystal were
621 exposed to solvent (Fig. 6c). Superposition of tetrameric molecules of FoADH and ZoADH in
622 the asymmetric unit shows an r.m.s.d. of 0.327-0.888 Å for whole C α atoms (Fig. 6d).

623 Structural homology search by DALI revealed that both FoADH and ZoADH share structural
624 similarities to the class II alcohol dehydrogenase (ADH4) from human (PDB code: 3COS,
625 Z-score= 45.8 for FoADH and 45.3 for ZoADH, sequence identity= 32% for FoADH [357 α
626 atoms] and 30% for ZoADH [357 α atoms]), an ADH from *E. coli* (PDB code: 5vm2, Z-score=
627 48.1 for FoADH and 38.1 for ZoADH, sequence identity= 28% for FoADH [329 α atoms] and
628 27% for ZoADH [328 α atoms]) as well as an ADH from *Thermotoga maritima* (PDB code: 3IP1,
629 Z-score= 35.8 for FoADH and 36.8 for ZoADH, sequence identity= 25% for FoADH [328 α
630 atoms] and 23% for ZoADH [332 α atoms]). Although these structural homologous ADHs share
631 low amino acid sequence similarities with less than 32% compared to FoADH and ZoADH, the
632 active site residues involved in the Zn²⁺ and NAD⁺ binding are highly conserved (Fig. S11). In
633 addition, the NAD⁺-binding domain exhibits a typical Rossmann fold motif and has the classical
634 conserved sequence "GXGXXG" as in other ADHs and the topologies of those ADHs are highly
635 similar (Fig. S11). The overall topology of those homolog structures was similar with FoADH
636 and ZoADH (Fig. S14). However, superimposition of those ADH structures revealed that there
637 is a large difference in conformation between catalytic and cofactor-binding domains with a
638 r.m.s.d. of 1.373-2.963 Å for FoADH and 1.376-2.191 for ZoADH (Fig. 6e), indicating that they
639 possess large distinct NAD⁺ and substrate-binding clefts. Meanwhile, ADHs from *E. coli* and
640 *T. maritima* also formed the tetrameric formation in crystal structures like FoADH and ZoADH
641 (Fig. S14). These ADHs have the similar tetrameric assembly, however the superimposition of
642 the tetrameric ADHs showed that these tetrameric assemble have low similarity with a r.m.s.d
643 of 17.68~29.94 Å.

644 NAD⁺ and Zn²⁺-binding sites of FoADH and ZoADH

645 While NAD⁺ is the required cofactor for alcohol oxidation, Zn²⁺ interacts with the alcohol
646 molecule in the active site. The electron density maps of a NAD⁺ molecule and two zinc ions
647 are clearly observed in a substrate binding cleft of both FoADH and ZoADH (Fig. S15). The
648 binding configuration of NAD⁺ and the Zn²⁺ ions of ZoADH and FoADH are highly similar
649 (Fig. 7a). The adenine ring of NAD⁺ was located in the hydrophobic pocket formed by
650 hydrophobic interaction (Ile219, Leu245, Thr268, Ile270 and Leu273 for FoADH, Ile220,
651 Leu246, Thr269, Ile271 and Leu274 for ZoADH). The adenine ribose appears to be in a C2'-
652 endo conformation, and the O2' and O3' -hydroxyl group of ribose forms a hydrogen bond with
653 the side chain of aspartate (Asp218 for FoADH and Asp219 for ZoADH). The pyrophosphate

654 moiety of the NAD⁺ interacts with the nitrogen atoms of the main chain of glycine-valine residue
655 (Gly197-Val198 for FoADH and Gly198 and Val199 for ZoADH) that forms the loop between
656 strand β 5 and helix α 4. The nicotinamide ribose is in a C2'-endo conformation, and hydrogen
657 bonds are formed between the ribose O2' -hydroxyl group and threonine (Thr43 for FoADH
658 and ZoADH). The nicotinamide ring is in the anti-conformation. The carboxamide nitrogen
659 atom of the nicotinamide ring interacted with main-chain of proline (Pro313 for FoADH and
660 Pro314 for ZoADH) and valine (Val290 for FoADH and Val291 for ZoADH). The carboxamide
661 oxygen atom of the nicotinamide ring interacted with main-chain of tyrosine (Tyr315 for FoADH
662 and Tyr316 for ZoADH). Therefore, in both FoADH and ZoADH, the NAD⁺ molecules are
663 stabilized by hydrophobic and hydrogen bonds interactions.

664 In both FoADH and ZoADH, two zinc ions are commonly observed in the active site (Zn1 site)
665 and in a loop between α 2 and β 7 (Zn2 site) (Fig. 7a and Fig. S15). The zinc ion at Zn1 site is
666 coordinated by conserved cysteine and histidine residues (Cys41, His58, and Cys169 for
667 FoADH and Cys41, His58, and Cys170 for ZoADH) in the catalytic domain. The zinc ion at Zn2
668 site is involved in the protein stability and is tetrahedrally coordinated by conserved cysteine
669 residues (Cys88, Cys91, Cys94, and Cys102 for both enzymes) (Fig. S15). There result
670 indicated that ZoADH and FoADH showed high structural similarity for the NAD⁺ and zinc
671 binding configuration.

672 Different structural conformations were observed between monomeric ADHs in the tetrameric
673 formation of FoADH and ZoADH (Fig. 6b), indicating that they exhibit structurally different
674 substrate binding cleft and active site. In both results of superimposition of the active sites of
675 FoADH and ZoADH, the positions of the NAD⁺ and Zn2 sites were similar, whereas a
676 significant difference was observed in the positions of the catalytic Zn1 sites (Fig. 7b). In
677 FoADH and ZoADH, the maximum distances between metals from the Zn1 site were 2.57 and
678 2.60 Å, respectively, from closed and open conformation of two domains of ADHs (Fig. 7b).

679 Since the substrate binds to the Zn1 site and a dehydrogenase reaction occurs through the
680 interaction of NAD⁺ with the hydroxyl group, the size of the space between NAD⁺ and Zn1 is
681 involved in substrate selectivity. The closest/longest distance between the Zn²⁺ and C5 atom
682 of the nicotinamide ring of NAD⁺ in FoADH and ZoADH were 3.21/4.91 Å, and 3.46/5.49 Å,
683 respectively (Fig. 7c). These different distance between Zn²⁺ and NAD⁺ were caused by
684 different from closed and open conformation of FoADH and ZoADH.

685 The electrostatic surfaces of FoADH and ZoADH showed that the substrate binding sites
686 commonly exhibited a hydrophobic surface (Fig. 7c). The space of the substrate binding site
687 of FoADH in closed and open conformation were approximately 3.4 x 4.2 Å and 3.9 x 5.4 Å,
688 respectively (Fig. S15). In the closed and open conformation of FoADH, His42 and Ala270 are
689 apart by 3.60 and 5.60 Å, respectively, showing the both surface structure surrounding the

690 NAD⁺ (Fig. 7c). ZoADH also exhibits open and closed conformations similar to FoADH, but the
691 distance of open conformation is relatively wide. The space of the substrate binding site of
692 ZoADH in closed and open conformation were approximately 3.0 x 3.8 Å and 3.8 x 4.9 Å,
693 respectively (Fig. S15). In the closed conformation of ZoADH, the catalytic domain and the
694 cofactor domain are close to each other, especially His42 and Ala270 by a distance of 3.88 Å,
695 indicating the surface structure surrounding the NAD⁺ (Fig. 7c). On the other hand, in the open
696 conformation of ZoADH, His42 and Ala270 are apart by 6.81 Å, and accordingly, the entire
697 NAD⁺ molecule in the surface structure is exposed to the solvent (Fig. 7c).

698

699 Discussion

700 In the present work, FoADH from *F. agariphila* KMM 3901^T and ZoADH from *Z. galactanivorans*
701 Dsij^T were characterized in detail to draw conclusions about their biological function. Three
702 main conclusions regarding the biological function can be derived by the knockout of the genes
703 encoding for ZoADH and CYP in *Z. galactanivorans* and subsequent growth studies on
704 D-galactose and G6Me. First, we confirmed the hypothesis of Reisky et al. that in the absence
705 of CYP-catalyzed oxidative demethylation, a G6Me utilization as sole carbon source is
706 infeasible for the organism (Reisky et al. 2018). Surprisingly, knockout of the ZoADH gene also
707 caused diminished growth of *Z. galactanivorans* in the presence of G6Me. Second, due this
708 observation, we can conclude a significant role of this ADHs in G6Me utilization in these marine
709 bacteria. From an ecological perspective, this has additional importance for the marine
710 carbohydrate degraders. G6Me can occur up to 28% within the porphyran chain (Rees and
711 Conway 1962). Thus, a reduced utilization of G6Me would represent a substantial potential
712 loss as a carbon source for the organism. Third, since normal growth was observed in the
713 presence of D-galactose as sole carbon source, a function in D-galactose metabolism can be
714 excluded. This was also supported by the observation that both ADHs lacked activity for
715 D-galactose. The ADHs are therefore probably involved in oxidative demethylation or a
716 subsequent reaction. Since no activity was observed for G6Me, the substrate of oxidative
717 demethylation could be excluded. Consequently, we hypothesized that the ADHs are involved
718 in the detoxification of formaldehyde, which is a by-product of the oxidative demethylation
719 reaction. This was also supported by the resistance of both ADHs to formaldehyde exposure.
720 Formaldehyde is a toxic metabolite due to its properties as a highly reactive electrophile. It can
721 react with free amino and thiol groups of proteins and nucleic acids, leading to protein and
722 DNA damages as well as cross-link formations (Chen et al. 2016; Shishodia et al. 2018; Tayri-
723 Wilk et al. 2020). It has been shown that higher concentrations of formaldehyde can negatively
724 affect the growth of *Z. galactanivorans* (Brott et al. 2022). Thus, a reduced growth of the ADH
725 knock-out strain could be explained by the potential accumulation of formaldehyde. There are

726 numerous metabolic pathways in which formaldehyde can be detoxified (Yurimoto et al. 2005;
727 Klein et al. 2022). However, in the thiol-dependent formaldehyde detoxification, a zinc-
728 dependent ADH and an esterase perform the key reactions (Sanghani et al. 2000; Gonzalez
729 et al. 2006). Genome neighborhood analysis revealed that in most marine bacteria that
730 possess the ADH gene, it was located in close proximity to a gene encoding for an esterase in
731 addition to the CYP gene. We therefore investigated whether the ADH catalyzed a thiol-
732 dependent detoxification of formaldehyde. However, with glutathione, mycothiol, and
733 bacillithiol as thiol cofactors, no activity was detected for either ADH. These observations can
734 be further explained with the crystal structures of both ADHs; sterically demanding compounds
735 such as mycothiol or bacillithiol cannot fit into the narrow active site of these enzymes. These
736 observations are also consistent with the results from the sequence similarity network, in which
737 glutathione- and mycothiol-dependent formaldehyde dehydrogenases were predominantly
738 present in different clusters (main clusters 1 and 4) than the ADHs (main cluster 2). Since no
739 activity could be detected with literature-known cofactors, additional thiols were considered;
740 however, no activity could be observed either. Thiol cofactors are still being discovered
741 (Newton and Rawat 2019), perhaps marine organisms also possess an unidentified thiol,
742 which can serve as a cofactor for this reaction. Since no activity was observed for
743 formaldehyde without an additional thiol cofactor, the biological function of a thiol-independent
744 formaldehyde dehydrogenase was excluded. In addition, some ADHs can possess dismutase
745 activities (Trivić et al. 1999). A formaldehyde dismutase catalyzes the disproportionation of
746 formaldehyde to methanol and formic acid in the presence of a covalent-bound NAD^+
747 (Yonemitsu and Kikuchi 2018). However, this reaction could not be detected. Both organisms
748 harbor other metabolic pathways for the detoxification of formaldehyde (Brott et al. 2022). For
749 instance, in *Z. galactanivorans*, the genes encoding for the key enzymes of the ribulose
750 monophosphate pathway are upregulated in the presence of porphyran (Brott et al. 2022), so
751 an accumulation of formaldehyde is unlikely. Eventually, the ADHs might have a completely
752 different biological function such as the regeneration of NADH (Hilberath et al. 2021; Kokorin
753 et al. 2021). In the oxidative demethylation reaction, NADH is oxidized to NAD^+ , a reduced
754 growth in the ADH knockout strain due to cofactor depletion might be possible. NADH could
755 be regenerated by oxidation of an unknown component or by the thiol-dependent
756 formaldehyde detoxification pathway. However, it is doubtful that the loss of one single enzyme
757 would cause such a tremendous effect on NADH/ NAD^+ homeostasis. Additionally, the ADHs
758 displayed predominantly activity for the reduction of aldehydes under NADH consumption,
759 therefore recycling of a cofactor is improbable.

760 Both ADHs possessed predominantly activity for aromatic substances, resulting in a substrate
761 specificity resembling partially those of cinnamyl alcohol and/or benzyl alcohol
762 dehydrogenases (Larroy et al. 2002; Willson et al. 2022). However, the highest activity was

763 observed for pyridine-3-carbaldehyde and furan derivatives. Furfural is generally produced as
764 a side product by pretreating lignocellulosic biomass for the production of bioethanol. Under
765 acidic conditions and high temperatures, dehydration of pentoses and hexoses proceeds,
766 leading to the formation of furfural or hydroxymethylfurfural. Furfural acts as an inhibitor in
767 subsequent bioethanol-producing fermentations by bacteria by prolonging the lag phase of
768 growth and thereby the fermentation time (Mariscal et al. 2016). Consequently, these marine
769 bacteria possess ADHs that catalyze the potential removal of furfural although the biological
770 function may be different. The ADHs lacked activity for various sugar substrates, which
771 excluded a polyol dehydrogenase activity. Activity for any other monosaccharides,
772 disaccharides or even oligosaccharides formed during porphyran degradation is unlikely as
773 well, considering the substrate specificity of the enzymes based on the narrow active site. The
774 data from biochemical characterizations are discussed in the SI.

775 We have determined the crystal structures of FoADH and ZoADH complexed with NAD⁺ and
776 two zinc ions. These ADHs showed high structural similarity in terms of topology and assembly.
777 On the one hand, these two ADHs showed similarities in topology with other ADHs from human,
778 *E. coli* and *T. maritima*, but showed distinct conformation between the cofactor and catalytic
779 domains of those ADHs. On the other hand, the crystal structures of FoADH and ZoADH
780 showed open and closed conformations, indicating that the conformation between the two
781 domains can change in the state where the substrate is not bound. These distinct
782 conformations of FoADH and ZoADH represent different substrate binding pockets. When they
783 exhibit an open conformation between the two domains of FoADH and ZoADH, they form a
784 broadened substrate-binding pocket. Accordingly, in terms of substrate accessibility, we
785 consider that substrate accessibility will be easier when FoADH and ZoADH have an open
786 conformation.

787 During substrate recognition, when the converting functional group from the substrate
788 approaches the Zn1 site on the substrate binding pocket of FoADH and ZoADH, the rest of the
789 substrate is exposed to the nicotinamide of NAD⁺ or the hydrophobic surface. Considering that
790 the nicotinamide group of NAD⁺ is involved in the oxidoreductase mechanism of the ADH, the
791 substrate would prefer to be located to the hydrophobic surface rather than the nicotinamide
792 group of NAD⁺. Accordingly, FoADH and ZoADH may prefer substrates having a hydrophobic
793 body. Our biochemical studies showed that both enzymes prefer aromatic substrates. We
794 expected that the aromatic ring of the substrate may be located on a hydrophobic surface
795 nearby the substrate binding pocket of FoADH and ZoADH. In this case, the aromatic ring of
796 the substrate could interact with the Phe136 residue in the hydrophobic surfaces of the
797 enzymes. Based on the active site structures of both ADH computational docking of a substrate
798 will be able to provide an insight into the molecular mechanism and substrate specificity.

799 However, from the results of this study, ZoADH and FoADH have various conformations
800 between catalytic and cofactor binding domain in NAD⁺ and two zinc ion binding states,
801 indicating the computational docking results could be different depending on the applied model
802 structure. Also, based on our results, we concluded that the docking results may be different
803 from biochemical experiments if the active sites of ZoADH and FoADH may have different
804 conformations. Therefore, to better understand the substrate specificity, the crystal structures
805 of ZoADH and FoADH in complex with the biological substrate will be needed in the future.

806 In summary, in this study we determined the putative functions of conserved ADH from marine
807 *Flavobacteriia*. Additionally, we provided the crystal structures of the enzymes of *F. agariphila*
808 and *Z. galactanivorans*. Enzymatic studies revealed the preferential conversion of aromatic
809 aldehydes. We revealed that these enzymes are not involved in formaldehyde detoxification
810 or in subsequent reaction of the oxidative demethylation of G6Me. Based on gene knockouts,
811 we demonstrated the essential role of these ADHs in the utilization of marine algal sugars. Our
812 study indicates a potential auxiliary activity of these ADHs in the utilization of marine sugars
813 by marine *Flavobacteriia*.

814 **Declarations**

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822 **Author contributions:** M.G., T.S. and U.T.B. initiated the study and directed the project. F.T.
823 conducted the growth studies and created the knock-out strain. K.H.N performed the
824 crystallization and structural analyses. T.D. performed the computational analysis. L. R. and
825 H.C.G. performed the cloning and initial experiments on enzyme function of FoADH. S.B. and
826 M.B. expressed and purified the enzymes and performed further experiments on enzyme
827 function and characterization. S.B. and K.H.N prepared the main manuscript, which was
828 revised by F.T., T.D, H.C.G., L. R., M.G, T.S. and U.T.B. and was approved by all authors.

829 **Ethical approval:** Not applicable.

830 **Data availability:** The datasets generated during and/or analyzed during the current study are
831 available from the corresponding author on reasonable request.

832

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1093 **Figure legends**

1094 **Fig. 1** Porphyrans contain 6-O-methyl-D-galactose, which can be metabolized by marine
1095 bacteria via oxidative demethylation. **a)** Porphyrans, the common name of the galactan of red
1096 algae of the genus *Porphyra*, consist of chains composed mainly of the alternating
1097 monosaccharide units 4-linked- α -L-galactose-6-sulfate (L6S) and 3-linked- β -D-galactose (Gal)
1098 or 3,6-anhydro- α -L-galactose (LA). Furthermore, O-methylation of D-galactose results in the
1099 formation of 6-O-methyl-D-galactose (G6Me). **b)** The oxidative demethylation of G6Me is
1100 catalyzed by a cytochrome P450 monooxygenase in combination with its redox partners
1101 ferredoxin and ferredoxin reductase, producing D-galactose and formaldehyde in equimolar
1102 amounts. **c)** In *Formosa agariphila* KMM 3901^T and **d)** *Zobellia galactanivorans* Dsij^T, genes
1103 encoding for the key enzymes of oxidative demethylation are located in close proximity to a
1104 gene encoding for a zinc-dependent alcohol dehydrogenase. *BN863_, for example *21030
1105 refers to locus tag *BN863_21030* for *F. agariphila* while *zgal*, for example *zgal_4674* refers to locus
1106 tag *zgal_4674* for *Z. galactanivorans*.

1107 **Fig. 2** Knockout of the ADH gene in *Z. galactanivorans* leads to impaired growth on G6Me.
1108 Different *Z. galactanivorans* strains (wild type (WT), gene knockout ADH (Δ ADH), and gene
1109 knockout CYP (Δ CYP)) were incubated in minimal medium amended with D-galactose or
1110 G6Me for 3 days at RT.

1111 **Fig. 3** Thiol-dependent detoxification of formaldehyde catalyzed by an ADH and an esterase.
1112 **a)** Principle of thiol-dependent detoxification of formaldehyde and **b)** investigated thiols.

1113 **Fig. 4** Influence of pH and buffer components on the ADH activity. pH optimum for the
1114 reduction reaction of **a)** FoADH and **b)** ZoADH as well as the pH optimum for the oxidation
1115 reaction catalyzed by **d)** FoADH and **e)** ZoADH. **c)** Reduction of benzaldehyde and **f)** oxidation
1116 of benzyl alcohol by the ADHs at the respective pH optima using various buffers. A pH of 6.5
1117 was employed for the reduction reaction and a pH of 8.5 for the oxidation reaction; all buffers
1118 had a concentration of 50 mM. Since some buffers including Bicine, Tricine, Tris, MOPSO and
1119 HEPES contain hydroxyl groups, a falsified activity due to turnover of these substances was
1120 excluded by a measurement without additional substrate. However, no activity was observed
1121 for any buffer component. All measurements (**a-f**) were performed under following conditions:
1122 a final substrate concentration of 10 mM benzyl alcohol or benzaldehyde, 3.5% (v/v) DMSO
1123 and 0.5 mM NAD⁺ or NADH was used. The reaction was started by the addition of ADH at a
1124 final enzyme concentration of 0.1 mg mL⁻¹. The measurement was performed at 25 °C in the
1125 respective buffers with concentrations of 50 mM. The maximum relative activity (100%)
1126 corresponds to the measurements in the 50 mM NaPi buffers pH 6.5 for reduction and pH 8.5
1127 for oxidation reactions. All measurements were performed as triplicates, the mean is given and
1128 the error bars indicate the standard deviation.

1129 **Fig. 5** Temperature optimum and thermostability of the ADHs. **a)** Influence of temperature on
1130 enzyme activity. The measurement was performed at various temperatures ranging from 20 to
1131 80 °C for 10 min. The maximum relative activity (100%) corresponds to the measurement at
1132 75 °C for both enzymes. Influence of temperature on enzyme stability for **b)** FoADH and **c)**
1133 ZoADH. The enzymes with a concentration of 1 mg mL⁻¹ were incubated at different
1134 temperatures between 20 and 80 °C for 1 or 4 hours, followed by the determination of residual
1135 activity. The measurement was performed at 40 °C. The maximum relative activity (100%)
1136 corresponds to a control incubated on ice for 1 or 4 h. All measurements (**a-c**) were performed
1137 under following conditions: a final substrate concentration of 10 mM benzyl alcohol and 0.5 mM

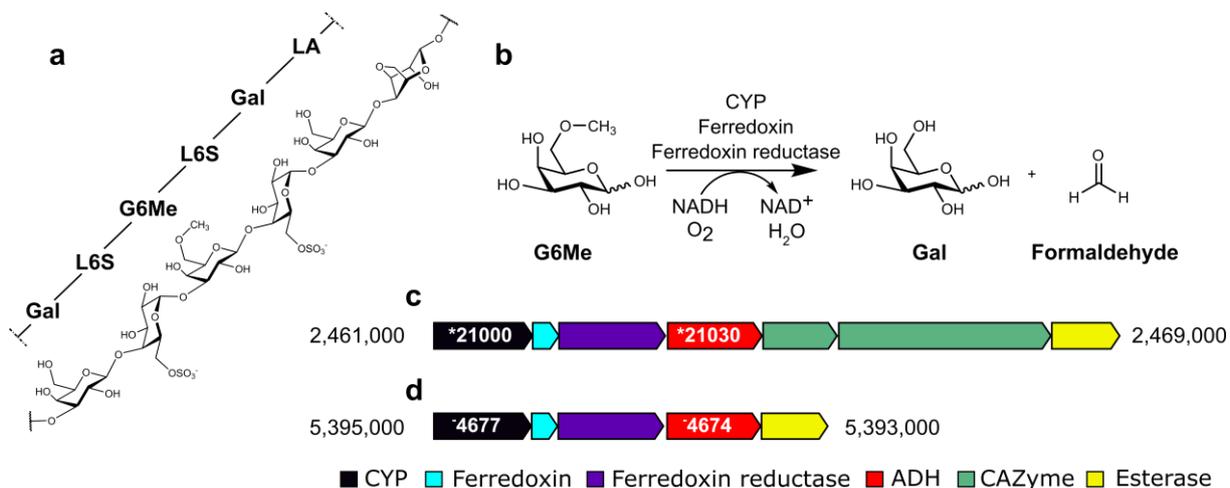
1138 NAD⁺ was used. The reaction was started by the addition of ADH at a final enzyme
 1139 concentration of 0.1 mg mL⁻¹. The measurements were performed in a 50 mM NaPi buffer
 1140 pH 7.5. All measurements were performed as triplicates, the mean is given and the error bars
 1141 indicate the standard deviation.

1142 **Fig. 6** Crystal structures of FoADH and ZoADH. **a)** Monomer structures of ZoADH and FoADH.
 1143 Catalytic and cofactor domain are indicated by cyan and green, respectively. NAD⁺ and zinc
 1144 ions are indicated by yellow stick and grey sphere, respectively. **b)** Superimposition of closed
 1145 (green) and open conformation between catalytic and cofactor-binding domains of ZoADH and
 1146 FoADH monomers. The superimposed cofactor-binding domain of ZoADH and FoADH are
 1147 indicated as grey cartoon. **c)** Tetrameric formation of ZoADH and FoADH. **d)** Superimposition
 1148 of tetrameric formation of FoADH (green) and ZoADH (cyan). **e)** Superimposition of monomer
 1149 structure of FoADH (green) and ZoADH (cyan) with all-trans-retinol dehydrogenase ADH4 from
 1150 *Homo sapiens* (pink, PDB code: 3COS), uncharacterized zinc-type alcohol dehydrogenase-
 1151 like protein YdjJ from *E. coli* (wheat, 5vm2), scyllo-inosose 3-dehydrogenase from *Thermotoga*
 1152 *maritima* (3IP1, yellow).

1153 **Fig. 7** Active sites of FoADH and ZoADH. **a)** Interaction of ZoADH and FoADH with NAD⁺ and
 1154 zinc ion at the Zn1 site. **b)** Superimposition of active site of open and closed conformation of
 1155 FoADH and ZoADH. **c)** Comparison of electrostatic surface structure of open and closed
 1156 conformations of FoADH and ZoADH

1157 Figures

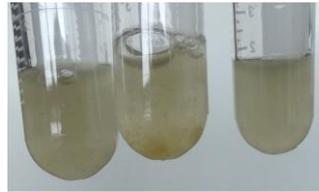
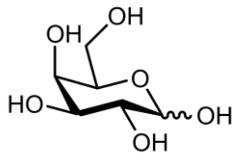
1158 Fig. 1



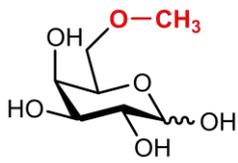
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1160 **Fig. 2**

D-Galactose



6-O-methyl-D-galactose

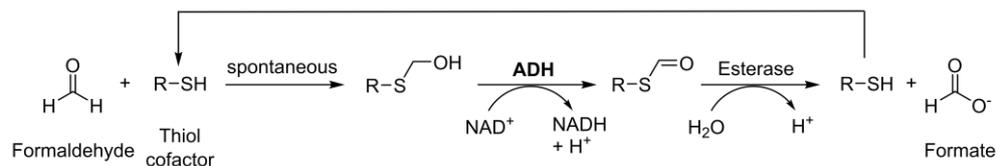
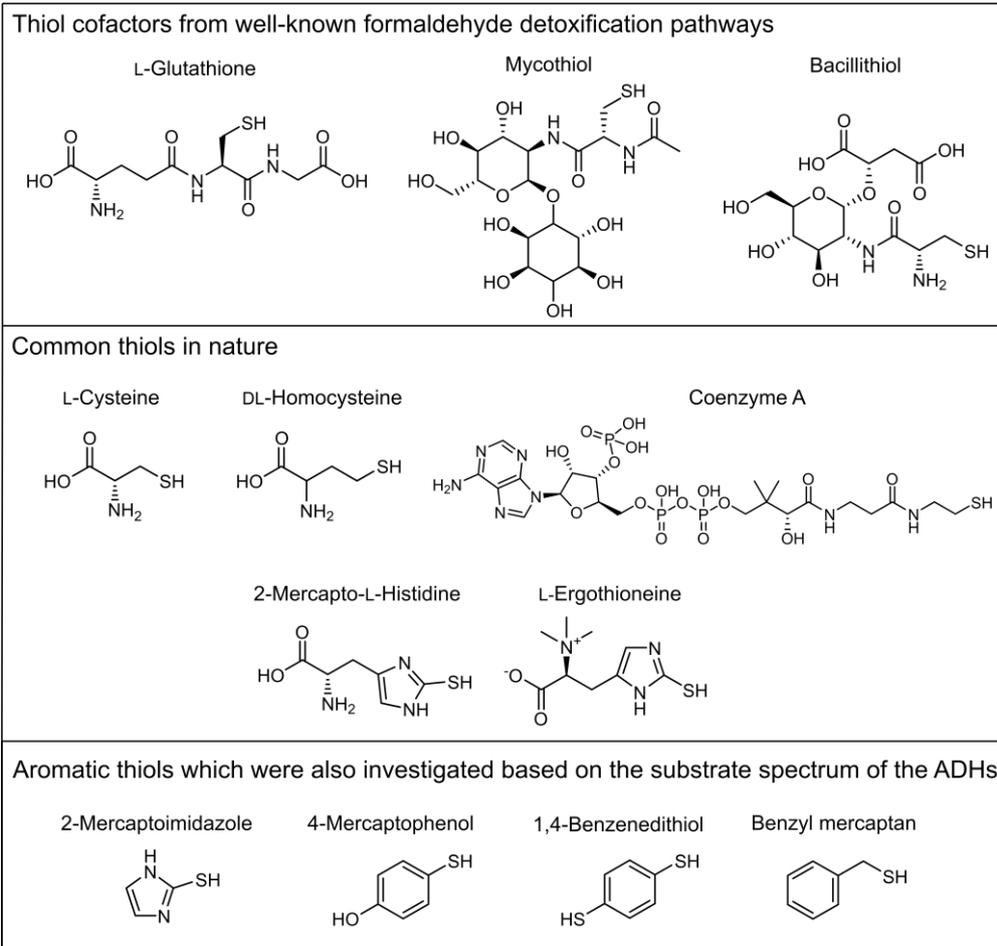


WT ΔADH ΔCYP



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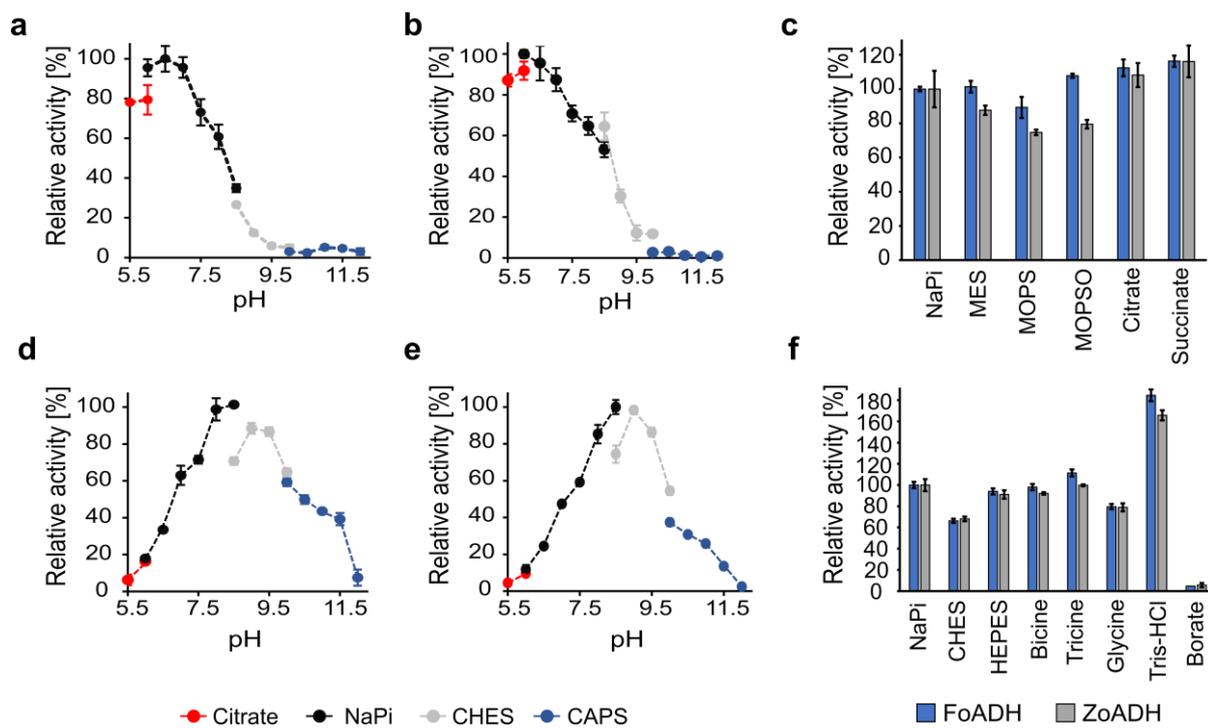
a**b**

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1167 **Fig. 4**

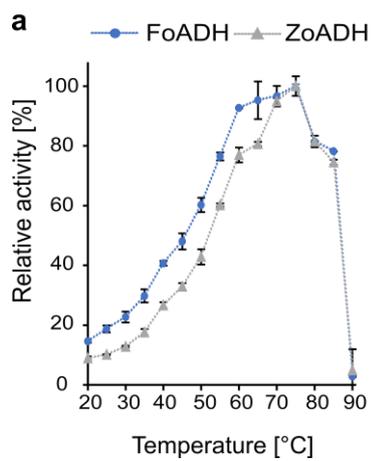


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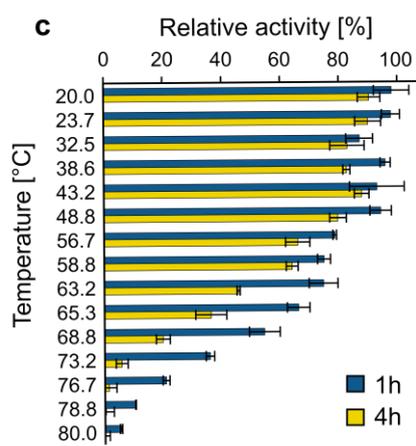
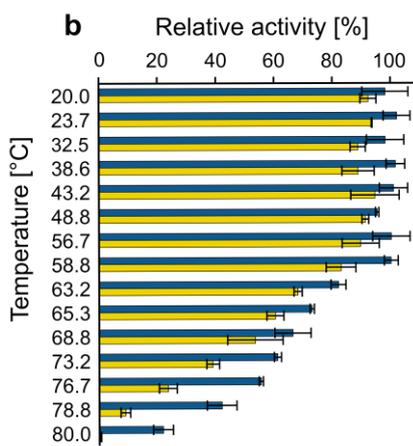
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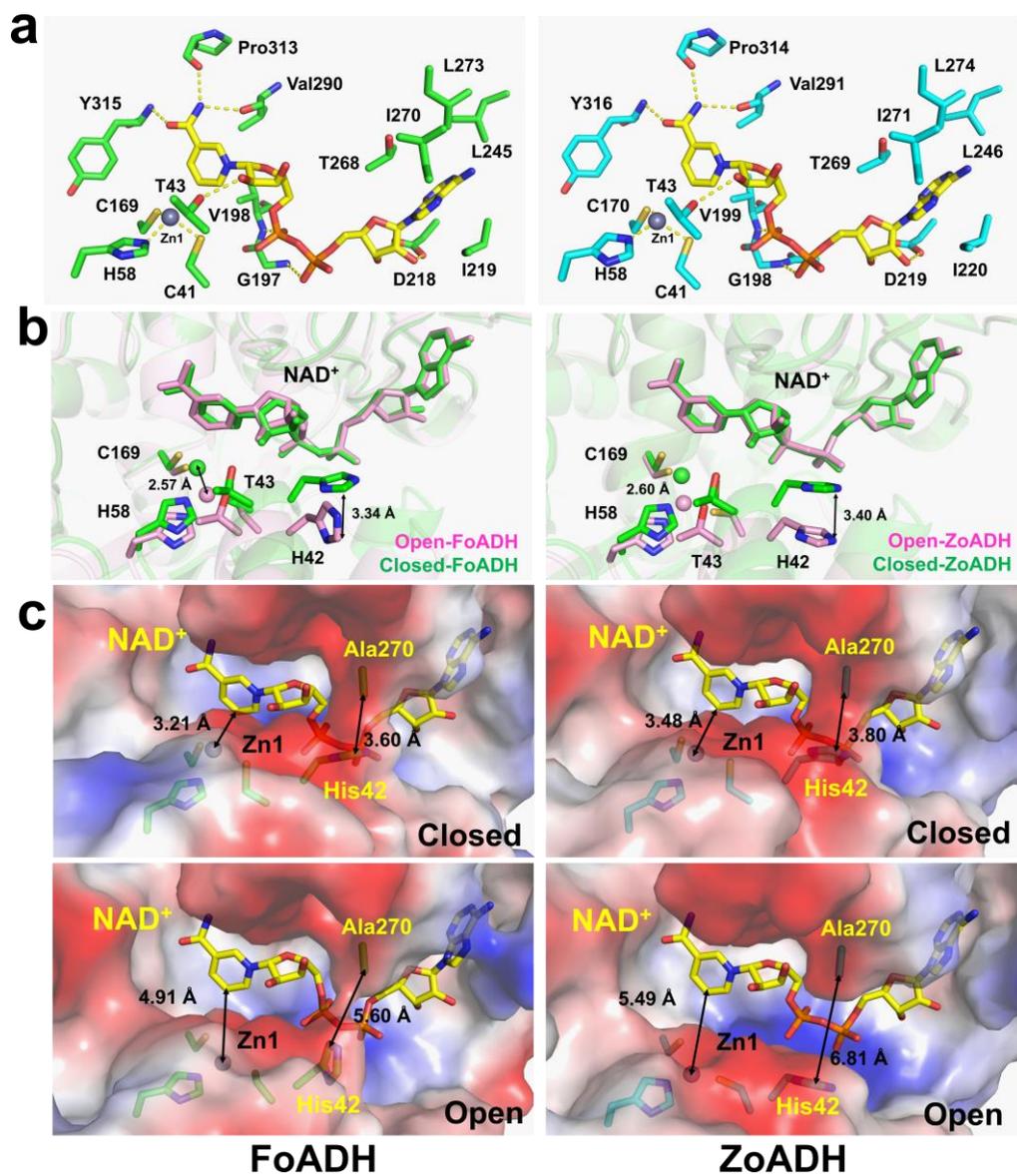
1171 **Fig. 5**



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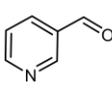
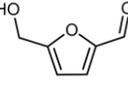
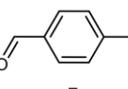
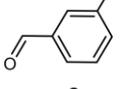
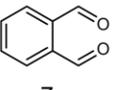
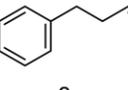
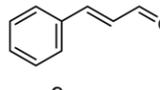
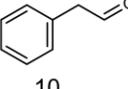
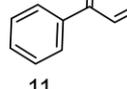
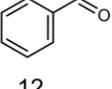
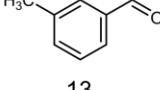
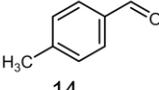
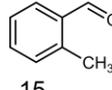
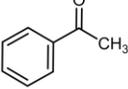
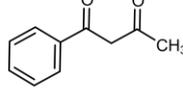
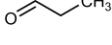
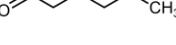
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1179 **Tables**

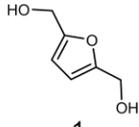
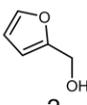
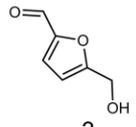
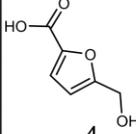
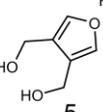
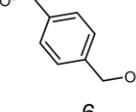
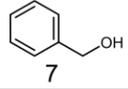
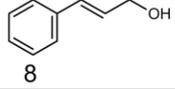
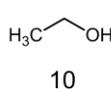
1180 **Table 1** Initial substrate screening of the ADH in the reduction direction revealed that they
 1181 preferentially convert aromatic aldehydes. Substrates were employed at a final concentration
 1182 of 10 mM. For NADH a concentration of 0.5 mM was used. The reaction contained 3.5% (v/v)
 1183 DMSO. The reaction was conducted in a 50 mM succinate buffer pH 6.5 at an incubation
 1184 temperature of 70 °C. All measurements were performed as triplicates, the mean and the
 1185 standard deviation is given.

Abbr.	Substrates	Specific activity [U/mg]		
		FoADH	ZoADH	
1	Pyridine-3-carbaldehyde	64.09 ± 2.39	54.85 ± 4.34	
2	Furfural	47.77 ± 1.19	44.78 ± 2.07	
3	5-(Hydroxymethyl)furfural	44.81 ± 2.16	38.29 ± 2.47	
4	Thiophene-3-carbaldehyde	37.45 ± 4.19	29.32 ± 2.59	
5	Terephthalaldehyde	30.05 ± 3.60	27.34 ± 4.68	
6	Isophthalaldehyde	26.71 ± 1.47	36.94 ± 3.68	
7	Phthalaldehyde	n.d. ^{a)}	n.d.	
8	Hydrocinnamaldehyde	26.00 ± 2.23	30.13 ± 2.32	
9	Cinnamaldehyde ^{b)}	12.57 ± 0.90	11.20 ± 0.11	
10	Phenylacetaldehyde	n.d.	n.d.	
11	Phenylglyoxal	n.d.	n.d.	
12	Benzaldehyde	5.14 ± 0.09	12.49 ± 0.73	
13	<i>m</i> -Tolualdehyde	6.30 ± 1.06	7.32 ± 0.32	
14	<i>p</i> -Tolualdehyde	4.71 ± 0.32	5.60 ± 0.44	
15	<i>o</i> -Tolualdehyde	0.04 ± 0.01	0.04 ± 0.01	
16	Acetophenone	n.d.	n.d.	
17	Benzoylacetone	n.d.	n.d.	
18	Formaldehyde	n.d.	n.d.	
19	Propionaldehyde	1.58 ± 0.10	0.92 ± 0.36	
20	Butyraldehyde	3.62 ± 1.04	4.02 ± 0.48	
21	Caproaldehyde	7.07 ± 0.64	6.62 ± 0.30	

a) n.d.: not detected

1186 b) Due to a high background absorption of the component, a substrate concentration of 1mM was employed.

1187 **Table 2** Both ADHs possess minor specific activities for the oxidation of alcohols.
 1188 Formaldehyde was also tested in a possible oxidation reaction to exclude thiol-independent
 1189 formaldehyde dehydrogenase activity. Substrates were employed at a final concentration of
 1190 10 mM. For NAD⁺ a concentration of 0.5 mM was used. The reaction contained 3.5% (v/v)
 1191 DMSO. The reaction was conducted in a 50 mM NaPi buffer pH 8.5 at an incubation
 1192 temperature of 70 °C. All measurements were performed as triplicates, the mean and the
 1193 standard deviation is given.

Abbr.	Substrate	Specific activity [mU/mg]		
		FoADH	ZoADH	
1	2,5-Bis(hydroxymethyl)furan	490 ± 50	290 ± 90	
2	Furfuryl alcohol	310 ± 10	194 ± 51	
3	5-(Hydroxymethyl)furfural	n.d. ^{a)}	n.d.	
4	5-(Hydroxymethyl)furan-2-carboxylic acid	n.d.	n.d.	
5	3,4-Bis(hydroxymethyl)furan	n.d.	n.d.	
6	1,4-Benzenedimethanol	86 ± 1.5	51 ± 3.0	
7	Benzyl alcohol	81 ± 0.7	53 ± 0.1	
8	Cinnamyl alcohol ^{b)}	11 ± 3.1	6 ± 2.9	
9	Methanol	n.d.	n.d.	
10	Ethanol	n.d.	n.d.	
11	Formaldehyde	n.d.	n.d.	

a) n.d.: not detected

1194 b) Due to a high background absorption of the component, a substrate concentration of 1mM was employed.

1195 **Table 3** Influence of various substances on the enzyme activity of both ADHs. The ADH was
 1196 incubated with the respective component for 1 h at RT prior to measurement. The maximum
 1197 relative activity (100%) corresponds to the measurement for the control, which contained no
 1198 additives. All measurements were performed under following conditions: a final substrate
 1199 concentration of 10 mM benzyl alcohol and 0.5 mM NAD⁺ was used. The reaction was started
 1200 by the addition of ADH at a final enzyme concentration of 0.1 mg mL⁻¹. The measurements
 1201 were performed in a 50 mM HEPES buffer pH 8.5 at 25 °C. All measurements were performed
 1202 as triplicates, the mean and the standard deviation is given.

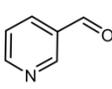
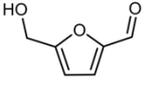
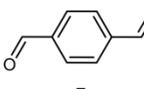
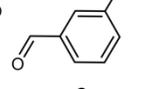
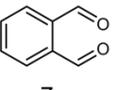
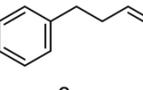
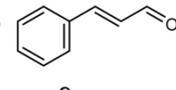
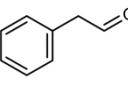
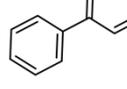
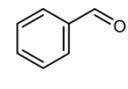
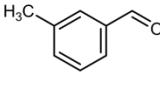
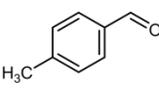
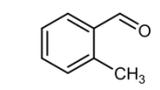
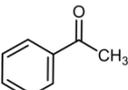
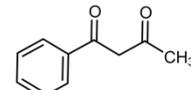
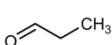
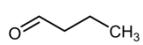
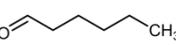
Chemical	Conc. [mM]	Relative activity [%]	
		FoADH	ZoADH
None	-	100 ± 1.3	100 ± 9.3
KCl	10	121 ± 1.9	154 ± 7.9
CaCl ₂	10	188 ± 6.3	306 ± 9.9
MgCl ₂	10	219 ± 7.2	328 ± 11.7
NiCl ₂	10	891 ± 13.8	1004 ± 6.6
CoCl ₂	10	1280 ± 38.1	1242 ± 25.4
MnCl ₂	10	1394 ± 58.5	973 ± 45.0
ZnCl ₂	10	n.d. ^{a)}	n.d.
CuCl ₂	10	n.d.	n.d.
FeCl ₃	10	n.d.	n.d.
EDTA	25	61 ± 1.9	61 ± 7.8
DTT	10	48 ± 4.6	85 ± 3.9
2-ME	10	19 ± 1.7	28 ± 4.1

1203

a) n.d.: not detected

1 **Tables**

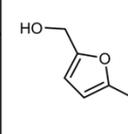
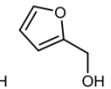
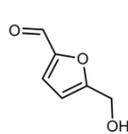
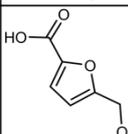
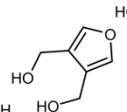
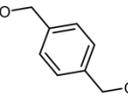
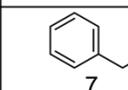
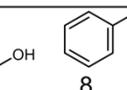
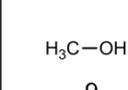
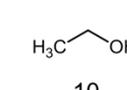
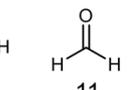
2 **Table 1** Initial substrate screening of the ADH in the reduction direction revealed that they
 3 preferentially convert aromatic aldehydes. Substrates were employed at a final concentration
 4 of 10 mM. For NADH a concentration of 0.5 mM was used. The reaction contained 3.5% (v/v)
 5 DMSO. The reaction was conducted in a 50 mM succinate buffer pH 6.5 at an incubation
 6 temperature of 70 °C. All measurements were performed as triplicates, the mean and the
 7 standard deviation is given.

Abbr.	Substrates	Specific activity [U/mg]		
		FoADH	ZoADH	
1	Pyridine-3-carbaldehyde	64.09 ± 2.39	54.85 ± 4.34	
2	Furfural	47.77 ± 1.19	44.78 ± 2.07	
3	5-(Hydroxymethyl)furfural	44.81 ± 2.16	38.29 ± 2.47	
4	Thiophene-3-carbaldehyde	37.45 ± 4.19	29.32 ± 2.59	
5	Terephthalaldehyde	30.05 ± 3.60	27.34 ± 4.68	
6	Isophthalaldehyde	26.71 ± 1.47	36.94 ± 3.68	
7	Phthalaldehyde	n.d. ^{a)}	n.d.	
8	Hydrocinnamaldehyde	26.00 ± 2.23	30.13 ± 2.32	
9	Cinnamaldehyde ^{b)}	12.57 ± 0.90	11.20 ± 0.11	
10	Phenylacetaldehyde	n.d.	n.d.	
11	Phenylglyoxal	n.d.	n.d.	
12	Benzaldehyde	5.14 ± 0.09	12.49 ± 0.73	
13	<i>m</i> -Tolualdehyde	6.30 ± 1.06	7.32 ± 0.32	
14	<i>p</i> -Tolualdehyde	4.71 ± 0.32	5.60 ± 0.44	
15	<i>o</i> -Tolualdehyde	0.04 ± 0.01	0.04 ± 0.01	
16	Acetophenone	n.d.	n.d.	
17	Benzoylacetone	n.d.	n.d.	
18	Formaldehyde	n.d.	n.d.	
19	Propionaldehyde	1.58 ± 0.10	0.92 ± 0.36	
20	Butyraldehyde	3.62 ± 1.04	4.02 ± 0.48	
21	Caproaldehyde	7.07 ± 0.64	6.62 ± 0.30	

a) n.d.: not detected

8 b) Due to a high background absorption of the component, a substrate concentration of 1mM was employed.

1 **Table 2** Both ADHs possess minor specific activities for the oxidation of alcohols.
 2 Formaldehyde was also tested in a possible oxidation reaction to exclude thiol-independent
 3 formaldehyde dehydrogenase activity. Substrates were employed at a final concentration of
 4 10 mM. For NAD⁺ a concentration of 0.5 mM was used. The reaction contained 3.5% (v/v)
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11	Formaldehyde	n.d.	n.d.	

a) n.d.: not detected

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1 **Table 3** Influence of various substances on the enzyme activity of both ADHs. The ADH was
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 3 relative activity (100%) corresponds to the measurement for the control, which contained no
 4 additives. All measurements were performed under following conditions: a final substrate
 5 concentration of 10 mM benzyl alcohol and 0.5 mM NAD⁺ was used. The reaction was started
 6 by the addition of ADH at a final enzyme concentration of 0.1 mg mL⁻¹. The measurements
 7 were performed in a 50 mM HEPES buffer pH 8.5 at 25 °C. All measurements were performed
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