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4 **Characterization of a novel type III alcohol dehydrogenase from *Thermococcus***
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6 ***barophilus* Ch5**
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Abstract

The genome of the hyperthermophilic and piezophilic euryarchaeon *Thermococcus barophilus* Ch5 encodes three putative alcohol dehydrogenases (Tba ADHs). Herein, we characterized Tba ADH₅₄₇ biochemically and probed its mechanism by mutational studies. Our data demonstrate that Tba ADH₅₄₇ can oxidize ethanol and reduce acetaldehyde at high temperature with the same optimal temperature (75°C) and exhibit similar thermostability for oxidation and reduction reactions. However, Tba ADH₅₄₇ has different optimal pH for oxidation and reduction: 8.5 for oxidation and 7.0 for reduction. Tba ADH₅₄₇ is dependent on a divalent ion for its oxidation activity, among which Mn²⁺ is optimal. However, Tba ADH₅₄₇ displays about 20% reduction activity without a divalent ion, and the maximal activity with Fe²⁺. Furthermore, Tba ADH₅₄₇ showcases a strong substrate preference for 1-butanol and 1-hexanol over ethanol and other alcohols. Similarly, Tba ADH₅₄₇ prefers butylaldehyde to acetaldehyde as its reduction substrate. Mutational studies showed that the mutations of residues D195, H199, H262 and H234 to Ala result in the significant activity loss of Tba ADH₅₄₇, suggesting that residues D195, H199, H262 and H234 are responsible for catalysis. Overall, Tba ADH₅₄₇ is a thermoactive ADH with novel biochemical characteristics, thereby allowing this enzyme to be a potential biocatalyst.

Keywords: Hyperthermophilic Archaea; Alcohol dehydrogenase; Biochemical characteristics

1 Introduction

Alcohol dehydrogenases (ADHs), which are members of the oxidoreductase family, are able to catalyze the cofactor-dependent oxidation of ethanol and other alcohols to produce the corresponding aldehydes and ketones [1]. Thus, ADHs play important roles in the detoxification and metabolism of primary and secondary alcohols. Based on their cofactor requirements, ADHs are classified into four types: nicotinamide adenine dinucleotide (NAD) dependent ADH; pyrroloquinoline quinone (PQQ) -dependent ADH; heme or cofactor F420 -dependent ADH; flavine adenine dinucleotide -(FAD) dependent ADH [2]. In addition, NAD-dependent ADHs are further subdivided into three types depending on the alcohols metabolized: short-chain ADHs exemplified by the drosophila ADH [3] (type I), medium-chain or zinc ADHs (type II or Zn-ADH) exemplified by the horse liver ADH [4], and long-chain or iron ADHs (type III or Fe-ADH) exemplified by the *Zymomonas* ADH2 [5, 6]. These three families display low similarity and arose independently throughout evolution, suggesting that these ADHs harbor different structures and mechanisms of reaction.

Several type III ADHs have been biochemically characterized since a type III ADH was first reported from *Zymomonas mobilis*, including *Saccharomyces cerevisiae* ADH4 [7], *Escherichia coli* L-1,2-propanediol oxidoreductase [8], *Clostridium acetobutylicum* ADH1 [9], *C. acetobutylicum* butanol dehydrogenases [10], and *Bacillus methanolicus* methanol dehydrogenase [11], and a human iron-activated ADH [12]. Crystal structures of the type III ADHs from bacteria and

1 archaea show that they possess a very highly conserved catalytic cleft where Fe²⁺
2
3 ion is coordinated with three His residues and one Asp residue [13-16]. Currently,
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5 several type III ADHs from hyperthermophilic *Thermococcus* have been reported since
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7 the first type III ADH was characterized from *Thermococcus litoralis* [17], including
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9 *Thermococcus* strain ES-1 [18-19], *Thermococcus* strain AN1 [20], *Thermococcus*
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11 *hydrothermalis* [21], and *Thermococcus thio还原ens* [15] and *Pyrococcus furiosus*
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13 [22]. All these reported type III ADHs from HA have one common characteristic:
14
15 high thermostability, which is distinct from other ADHs from mesophiles. Although
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17 several ADHs from HA have been found, thermostable ADHs are still in demand due
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19 to unique characteristics, demonstrating a variety of substrate specificities and
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21 physiological functions.
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31 *Thermococcus barophilus* Ch5, which was isolated from a deep-sea
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33 hydrothermal field of the Mid-Atlantic Ridge (Logachev field chimney, 3,020 m
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35 depth), is a thermo-piezophilic euryarchaeon, growing optimally at 88°C under
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37 40 MPa of hydrostatic pressure [23]. The genome of *T. barophilus* Ch5 encodes 2,679
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39 proteins, two thirds of which have no associated functions [24]. Furthermore, several
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41 thermostable enzymes from *T. barophilus* Ch5 have been characterized [25-29] and
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43 have been shown to have distinct characteristics from other homologues found in
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45 other extremophilic archaeal models. Thus, *T. barophilus* Ch5 is an important model
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47 for studying structural and function relationship of thermostable proteins or enzymes
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49 present in hyperthermophilic archaea as well as a great untapped source of novel extreme
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51 thermophilic enzymes for industrial processes.
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1 Three putative alcohol dehydrogenases (Tba ADHs) are encoded in the genome
2 of *T. barophilus* Ch5, including one short-chain ADH (GenBank: ALM74123) and
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6 two putative type III ADHs (GenBank: ALM74514 and ALM74601). Interestingly,
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9 these two putative Fe-ADHs from *T. barophilus* Ch5 have only 26% similarity in
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12 amino acid compositions, suggesting that they might harbor distinct characteristics.
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14 Currently, no ADHs from *T. barophilus* Ch5 has been biochemically characterized. In
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17 this work, we report the cloning, expression, biochemical characterization and
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20 mutational studies of the recombinant Tba ADH₅₄₇ encoded by gene TBCH5v1_0547
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23 (Genbank: ALM74514) and is conserved in all *Thermococcales* species, suggesting
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26 that this ADH might be a putative type III alcohol dehydrogenase with novel
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29 characterization.

30 **2 Materials and methods**

31 *2.1 Materials*

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36 The pET-30a(+) vector was purchased from Novagen (Merck, Darmstadt,
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38
39 Germany). Plasmid Extraction Kit, PCR Cycle Pure Kit, and Gel Extraction Kit were
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42 purchased from Omega (Guangzhou, China). *Escherichia coli* DH5a cells and *E. coli*
43
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45 BL21 (DE3) cells were purchased from Transgene (Beijing, China); dNTPs, T4 DNA
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48 ligase, *Nde*I, *Xho*I, and Pfu DNA polymerase were purchased from Thermo Scientific
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51 (Waltham, MA). Chemicals were purchased from Amresco (WA, USA).

52 *2.2 Phylogenetic analyses*

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56 The genes coding for Tba ADH₅₄₇ and Tba ADH₆₄₁ were used to identify their
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59 homologues in all *Thermococcales* species which genomes are sequenced. ADH
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1 sequences were aligned in Seaview with Muscle [30]. Conserved sites were selected
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3 with GBlocks. PhyML v3.696 was employed to construct a maximum likelihood
4
5 unrooted phylogenetic tree. Bootstrapping was performed with 1000 replicates. The
6
7 sequence accession numbers for each individual protein is labeled in the tree. The tree
8
9 was inferred with PhyML. The scale bar represents the average number of
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11 substitutions per site. Numbers at branches represent bootstrap values (1000 replicates,
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13 values > 50% are shown).
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20 *2.3 Sequence alignment*

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22 The target ADH sequences from archaea, bacteria and eukaryotes were retrieved
23
24 from NCBI. The sequences were aligned by DNAMAN software version 6. The
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26 alignment figure was output with a graphic file.
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31 *2.4 Cloning of the gene encoding Tba ADH₅₄₇*

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33 We amplified the TBCH5v1_0547 gene encoding a putative type III alcohol
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35 dehydrogenase using the *T. barophilus* Ch5 genomic DNA as a template and Pfu
36
37 DNA polymerase in the presence of the forward primer and the reverse primer. The
38
39 sequences of these two primers are listed in Table 1. The amplified PCR product and
40
41 the pET-30a(+) vector were digested by *Nde*I and *Xho*I, and the cleaved DNA
42
43 fragments were ligated by T4 DNA ligase to yield the recombinant plasmid
44
45 pET30a(+)-Tba ADH₅₄₇, and transformed into *E. coli* DH5 α cells. After sequence
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47 verification, the recombinant plasmid pET30a(+)-Tba ADH₅₄₇ was transformed into *E.*
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coli BL21 (DE3) cells for expression of the Tba ADH₅₄₇ protein harboring a 6 x
His-tag in its C-terminus.

2.5 Overexpression and purification of Tba ADH₅₄₇

For the Tba ADH₅₄₇ protein expression, the expression strain *E. coli* BL21 (DE3) harboring the pET30a(+)-Tba ADH₅₄₇ plasmid was cultured at 37°C in LB medium containing 10 µg/mL kanamycin until the OD₆₀₀ reached 0.6. Then, the expression of the Tba ADH₅₄₇ gene was induced by addition of isopropyl thiogalactoside (IPTG, 0.1 mM). Expression was performed for 12 hat room temperature.

Cells were harvested by centrifugation (5,000 g) at room temperature. The collected pellet was resuspended in Ni column buffer A containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 500 mM NaCl, 50 mM imidazole and 10% glycerol. Cells were disrupted by ultrasonication at 4°C. Cell debris was removed by centrifugation (16,000 g) at 4°C. The collected supernatant was heated at 70°C for 20 min to inactivate the *E. coli* proteins. After centrifugation at 16,000 g and 4°C, the resulting supernatant was loaded onto a HisTrap FF column (GE Healthcare, Uppsala, Sweden). The Tba ADH₅₄₇ protein was eluted with NCGTM Chromatography System (Bio-Rad, Hercules, CA, USA) by a linear gradient of 50–500 mM imidazole with a Ni column buffer B containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 500 mM NaCl, 500 mM imidazole and 10% glycerol. The collected fractions containing the His-tagged Tba ADH₅₄₇ protein were analyzed by migration on a 10% SDS-PAGE. The purified Tba ADH₅₄₇ protein fractions were combined and dialyzed against a storage buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 50% glycerol and 50 mM NaCl, and stored at -80°C. The Tba ADH₅₄₇ protein concentration was determined by measuring the absorbance at 280 nm. The theoretical molar extinction

1 coefficient of Tba ADH₅₄₇ protein is predicted to be 33,350 M⁻¹ cm⁻¹.
2

3 *2.6 Construction, overexpression, and purification of the Tba ADH₅₄₇ mutants*

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6 Following the manufacturer's instructions, the Tba ADH₅₄₇ mutants were
7
8 constructed by Site-Directed Mutagenesis Kit (Transgene, China) using the wild-type
9
10 Tba ADH₅₄₇ gene as a template. The sequences of the mutagenic primers are listed in
11
12 Table 1. The D195A, H199A, H262A, H266A, and H274A mutant plasmids were
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14 verified by sequencing, and the mutant proteins were overexpressed, purified and
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16 quantified as described for the wild-type protein.
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22 *2.7 Enzymatic assays of Tba ADH₅₄₇*

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25 The standard Tba ADH₅₄₇ enzymatic assay was performed in a total volume of 3
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27 ml containing 100 mM Tris-HCl (pH 8.5), 0.67 mM NAD⁺, 0.1 mM MgCl₂, 0.46 M
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29 ethanol and 500 nM Tba ADH₅₄₇ at 75°C for 30 min and was terminated by placing
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31 samples on ice. Alternatively, the standard reaction for the reductive reaction in a total
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33 volume of 3 ml including 100 mM Tris-HCl (pH 8.5), 0.3 mM NADH, 0.46 M
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35 acetaldehyde, 0.1 mM FeSO₄, and 500 nM Tba ADH₅₄₇ was conducted at 75°C for 30
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37 min and were terminated by placing samples on ice. Tba ADH₅₄₇ activity was
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39 determined spectrophotometrically at 340 nm by measuring the amount of NADH
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41 produced or reduced. The extinction coefficient (ϵ_{340}) was 6,220 M⁻¹ cm⁻¹. All
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43 reactions were performed in triplicate and the average values were reported. One unit
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45 of enzyme was defined as the amount of enzyme producing or reducing 1 μ mol of
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47 NADH per min.
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58 The optimal reaction temperature of the Tba ADH₅₄₇ activity was investigated in
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1 the standard conditions but varying temperature in a 40°C to 100°C range for
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3 oxidation and in a 40°C to 80°C for reduction. The optimal pH of the Tba ADH₅₄₇
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5 activity was determined at 75°C varying the pH of the reactions between 6.5 and 10
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7 for oxidation and between 6.0 and 8.0 for reduction. The varied pHs were adjusted
8
9 with five different buffers (all at 20 mM concentrations): sodium phosphate-NaOH
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11 (pH 6.0, pH 6.5 and pH 7.0), Tris-HCl (pH 7.5, pH 8.0, and pH 8.5 at the desired
12
13 temperature), and Gly-NaOH (pH 9.0, pH 9.5 and pH 10.0). The requirement for a
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15 divalent ion for the activity of Tba ADH₅₄₇ was assayed in presence of 10 mM EDTA
16
17 or 0.1 mM Fe²⁺, Mg²⁺, Mn²⁺, Co²⁺, Cu²⁺, Ca²⁺, Ni²⁺ or Zn²⁺. The substrate specificity
18
19 of the Tba ADH₅₄₇ activity was investigated with ethanol, methanol, isopentanol,
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21 isopropanol, ethylene glycol, 1-butanol, 1-hexanol or glycerol as the substrates for
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23 oxidation, and with acetone, butylaldehyde, caproaldehyde or acetaldehyde as the
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25 substrates for reduction.
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36 *2.8 Kinetic assays of Tba ADH₅₄₇*

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39 The kinetic parameters of Tba ADH₅₄₇ were obtained by performing reactions at
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41 different ethanol concentrations (1 mM, 5 mM, 10 mM, 20 mM, 40 mM, 80 mM, 100
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43 mM and 200 mM) for oxidation and at different acetaldehyde concentrations (1 mM,
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45 2.5 mM, 5 mM, 10 mM, 20 mM, 40 mM, 80 mM, and 100 mM) for reduction. The
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47 corresponding K_m and V_{max} values were calculated using the linear regression and the
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49 values given represented the calculated mean.
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56 *2.9 Circular dichroism (CD) measurements*

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58 The wild-type (WT), D195A, H199A, H262A, H266A and H274A mutant Tba
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1 ADH₅₄₇ proteins were dialyzed into 50 mM PBS (phosphate-buffered saline) pH7.5
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3 for CD analysis. The CD spectra were recorded at 20°C from 200 nm to 250 nm using
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5 a J-810 spectropolarimeter (JASCO, Japan) and a cuvette of path length 0.2 cm. The
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7 spectra were generated with 0.2 mg/ml WT and mutant Tba ADH₅₄₇ proteins at a
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9 scanning rate of 50 nm/min, and triplicate spectrum readings were collected per
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11 sample. The CD spectral data were reported as mean residue ellipticity [θ] by using
12
13 the program KaleidaGraph (Synergy Software).
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20 **3 Results**

21 *3.1 Tba ADH₅₄₇ is a putative type III alcohol dehydrogenase*

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23 Two putative NAD-ADHs of type III (Fe-ADH) are encoded in the genome of *T.*
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25 *barophilus* Ch5: Tba ADH₆₄₁ and Tba ADH₅₄₇. As shown in Fig. 1A, Tba ADH₅₄₇ and
26
27 Tba ADH₆₄₁ are clustered in different branches. Tba ADH₅₄₇ is conserved in all
28
29 *Thermococcales* species (Fig. 1B), including *Thermococcus*, *Pyrococcus* and
30
31 *Palaeococcus* genera. Tba ADH₅₄₇ is the most closely related to ADH from *T.*
32
33 *paralvinellae* (Fig. 1B), and this ADH displays a relative closer relation with the ADH
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35 homologues from *Palaeococcus* than the ADH homologues from *Pyrococcus* in
36
37 *Thermococcales*. Surprisingly, Tba ADH₅₄₇ is not conserved in other Archaea (Fig.
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39 1B). It is only distantly related to bacterial homologues (Fig. 1B). Tba ADH₅₄₇
40
41 displays 26~30% similarity with Tba ADH₆₄₁ and the homologues from
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43 *Thermococcus* strain ES-1, *T. hydrothermalis*, *T. litoralis*, *Thermococcus* strain AN1,
44
45 *Thermococcus kodakarensis* (ADH2), *Pyrococcus furiosus*, *Z. mobilis* and *S.*
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47 *cerevisiae*. In contrast, Tba ADH₅₄₇ exhibits 71~75% similarity with the homologues
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1 from *Thermococcus* strain AN1, *T. thio还原ens* and *T. kodakarensis* (ADH1),
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3 suggesting that Tba ADH₅₄₇ might be more similar in function to *T. zilligii*, *T.*
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6 *thio还原ens* and *T. kodakarensis* (ADH1) ADHs. Thus, the low similarity of two type
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8
9 III ADHs from *T. barophilus* Ch5 suggests that they might have distinct biochemical
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11 characteristics.

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13
14 As shown in Fig. 2, Tba ADH₅₄₇ possesses three conserved motifs: motif I
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16 (GGGS), motif II (DxxxH), and motif III (a conserved motif with three histidine
17
18 residues), which are present in type III ADHs. However, the amino acid variations
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20 and three residues are missing in the highly conserved motif III in Tba ADH₅₄₇ and
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22
23 ADHs from *T. thio还原ens*, *T. kodakarensis* (ADH1) and *Thermococcus* strain AN1.
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26 Furthermore, thirty four residues are strictly conserved in all thirteen proteins (Fig. 2),
27
28 including seven glycine residues, six proline residues, six alanine residues, four
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30 histidine residues, three aspartic acid residues, two lysine residues, two glutamic acid
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32 residues, one threonine, one leucine and one isoleucine. Overall, Tba ADH₅₄₇
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34 possesses the conserved motifs present in type III ADHs, however, displays low
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36 similarity with the homologues exemplified by Tba ADH₆₄₁ ADH.
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45 3.2 Cloning, expression and purification of Tba ADH₅₄₇ protein

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47 In this work, we cloned the Tba ADH₅₄₇ gene encoded by TBCH5v1_0547
48
49 (Genbank: ALM74514) into a pET30a(+) expression vector and overexpressed it in *E.*
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51 *coli* BL21 (DE3) cells. As shown in Fig. 3A, the gene encoding the Tba ADH₅₄₇
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53 protein was successfully induced after addition of IPTG and the expressed protein was
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55 recovered in the supernatant after ultrasonication disruption. After heat treatment at
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1 70°C for 30 min, most of *E. coli* proteins were inactivated and removed after
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3 centrifuge, however, the Tba ADH₅₄₇ protein can withstand this heat treatment. Last,
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5 the recombinant Tba ADH₅₄₇ protein was purified to near homogeneity after
6
7 Ni-column affinity purification (Fig. 3A). The purified 6 x His-tagged protein
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9 displayed an approximate MW of 42 kDa (Fig. 3A), consistent with the deduced
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11 amino acid sequence.
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16 3.3 Effect of temperature on Tba ADH₅₄₇ activity

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18 Since *T. barophilus* Ch5 is a hyperthermophile [23], we expect its proteins or
19
20 enzymes to be highly thermophilic and thermostable. We first investigated the
21
22 thermophilicity of Tba ADH₅₄₇ by performing enzyme activity assays at varied
23
24 temperatures ranging from 40°C to 100°C. As shown in Fig. 3B, Tba ADH₅₄₇ only
25
26 retained about 10% and 30% relative activity at 40°C and 100°C, respectively. Tba
27
28 ADH₅₄₇ displayed maximal activity at 75°C, which makes it a thermophilic enzyme.
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35 Next, we determined whether Tba ADH₅₄₇ has reduction activity. Using
36
37 acetaldehyde as the substrate, we performed the reduction reaction catalyzed by Tba
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39 ADH₅₄₇ in the presence of NADH at various temperatures ranging from 40°C to 80°C
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41 since NADH is unstable at temperatures higher than 80°C. We found that Tba ADH₅₄₇
42
43 has the reduction activity, and also displays maximal activity for acetaldehyde
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45 reduction at 75°C (Fig. 3B). Thus, Tba ADH₅₄₇ has the same optimal reaction
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47 temperature for both ethanol oxidation and acetaldehyde reduction under our standard
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49 reaction conditions.
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57 3.4 Thermostability of Tba ADH₅₄₇

1 To probe the thermostability of Tba ADH₅₄₇, we incubated this ADH at varied
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3 temperatures for different times and then used the heated enzyme to perform the
4
5 ethanol oxidation and acetaldehyde reduction reactions. We selected the heated Tba
6
7 ADH₅₄₇ at 90, 95 and 100°C for 30 min for analyzing its reduction activity. As shown
8
9 in Fig. 4A, the reduction activity of Tba ADH₅₄₇ decreased as the heating temperature
10
11 increased. However, this ADH still retained 20% reduction activity even after this
12
13 ADH was heated at 100°C for 30 min.
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20 On the other hand, we employed the heated Tba ADH₅₄₇ at 100°C at varied times
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22 to analyze its oxidation reaction. Compared with the control reaction without the
23
24 heating treatment, the oxidation activity of Tba ADH₅₄₇ was lowered as the heating
25
26 time extended, (Fig. 4B). However, Tba ADH₅₄₇ still harbored about 20% oxidation
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28 activity after heating at 100°C for 30 min, which is consistent with the observation for
29
30 the reduction activity of the enzyme heated at 100°C for 30 min. Overall, Tba ADH₅₄₇
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32 is a thermophilic and thermostable ADH, suggesting its potential application in ethanol
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34 oxidation and acetaldehyde reduction at high temperature.
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42 *3.5 Effect of pH on Tba ADH₅₄₇ activity*

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44 Next, we investigated the optimal pH for ethanol oxidation and acetaldehyde
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46 reduction of Tba ADH₅₄₇. As shown in Fig. 5A, no Tba ADH₅₄₇ activity was observed
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48 below pH 6.5 and only a residual activity was detected at pH 10. Tba ADH₅₄₇ can
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50 oxidize ethanol in the pH range from 7.0 to 10.0. At pH 8.5, Tba ADH₅₄₇ displayed
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52 maximal activity, suggesting that pH 8.5 is the optimal pH for the reduction reaction
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54 of this ADH.
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1 The effect of pH on reduction reaction of Tba ADH₅₄₇ was also determined using
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3 varied pH buffers ranging from 6.0 to 8.0 since NADH is unstable at above pH 8.0
4
5 (Fig. 5A). Tba ADH₅₄₇ exhibited maximal reduction activity at pH7.0, suggesting that
6
7 its optimal reaction pH is 7.0, which is lower than that of its oxidation reaction. Thus,
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9 Tba ADH₅₄₇ displays distinct optimal pH adaptation for oxidation and reduction
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11 reactions.
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16 *3.6 Effect of divalent ions on Tba ADH₅₄₇ activity*

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18 The metal requirements of archaeal NAD-dependent ADHs are very variable,
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20 thus, we investigated the metal dependency of Tba ADH₅₄₇ activity for ethanol
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22 oxidation. In the absence of the addition of a divalent ion or in the presence of 10 mM
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24 EDTA (the latter should chelate the divalent ions eventually co-purified with the Tba
25
26 ADH₅₄₇ enzyme), Tba ADH₅₄₇ yielded no measurable activity (Fig. 5B). The activity
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28 in Tba ADH₅₄₇ is restored to some extent upon addition of all the divalent ions tested.
29
30 Maximum Tba ADH₅₄₇ activity was observed in the presence of 0.1 mM Mn²⁺. By
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32 contrast, the lowest activity of Tba ADH₅₄₇ was found in the presence of 0.1 mM Zn²⁺,
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34 Cu²⁺, Ca²⁺, Ni²⁺, Co²⁺, Mg²⁺ or Fe²⁺, which stimulated Tba ADH₅₄₇ activity with less
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36 than 50% of that observed with Mn²⁺. Thus, Tba ADH₅₄₇ is dependent on a divalent
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38 ion for ethanol oxidation, which is sharply contrasted to the currently reported ADHs.
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50 Compared with the oxidation reaction, Tba ADH₅₄₇ displays about 30%
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52 reduction activity in the absence of divalent ion or in the presence of EDTA (Fig. 5B).
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54 Surprisingly, this ADH exhibits maximal reduction activity in the presence of Fe²⁺,
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56 suggesting that Fe²⁺ is the optimal divalent ion for its reduction activity, which is
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1 sharply contrasted with the observation that Mn^{2+} is the optimal ion for the oxidation
2 activity of this ADH. Compared with the control reactions without divalent ion or
3 without EDTA, Ca^{2+} slightly inhibited the reduction activity whereas Cu^{2+} , Mg^{2+} , or
4 Ni^{2+} stimulated the reduction activity of this ADH with varied degrees. On the other
5 hand, Zn^{2+} or Mn^{2+} enables this ADH to retain similar reduction activity to the control
6 reactions. Thus, Tba ADH₅₄₇ displays distinct divalent ion adaptation for acetaldehyde
7 reduction.

8 *3.7 Substrate specificity of Tba ADH₅₄₇*

9 We determined the substrate specificity of Tba ADH₅₄₇ under the optimal
10 conditions (75°C, pH 8.5 and 0.1 mM Mn^{2+}) for oxidation. Quite strikingly, we found
11 that the recombinant Tba ADH₅₄₇ exhibited the highest activity in the presence of
12 1-butanol or 1-hexanol and not in the presence of ethanol, suggesting that 1-butanol
13 and 1-hexanol are more suitable substrates for this enzyme than is ethanol (Fig. 6A).
14 Only a few other primary alcohols were possible substrates for Tba ADH₅₄₇, including
15 ethylene glycol (40% activity), isopentanol (25% activity), isopropanol (10% activity)
16 and glycerol (5% activity). In contrast, we observed that Tba ADH₅₄₇ is fully inactive
17 on methanol. Thus, Tba ADH₅₄₇ displays a preference for substrate in the order from
18 high to low: 1-butanol > 1-hexanol > ethanol > ethylene glycol > isopentanol >
19 isopropanol > glycerol. Combining all these observations, we can conclude that the
20 optimal reaction conditions for Tba ADH₅₄₇ activity with 1-butanol as a substrate are:
21 75°C, pH 8.5 and 0.1 mM Mn^{2+} . Under the optimal reaction conditions, we measured
22 the specific activity of Tba ADH₅₄₇ to be 931 U/mg for 1-butanol oxidation.

1 Next, we determined the substrate specificity of reduction reaction of Tba
2
3 ADH₅₄₇ using acetone, acetaldehyde, caproaldehyde and butylaldehyde as the
4
5 substrates. Similar to the oxidation reaction with 1-butanol as a preference substrate,
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7 Tba ADH₅₄₇ displayed higher reduction activity in the presence of butylaldehyde than
8
9 in the presence of acetaldehyde (Fig. 6B), suggesting that butylaldehyde is more
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11 suitable substrate than acetaldehyde for reduction reaction of this ADH. Compared
12
13 with the acetaldehyde reduction reaction, Tba ADH₅₄₇ retained significantly lowered
14
15 reduction activity for acetone and caproaldehyde reduction, suggesting that acetone
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17 and caproaldehyde are also possible substrates for reduction activity of this ADH.
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19 Thus, the optimal reaction conditions for Tba ADH₅₄₇ reduction activity with
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21 butylaldehyde as a substrate are: 75°C, pH 7.0 and 0.1 mM Fe²⁺. The specific activity
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23 of Tba ADH₅₄₇ was measured to be 3924 U/mg for butylaldehyde reduction,
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25 suggesting that this ADH has a higher activity for aldehyde reduction than alcohol
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27 oxidation.
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39 *3.8 K_m and V_{max} for Tba ADH₅₄₇*

41 We determined the kinetic parameters of Tba ADH₅₄₇ using the ethanol at varied
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43 concentrations ranging from 1 mM to 200 mM as the substrates under the optimal
44
45 oxidation conditions as described above. The K_m and V_{max} values were calculated to
46
47 be 92 mM and 8×10^{-3} mM/min from the fitted Lineweaver-Burk plot (Fig. 7A),
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49 respectively. Considering that 500 nM Tba ADH₅₄₇ was employed in oxidation
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51 reaction, the K_{cat} was further measured to be 0.27 s⁻¹.
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58 Furthermore, we examined the kinetic parameters of Tba ADH₅₄₇ using the
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1 acetaldehyde at varied concentrations ranging from 1 mM to 80 mM as the substrates
2
3 under the optimal reaction condition (75°C, pH 7.0 and 0.1 mM Fe²⁺). As shown in
4
5 Fig. 7B, the K_m and V_{max} values of Tba ADH₅₄₇ for acetaldehyde reduction were
6
7 estimated to be 34.5 mM and 0.266 mM/min from the fitted Lineweaver-Burk plot,
8
9 respectively. The corresponding K_{cat} for acetaldehyde reduction was further calculated
10
11 to be 69 s⁻¹, which is about 256-fold higher than that for ethanol oxidation. By
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13 contrast, the K_m value for acetaldehyde reduction of Tba ADH₅₄₇ is about 2.7-fold
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15 lower than that for ethanol oxidation. Thus, Tba ADH₅₄₇ displays higher catalytic
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17 efficiency for acetaldehyde reduction than for ethanol oxidation.
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25 *3.9 Mutational studies of Tba ADH₅₄₇*

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27 As shown in Fig. 2A, Tba ADH₅₄₇ harbors three conserved motifs with several
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29 conserved amino acid residues present other ADHs. To reveal key amino acid residues
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31 for catalysis of Tba ADH₅₄₇, we constructed the D195A, H199A, H262A, H266A, and
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33 H274A mutants. Following the wild-type protein expression and purification, we
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35 purified five Tba ADH₅₄₇ mutant proteins (Fig. 8A).
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42 To investigate whether the D195A, H199A, H262A, H266A, and H274A mutants
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44 cause the overall structure change of Tba ADH₅₄₇, we performed the CD analysis of
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46 the WT and mutant enzymes. The D195A, H199A, H262A, H266A, and H274A
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48 mutants displayed different conformational change from the wild-type Tba ADH₅₄₇
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50 with varied degrees (Fig. 8B). Specifically, the D195A, H266A and H274A mutants
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52 exhibited similar overall structure (Fig. 8B), which are distinct from the WT enzyme.
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58 Furthermore, the H199A substitution caused maximal change of overall structure of
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1 the WT enzyme, followed by three substitutions (D195A, H266A and H274A) and the
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3 H262A substitution (Fig. 8B). Thus, our observations demonstrate that the D195A,
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5 H199A, H262A, H266A, and H274A substitutions disrupt the overall structure of the
6
7 WT enzyme.
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11 Next, we investigated the ethanol oxidation and acetaldehyde reduction activities
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13 of these five mutants. Compared with the wild-type protein, the D195A mutant
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15 displayed no reduction activity and the slight weak oxidation activity (Fig. 8C),
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17 suggesting that D195 is a key residue for catalysis of this ADH. Similarly, the H262A
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19 mutant harbored the weak oxidation activity (2.9%) and reduction activity (6.4%),
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21 thereby indicating that H262 is also a key residue for catalysis. However, the H266A
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23 mutant had the higher reduction activity (134%) but the lower oxidation activity (36%)
24
25 than the wild-type protein, suggesting that the mutation of H266 to Ala stimulates its
26
27 reduction activity but suppresses its oxidation activity. Furthermore, the H199A and
28
29 H274A mutants retained 10%~20% oxidation and reduction activity, demonstrating
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31 that residues H199 and H274 are involved partially in catalysis. Overall, residues
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33 D195 and H262 in Tba ADH₅₄₇ are essential for catalysis, and residues H199 and
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35 H274 are also involved partially in catalysis.
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47 **4. Discussion**

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49 In this work, we present the biochemical characteristics and mutational studies of
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51 Tba ADH₅₄₇ which is one of the ADHs of the hyperthermophilic and piezophilic
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53 euryarchaeon *T. barophilus* Ch5. Tba ADH₅₄₇ belongs to a cluster of type III archaeal
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55 ADHs (Table 2) since this enzyme harbors three conserved motifs present in
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1 iron-containing ADHs. However, Tba ADH₅₄₇ displays low amino acid similarity
2
3 with the reported iron-containing ADHs from hyperthermophiles, including *T.*
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6 *litoralis* [17], *T. hydrothermalis* [21], *Thermococcus* strain ES-1 [18], *P. furiosus* [22]
7
8 and *Thermotoga hypogea* [31], suggesting this ADH might be a novel type III ADH.
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10 Comparison of biochemical characteristics of iron-containing ADHs from
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12 hyperthermophiles is summarized in Table 2, displaying distinct properties for
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14 oxidation or reduction.
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20 As expected, Tba ADH₅₄₇ displays maximal enzyme activity at high
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22 temperatures since it originates from the hyperthermophilic euryarchaeon *T.*
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24 *barophilus* Ch5. In comparison to other thermostable type III ADHs, Tba ADH₅₄₇
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26 displays thermophilicity 5~10°C degrees lower than that reported for ADHs from *T.*
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28 *hydrothermalis* (80°C) [21], *T. litoralis* (85°C) [17] and *Thermococcus* strain AN1
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30 (85°C) [20]. Interestingly, the optimal temperatures of ADHs from *Thermococcus*
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32 strain ES-1, *P. furiosus* and *T. hypogea* exceeded 100°C and could not be clearly
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34 determined [18, 22, 31]. To our knowledge, we first measured the optimal temperature
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36 of acetaldehyde reduction, demonstrating the same optimal temperature for ethanol
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38 oxidation under our assay conditions. Overall, Tba ADH₅₄₇ harbors a relatively low
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40 optimal temperature for its activity, compared with the reported type III ADHs from
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42 hyperthermophiles.
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53 In this work, we revealed that Tba ADH₅₄₇ retains about 40% and 20% activity
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55 after being heated at 95°C and 100°C for 30 min, respectively. The half-life of *P.*
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57 *furiosus* ADH was reported to be 7 h at 95°C [22] while the half-life of *T. litoralis*
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1 ADH was about 0.3 h at 95°C [17]. *Thermococcus* strain ES-1 and *T. hypogea* ADHs
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3 retain 50% activity after being heated at 95°C for 4 h and at 90°C for 2 h, respectively
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6 [32, 34]. A half-life of *Thermococcus* strain AN1 ADH was measured to be only 16
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9 min at 80°C [20]. Furthermore, *T. hydrothermalis* ADH abolishes 50% activity at
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12 80°C for 0.25 h [21]. Thus, the type III ADHs from hyperthermophiles exhibits strong
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14 thermostability, but their degree of thermostability varies with different their host
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17 sources.

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20 Maximal activity of Tba ADH₅₄₇ for ethanol oxidation was observed at pH 8.5,
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22 which is similar to other type III ADHs from *T. litoralis* and *Thermococcus* strain
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24 ES-1 [17-18]. Tba ADH₅₄₇ differs significantly from other extremophilic type III
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26 ADHs displaying a lower pH optimum than the type III-ADHs from *T. hydrothermalis*,
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28 *P. furiosus* and *T. hypogea* [21-22, 31]. However, maximal activity of Tba ADH₅₄₇ for
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30 acetaldehyde reduction was measured at pH 7.0, suggesting that this ADH displays
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32 various optimal pH for oxidation and reduction reactions. Similar observations are
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34 found in the reported ADHs from *Thermococcus* strain ES-1 [18], *T. hydrothermalis*
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36 [21], *P. furiosus* [22], and *T. hypogea* [31].

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39 Although the members of the Fe-ADH family are described as iron-activated
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41 alcohol dehydrogenases, these enzymes can be activated by a range of divalent
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43 cations, including zinc, nickel, magnesium, copper, cobalt, or manganese (e.g.,
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45 [32-35], besides iron. To date, divalent cations are needed for a type III ADH activity,
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47 among which Fe is essentially ADHs from *Thermococcales*. However, we found that
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50 Tba ADH₅₄₇ is the only known example of a type III archaeal ADH that is dependent
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1 on a divalent ion for its oxidation activity. Interestingly, this ADH displays maximal
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3 oxidation activity in the presence of added Mn^{2+} . However, Tba ADH₅₄₇ can catalyze
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5 acetaldehyde reduction reaction in the absence of a divalent ion and displays maximal
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7 activity in the presence of Fe^{2+} for acetaldehyde reduction.
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11 Among the characterized archaeal ADHs capable of oxidizing primary alcohols,
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13 all others isolated from *Thermococcales* species exhibit a greater preference for
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15 1-butanol over ethanol as a substrate as observed for Tba ADH₅₄₇. Similar to the
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17 ADHs from *Thermococcus* strain AN1 [20], *T. litoralis* [17] and *Thermococcus* strain
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19 ES-1 [18], Tba ADH₅₄₇ shows a higher preference for primary alcohols of longer
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21 chain such as 1-hexyl alcohols. However, AdhD from *P. furiosus* essentially cannot
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23 process ethanol and has a strong preference for 1-butanol [36], and the ADH from *T.*
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25 *litoralis* has a marked preference for aliphatic primary alcohols [17]. In contrast to
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27 Tba ADH₅₄₇, *Thermococcus sibiricus* ADH, which belongs to the short-chain ADH
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29 family, demonstrates a preference for ethanol over 1-butanol, but is most probably a
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31 secondary alcohol dehydrogenase as shown by its strong preference for 2-butanol
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33 over 1-butanol [37]. In addition, similar to the ADHs from all the *Thermococcales*
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35 species listed above, Tba ADH₅₄₇ cannot utilize methanol as a substrate. In this
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37 respect, Tba ADH₅₄₇ and ADHs from the other *Thermococcales* differ from the ADHs
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39 found outside the *Thermococcales* order, such as *Picrophilus torridus* ADH [38] or
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41 *Thermoplasma acidophilum* ADH [39], which retain some level of activity with
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43 methanol.
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58 As shown in Table 2, the K_m values of Tba ADH₅₄₇ for ethanol oxidation and
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1 acetaldehyde reduction are higher than those of the reported ADHs from the other
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3 *Thermococcales* order. Furthermore, the K_{cat} of Tba ADH₅₄₇ for ethanol oxidation is
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5 lower than that of the reported ADHs from the other *Thermococcales* order. However,
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7 the K_{cat} of Tba ADH₅₄₇ for acetaldehyde reduction is maximum among the all the
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9 reported ADHs listed above. Importantly, the K_{cat}/K_m of Tba ADH₅₄₇ for acetaldehyde
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11 reduction is 670-fold higher than that for ethanol oxidation, suggesting that this ADH
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13 displays higher catalytic efficiencies for acetaldehyde reduction than ethanol
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15 oxidation as suggested by Fernandez et al. who proposed that type III ADHs from
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17 microorganisms contributed to aldehyde detoxication rather than alcohol oxidation
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19 [40]. Similarly, 13-fold, 49-fold and 3.3-fold higher K_{cat}/K_m values for acetaldehyde
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21 reduction than ethanol oxidation are observed for the ADHs from *Thermococcus*
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23 strain ES-1, *P. furiosus* and *T. hypogea* [18, 22, 31], respectively. Thus, Tba ADH₅₄₇
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25 displays higher K_{cat}/K_m values for acetaldehyde reduction over ethanol oxidation than
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27 other ADHs from hyperthermophiles listed above. Since *T. barophilus* Ch5 is an
28
29 anaerobic euryarchaeon [23], the catalytic activity of Tba ADH₅₄₇ for ethanol synthesis
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31 is far greater than that for ethanol oxidation, which might recover the NAD⁺ required
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33 for another Entner–Doudoroff glycolytic (ED) pathway under anaerobic conditions.
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36 Alignment of amino acid sequences from type III ADH demonstrates that they
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38 possess a conserved motif III with the three highly conserved histidine residues.
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40 Mutational studies show that the H263R, H267A and H277A mutations resulted in the
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42 *E. coli* propanediol oxidoreductase without catalytic activity [41]. Crystal structure of
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44 this enzyme shows that His26 and His277 are strictly essential metal-ion ligands, and
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1 His267 could interact with the substrate, and therefore should be involved in catalysis,
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3 but not in metal-binding [13]. Similar to *E. coli* propanediol oxidoreductase, Tba
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5 ADH₅₄₇ harbors the corresponding residues H262, H266 and H274 in the conserved
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7 motif III. Our mutational data show that the H262A, H266A and H274A Tba ADH₅₄₇
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9 mutants retain 5%, 40%, and 25% oxidation activity, respectively. Thus, these
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11 observations suggest that a combination of residues H262, H266 and H274 is essential
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13 for catalysis. Surprisingly, the mutation of H266 to Ala results in 1.4-fold increase in
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15 reduction activity while the mutations of H262 and H274 to Ala lead to 75% and 90%
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17 reduction activity loss, respectively, which might be caused by overall conformational
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19 disruption due to the H262A, H266A and H274A substitutions. To our knowledge, we
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21 first revealed the function the three highly conserved histidine residues in the motif III
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23 of type III ADH in acetaldehyde reduction using Tba ADH₅₄₇ as a target,
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25 demonstrating that residue H262 and H274 are involved in catalyzing acetaldehyde
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27 reduction reaction.
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39 In addition to the motif II, motif II (DxxxH) with a highly conserved His residue
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41 and an Asp residue is widely present in type III ADHs. The crystal structure of *T.*
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43 *thioreducens* ADH shows that the conserved residues Asp193 and His197 interact
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45 with Fe atom plus residues His260 and His272 located in the conserved motif III [15].
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48 The conserved His residue and an Asp residue in motif II are observed in the crystal
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50 structures of other type III ADHs. However, the biochemical function of the
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52 conserved His residue and an Asp residue in motif II has not been clarified. In this
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54 work, we constructed the D195A and H199A Tba ADH₅₄₇ mutants and investigated
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1 their activity for ethanol oxidation and acetaldehyde reduction. Note that residues
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3 D195 and H199 are analogous to residues D193 and H197 in *T. thioreducens* ADH.
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6 Our observations demonstrate that the D195A mutant retains slight weak activity for
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8 ethanol oxidation, and lacks acetaldehyde reduction activity. Furthermore, the H199A
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10 mutant harbors the compromised activity. Similar to the H262A, H266A and H274A
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12 substitutions, the D195A and H199A substitutions disrupt the overall structural
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14 change, which might cause the activity loss of the wild-type Tba ADH₅₄₇. Thus,
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16 residues D195 and H199 in Tba ADH₅₄₇ are responsible for catalysis and maintaining
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18 their structural roles.
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24 **5. Conclusion**

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26 Herein, we present biochemical characterization of Tba ADH₅₄₇, displaying same
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28 optimal temperature (75°C) for oxidation and reduction activities. However, this ADH
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30 has distinct optimal pH for ethanol oxidation and acetaldehyde reduction.
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32 Interestingly, Tba ADH₅₄₇ can catalyze ethanol oxidation with a divalent ion, which is
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34 sharply contrasted to the reported ADHs. Furthermore, Tba ADH₅₄₇ exhibits distinct
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36 divalent ion preference: Mn²⁺ for ethanol oxidation and Fe²⁺ for acetaldehyde
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38 reduction. Primary alcohols are the favored substrates for oxidation of Tba ADH₅₄₇,
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40 with 1-butanol yielding the highest efficiency. Furthermore, butyaldehyde is a more
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42 suitable substrate for reduction of Tba ADH₅₄₇ than acetaldehyde. We revealed that
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44 residue D195, H199, H262 and H234 in Tba ADH₅₄₇ are involved in catalysis by
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46 mutational studies. Overall, our findings suggest that Tba ADH₅₄₇ might have a
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48 biotechnological potential for the biotransformation reaction due to its unique
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50 biochemical characteristics and high activity at high temperature.
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Author Contributions

LZ and PO designed experiments; DJ, YL and LW performed experiments; LZ, KD, QL and PO analyzed data; LZ, KD, QL and PO wrote and revised the paper.

Declaration of Competing Interest

All authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure legends

Fig. 1. Phylogenetic analysis of Tba ADH₆₄₁ and Tba ADH₅₄₇. A. Phylogenetic tree of Tba ADH₆₄₁ and Tba ADH₅₄₇ among bacteria and archaea. B. Phylogenetic tree of Tba ADH₅₄₇ among bacteria and archaea.

Fig. 2. Sequence alignment of type III ADHs from bacteria, archaea and eukaryotes. Three conserved motifs (motif I: GGGS; motif II: DxxxH; motif III: the three highly conserved histidine residues) are boxed. The consensus residues are colored. The residues for engineering Tba ADH₅₄₇ mutants are marked by an asterisk. Tba: *Thermococcus barophilus* (GenBank: ALM74514.1 for ADH₅₄₇ and ALM74601 for ADH₆₄₁); Tan: *Thermococcus* strain AN1, (GenBank: WP_010477684); Tth: *Thermococcus thioreducens* (GenBank: ASJ12775); Tko: *Thermococcus kodakarensis*

1 (GenBank: WP_011249959 for ADH1 and WP_083755808 for ADH2); Tpa:
2
3 *Thermococcus paralvinellae* (GenBank: AHF80371); Thy: *Thermococcus*
4
5 *hydrothermalis* (GenBank: CAA74334); Tsp: *Thermococcus* sp. (GenBank:
6
7 AAB63011); Tli: *Thermococcus litoralis* (GenBank: AAB63011); Pfu: *Pyrococcus*
8
9 *furiosus* (GenBank: WP_011011187); Zmo: *Zymomonas mobilis* (GenBank:
10
11 TWE26216); Sce: *Saccharomyces cerevisiae* (GenBank: GAX68104).
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17 **Fig. 3.** Tba ADH₅₄₇ can catalyze ethanol oxidation and acetaldehyde reduction
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19 reaction at high temperature. A. Overexpression and purification of Tba ADH₅₄₇
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21 protein. M: protein marker. B. The optimal reaction temperature of Tba ADH₅₄₇
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23 activity. The ethanol oxidation reactions of Tba ADH₅₄₇ were performed in the
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25 presence of Mn²⁺ at different temperatures at pH 8.5. The acetaldehyde reduction
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27 reactions of Tba ADH₅₄₇ were performed in the presence of Fe²⁺ at different
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29 temperatures at pH 7.0.
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36 **Fig. 4.** Thermostability of Tba ADH₅₄₇ activity. A. The thermostability for reduction
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38 reaction catalyzed by Tba ADH₅₄₇. The heated Tba ADH₅₄₇ at varied temperatures for
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40 30 min was employed to perform the acetaldehyde reduction reactions in the presence
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42 of Fe²⁺ at 75°C at pH 7.0. CK: the reaction with the non-heated enzyme. B. The
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44 thermostability for oxidation reaction catalyzed by Tba ADH₅₄₇. The heated Tba
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46 ADH₅₄₇ at 100°C for varied times was employed to perform the ethanol oxidation
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48 reactions in the presence of Mn²⁺ at 75°C at pH 8.5. CK: the reaction with the
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50 non-heated enzyme.
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58 **Fig. 5.** Effects pH and divalent metal ions on Tba ADH₅₄₇ activity. A. Effect of pH on
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1 Tba ADH₅₄₇ activity. The ethanol oxidation reactions of Tba ADH₅₄₇ were performed
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3 in the presence of Mn²⁺ at 75°C at varied pHs. The acetaldehyde reduction reactions
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5 of Tba ADH₅₄₇ were performed in the presence of Fe²⁺ at 75°C at varied pHs. CK: the
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7 reaction without enzyme. B. Effects divalent metal ions on Tba ADH₅₄₇ activity. The
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9 ethanol oxidation reactions of Tba ADH₅₄₇ were performed in the presence of varied
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11 divalent metal ions at 75°C at pH 8.5. The acetaldehyde reduction reactions of Tba
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13 ADH₅₄₇ were performed in the presence of varied divalent metal ions at 75°C at pH
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15 7.0. CK: the reaction without enzyme. EDTA: the reaction with 10 mM EDTA.
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23 **Fig. 6.** Substrate specificity of the oxidation activity and the reduction activity of Tba
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25 ADH₅₄₇. A. Substrate specificity of the oxidation activity of Tba ADH₅₄₇. The
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27 oxidation reactions of Tba ADH₅₄₇ were performed in the presence of Mn²⁺ at 75°C at
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29 pH 8.5 using varied alcohols as the substrates. B. Substrate specificity of the reduction
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31 activity of Tba ADH₅₄₇. The reduction reactions of Tba ADH₅₄₇ were performed in the
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33 presence of Fe²⁺ at 75°C at pH 7.0 using varied aldehydes as the substrates.
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39 **Fig. 7.** The Lineweaver-Burk plots for ethanol oxidation and acetaldehyde reduction
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41 of Tba ADH₅₄₇. (A) The Lineweaver-Burk plot for ethanol oxidation of Tba ADH₅₄₇.
42
43 The oxidation reactions of Tba ADH₅₄₇ were performed in the presence of Mn²⁺ at
44
45 75°C at pH 8.5 using ethanol with varied concentrations as the substrates. The
46
47 Lineweaver-Burk curve was generated by plotting the reciprocal of each ethanol
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49 concentration as a horizontal ordinate and the reciprocal of each reaction rate as a
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51 vertical coordinate with a linear regression. (B) The Lineweaver-Burk plot for
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53 acetaldehyde reduction of Tba ADH₅₄₇. The reduction reactions of Tba ADH₅₄₇ were
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1 performed in the presence of Fe^{2+} at 75°C at pH 7.0 using acetaldehyde with varied
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3 concentrations as the substrates. The Lineweaver-Burk curve was generated by
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5 plotting the reciprocal of each acetaldehyde concentration as a horizontal ordinate and
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7 the reciprocal of each reaction rate as a vertical coordinate with a linear regression.
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10 **Fig. 8.** Purification and the activity assays of the Tba ADH₅₄₇ mutants. A. Purification
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12 of the WT, D195A, H199A, H262A, H266A and H274A mutant Tba ADH₅₄₇ proteins.
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14 WT: wild-type; M: protein marker. B. CD analysis of the WT and mutant Tba ADH₅₄₇
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16 proteins. The mean residue ellipticity was recorded with different color line for the
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18 WT and mutant Tba ADH₅₄₇ proteins as indicated by monitoring changes in
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20 secondary structure with scanning from 200nm to 250 nm. The CD spectra of the WT,
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22 D195A, H199A, H262A, H266A and H274A mutant Tba ADH₅₄₇ proteins are colored
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24 with black, red, yellow, green, cyan and blue, respectively. C. The activity assays of
25
26 the Tba ADH₅₄₇ D195A, H199A, H262A, H266A and H274A mutants. The oxidation
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28 reactions of the Tba ADH₅₄₇ mutants were performed in the presence of Mn^{2+} at 75°C
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30 at pH 8.5 using ethanol as the substrate. The reduction reactions of the Tba ADH₅₄₇
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32 mutants were performed in the presence of Fe^{2+} at 75°C at pH 7.0 using acetaldehyde
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34 as the substrate.
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Table 1 Sequences of the oligonucleotides used to clone the Tba ADH₅₄₇ gene and construct its mutants

Name	Sequence (5'-3')
Tba ADH ₅₄₇ F	GGGAATTC <i>CATATG</i> CAGTTCTTCAGCTTAAAGAC
Tba ADH ₅₄₇ R	CCGCTC <i>GAGATCATAGA</i> AGGCTCTCTTATAC
D195A F	AAGGAACAGCGGACTGGCTGTTCTGGTGCAC
D195A R	<u>G</u> CCAGTCCGCTGTTCCCTTGCAACTTCTTTTG
H199A F	GACTGGATGTTCTGGTGGCCGGAATTGAAGCT
H199A R	<u>G</u> CCACCAGAACATCCAGTCCGCTGTTCCCTTGC
H262A F	CCCGTTTAGGCTTGTGCGCCAGCTTAAGCCAT
H262A R	<u>G</u> CGCACAAGCCTAAACGGGCATTAAGAAAAGC
H266A F	TGTGCCACAGCTTAAGCGCTAAAGCGGCTTGG
H266A R	<u>G</u> CGCTTAAGCTGTGGCACAAGCCTAAACGGGC
H274A F	CGGCTTGGATTGCCCTGCGGCTTGTAAAT
H274A R	<u>G</u> CAGGGGCAATCCAAGCCGCTTTATGGCTTAA

The italic bases represent restriction sites.

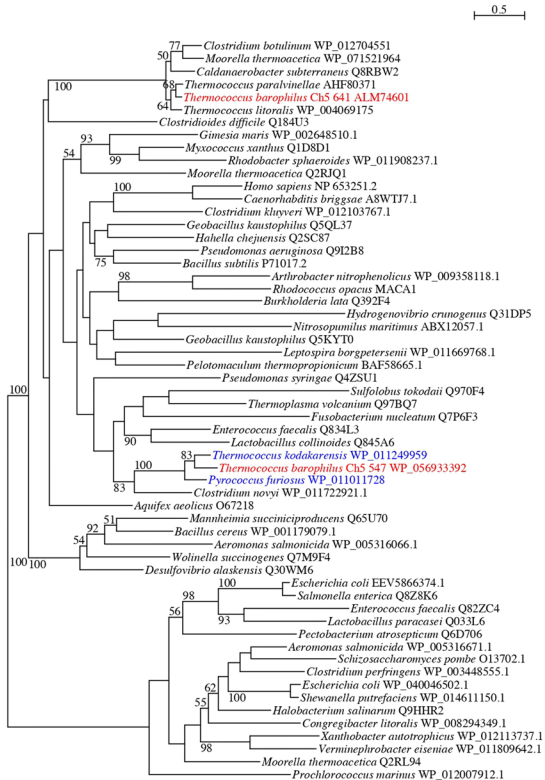
The substitution bases are underlined.

Table 2 Comparisons of biochemical characteristics of iron-containing ADH members from hydrothermophiles.

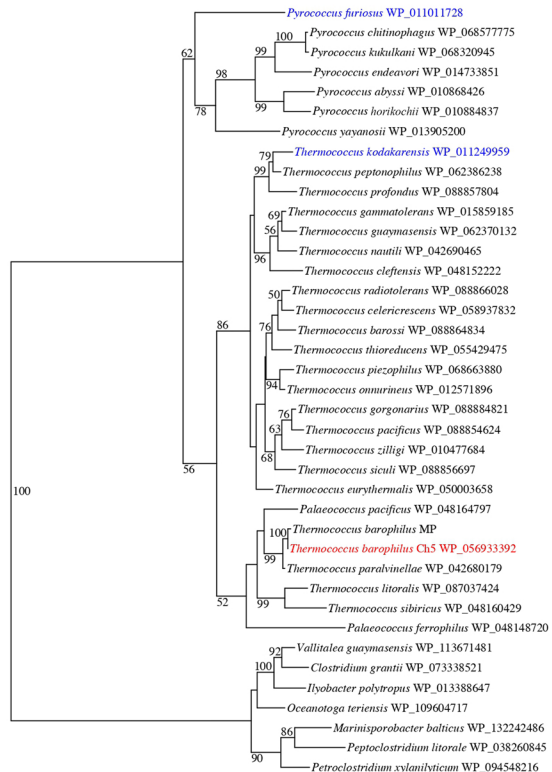
Organism		T/°C optimum	pH optimum	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ M ⁻¹)	Reference
<i>T. barophilus</i>	Oxidation ^a	75	8.5	92	0.27	2.9	This work
	Reduction ^b	75	7.0	34.5	69	2.0 x 10 ³	
<i>Thermococcus</i> strain AN1	Oxidation	85	6.8-7.0	ND	ND	ND	[20]
	Reduction	ND	7.0	0.12	ND	ND	
<i>T. litoralis</i>	Oxidation	85	8.8	11	26	2.3 x 10 ³	[17]
	Reduction	ND	ND	0.4	ND	ND	
<i>T. hydrothermalis</i>	Oxidation	80	10.5	ND	ND	ND	[21]
	Reduction	ND	7.5	ND	ND	ND	
<i>Thermococcus</i> strain ES-1	Oxidation	>95	8.8-10.4	8.0	48	6.0 x 10 ³	[18]
	Reduction	ND	ND	0.25	19	7.6 x 10 ⁴	
<i>P. furiosus</i>	Oxidation	>95	9.4-10.2	29.4	19	6.5 x 10 ²	[22]
	Reduction	ND	ND	0.17	5.5	3.2 x 10 ⁴	
<i>T. hypogea</i>	Oxidation	>95	11.0	9.7	14	1.5 x 10 ³	[31]
	Reduction	ND	8.0	3.1	15	5.0 x 10 ³	

a: Ethanol oxidation reaction; b: Acetaldehyde reduction; ND: Not determined

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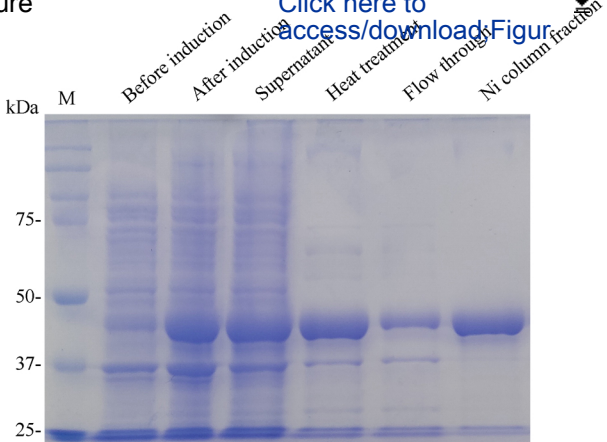
Click here to access/download;Figure;Fig-1.pdf



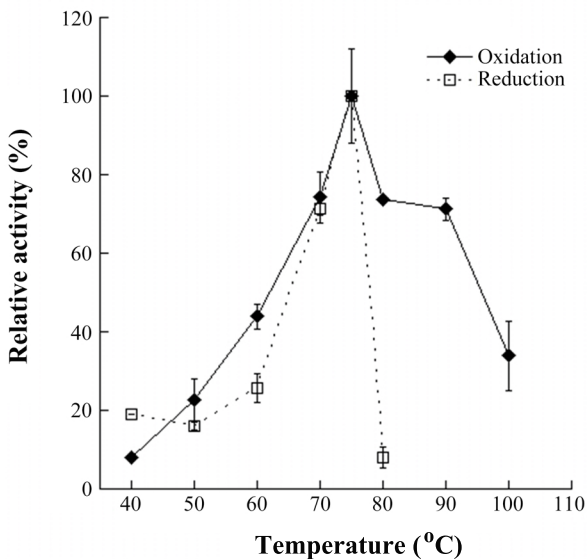
Tba_ADH547MFFSLKTRIVL..GEGSLKYSVAKKHSR.....VLIFSSSKSMRVHGFNLNSTMNYVEDANAEEVETLGGVPAE...FVEEYVF	74
Tan_ADHMFQLKTRIII..GEGSLKALSREVRGYER.....VLILSSSSMKRHGFLGEVEDCVREAGAEEVFSVTNLPAEI...GVTVE	72
Tth_ADHMFWLKTRIII..GEGSLRSLREVKGHER.....VLILASGSMKRHGFLSEAEDYVKEAGAEEVFSIAGLPAEI...SVEVIE	72
Tko_ADH1MFWLKTRISE..GEDSLESLEKDEAKDHER.....VLILASGSMKRHGFLSEAEDYVREAGAEEVMSISGLPAEI...SVEVIE	72
Tba_ADH641MLWESQIPINQIFEMRCKTTNYFVGVAINKFYDIAKDLKE..RGITKVILVTGRSSYKKGCAWDVVKPAALENGIEYVHYDKIGPN...TVDMDV	91
Tpa_ADHMLWESQIPINQIFELRCRTIDYFVGVAINKFYDIAKDLKENRGISRVILVTGKSSYKKGCAWDVVKPAALENGIEYVHYDKVGN...TVDMDV	92
Thy_ADHMVWESHVSINQVFEMRCKTTNYFGLCAIHKFNDIVRELK..KGVDRVILVTGXSSYKKGCAWDVVRPALENGVEYVHYDKVGAN...TVDMDI	91
Tsp_ADHMVWESHVPINQVFELRCRTTIDYFGLCAIHKFNDFVRELK..KGVDRVILVTGSSSYKKGCAWDVVRPALENGVEYVHYDKVGAN...TVDMDI	91
Tli_ADHMLWESRLPINQVFELRCRTIDYFVGVAINKFYDIAKDLKENRDIDKVLVLTGKSSYKKGCAWDVVKPALEKYGIEYVHYDKVGN...TVDMDI	92
Tko_ADH2	MAGQSFDLNFHWPSFFLPLQLNCDSSNKNFGRKRLPELLER..HGLSG.....KALMVTGRSSTKKTGLLYRVQELLKAGVESIVFDRVIPN...ISTHVD	94
Pfu_ADHMFEISIYLP..TEIVFGPGK...LEMLPKLVKK..HGLSG.....KALIVTGRSTKETGVLYRVQELLKQAGVESIVFDKIIPN...ISTHVD	80
Zmo_ADHMASST..FYIPFVNEMGEGSLEKAIKDLNGSGF.....KNALIVSDAFMNKSGVVKQVADLLKQAQINSAVYDGVMPN...TVTAVL	78
Sce_ADHMSSVTGFYIPPIISFFGEGALEETADYIKNKDY.....KALIVTDPGIAAIGLSGRVQKMLEERGLNVAIYDKTQPN...NIANVT	79
Consensus	an	
Tba_ADH547	SIMPKVR.EFQPDLIVALGGGSGVIIDVAKAVKVFYDAPELKFEVAFISRFEKPKFIPKLTPLIAIPSTISGAGSEVSAASVLKKGDIKYN..LVSFEIAF	171
Tan_ADH	ELLPKLREEFQPDLVALGGGSGVIIDAKALKVFYDAPEVKFEEIAFIDRFKSKPKFVPKLKTPLIAIPSTISGAGSEVSASSVLKKGVEVKYN..IVSLEIAF	170
Tth_ADH	EFLPKVR.EFGPDLVAMGGGSGVIIDTKALKVFYDAPELNFGIEIAFIDRFKSKPKFVPRKLTLLIAIPSTISGAGSEVSGASVLKKGVEVKYN..IVTPEIAF	169
Tko_ADH1	EFLPKVR.EFGPDLIAMGGGSGVIIDTKALKVFYDAPELVFEEIAFINRFSRPFKFPKLTPLIAIPSTISGAGSEVSAASVLKKGKGIKYN..IVTPEIAF	169
Tba_ADH641	EAAQMGR.EFGAQAVIDGGGSGPIIDSAKVAIILEYTDKDARQLYELK.....FTPKKAKPVIAINTHGTGTVEVDRFAVASILEKEYKPAIAYDCIYF	184
Tpa_ADH	EAAQMGR.EFGAQAVIDGGGSGPIIDTAKSVAIILEYTDKTARELYKQ.....FSRPAKPIIAINTHGTGTVEVDRFAVASILEEYKPAIAYDCIYF	185
Thy_ADH	EAAEMGR.EFGAQAVIDGGGSGPIIDSAKVAIILEYDPTARDLYEFR.....FTPVKAKPIIAINTHGTGTVEVDRFAVASIPEKEYKPAIAYDCIYF	184
Tsp_ADH	EAAEMGR.EFGAQAVIDGGGSGPIIDSAKVAIILEYDPTARDLYEFR.....FTPVKAKPVIAINTHGTGTVEVDRFAVASIPEKEYKPAIAYDCIYF	184
Tli_ADH	EAKEMGV.EFGAQAVIDGGGSGPIIDTAKSVAIILEYDPTKGDLYEFK.....FKPTKAKPIIAINTHGTGTVEVDRFAVASILEKEYKPAIAYDFIYF	185
Tko_ADH2	EAAELAR.RENVDFVVGLGGGSAIDSAKAIAMTAKSGGKYWDYVPAVGGG...KKPSGALPIVAIPTHTGTVEADPYAVITNPETKEKQGIGYDVLFF	189
Pfu_ADH	EGAEIAR.KENVSFVVGLGGGSAIDSAKAIAMTAAAGGKYWDYVPAVGGG...KKPTGALPIVAIPTHTGTVEADPYAVITNPETKEKQGIGYDVLFF	175
Zmo_ADH	EGLKILK.DNNSDFVISLGGGSPHICAKAIALVATNGGEVVDYEGIDK.....SKKPALPLMSINTIAGTASEMTRFCIITDEVHRVKMAIVDRHVTF	170
Sce_ADH	AGLKVLK.EQNSEIVVSI GGGAHINAKAIALLATNGGEIGDYEGVNC.....SKKAALPLFAINTIAGTASEMTRFTIISNEEKKIKMAIIDNNVTF	171
Consensus	gggs d k i t g e III p	
Tba_ADH547	EFAILDLRPLRPTMPKEVARNNSGLDVLVHGIEAYTTTAATFSDAMAIIKAIKLVYKWLPLSVQG..DEKARENHYAATMGIAFLNARLGLCHSLSKAA	269
Tan_ADH	EVAILLRPLRPTMPGEVARNNSGLDVLVHGIEAYTTKAANFSDTMAINAIAIKTVYFWLPLSVKG..DEEARARVHYAATMGIAFLNARLGLCHAMSKAA	268
Tth_ADH	DVAILLRPLRPTMPPEVARNNSGLDVLVHGIEAYTTKVASFSDAMAIIKAIKTVYFWLPLSVKG..DEEARARVHYAATMGIAFLNARLGLCHAMSKAA	267
Tko_ADH1	EVAILLRPLRPTMPREVARNNSGLDVLVHGIEAYTTKVASFSDAMAIIKAIKTVYFWLPLSVKG..DPEARARMHYAATMGIAFLNARLGLCHAMSKAA	267
Tba_ADH641	LYAIDDLALTVKLPADQTRYVTIDALNHVNEAATTKVAVSYSILLAQETARLIFKYIPEALNHPDNLQARYYLLYSAIAGISFDNGLLHFTHALEHPLS	284
Tpa_ADH	LYSIDDLTLMTKLPADQTRYVTIDALNHVNEAATTKVAVSYSILLAKETARLIFIYIPEALIHDPDNLQARYYLLYSAIAGISFDNGLLHFTHALEHPLS	285
Thy_ADH	LYAIDDLALTTKLPPEQTLVYTDALNHVTEAATTKVANYSILLAKEAARLIFTYIPEALNHPDNLQARYALLYSAIAGISFDNGLLHFTHALEHPLS	284
Tsp_ADH	LYAIDDLSLTVKLSPEQTLYLTIDALNHVTEAATTKLANYSILLAKEAARLIFTYIPEALKHPDNLQARYALLYSAIAGISFDNGLLHFTHALEHPLS	284
Tli_ADH	LYAIDDLALMTKLPANQTRYVTIDALNHVNEAATTKATNYSILLAQETVRLISKYIPEALAHPENLQARYYLLYSAIAGISFDNGLLHFTHALEHPLS	285
Tko_ADH2	KFSIVDEEVMVTLPCNQTVYTSMAFYYSIEAFLNVRACYSVDVLDLSMRRVVYIPIAYENLQDLQARTQLAWASTEAGITETLTGVIANHALEHGLS	289
Pfu_ADH	KFSIVDEELMLTLPKQTVYTSMAFYYSIEAFLNVRACYSVDVLDLSMRRVVYIPIAYENLRNLEARTQLAWASTEAGITETVTGVVANAHALEHGLS	275
Zmo_ADH	MVSVNDELMLMVGMPKGLTAATGMALTAFAFAYSSTAATEITDACALKAASMIAKNKTACDNGKDMPEAREAMAYQFLAGMAFNNASLGYVHAMACQLG	270
Sce_ADH	AVAVNDELSTMFGLPPALTAATGLTALTAFCIEAYVSTASNETITDACALKGIDLINESTVAAYKDGKDKKRTDMCYEYLAGMAFNNASLGYVHALACQLG	271
Consensus	dp d h ea p a l a a ag h h	
Tba_ADH547	WIA...FHGLLNNAIFLYVMEFN..MRSEYARKRYAEIARELG...FNTAQELIEVIREFNEMLGVPK...SELVSEEEFSLKLDSEMSEK..AYHDPLINF	358
Tan_ADH	WIG...FHGLLNNAIFLYVMEFNA.ERSEYARKRYAEIARELG...FNTAKDLIEVVKELNEMLGVPG...SELVDEEAFVSKVEEMANK..AYRDGLIPF	358
Tth_ADH	WIG...FHGLLNAVFLYVMEFNA.SKSDYARRRYAEIARELG...FQAKDLIEVVKELNEMLGVPK...GELVDEETFASKVEEMAER..TYHDGLIAF	357
Tko_ADH1	WVG...FHGLLNNAIFLYVMEFNA.ERSEYARKRYAEIARELG...LKSAAELIKAVRELNERLGVPK...SELVDEETFVERVEEMAER..AYRDGLIAF	357
Tba_ADH641	AVKPELHGLGLAMLLAVIKHIYPATAPILAIEVYRPLVPDAKG.VPG.EALKVAKAVEKWLFSIGITEK...TDVGFGEDDVKKLAEAMTTPSLDLLLLSL	382
Tpa_ADH	AVKPDHGLGLAILLAVIKHIYPATARILAEIYRPLVPEVKG.VPG.EAELVAKKVEEWFSAIGITQK...TDVGFSEDDVKKRLTELAMTTPSLNLLLLSL	383
Thy_ADH	AVKPDHGLGLAMLLAVIRQIYPATAKILAIEVYRPLVPEAKG.VPG.EVELVARRVEEWFSAIGITEK...ADVGFTEGDVDKLTQLAMTTPSLDLLLLSM	382
Tsp_ADH	AVKPDFHGLIGLAMLLAVIRHIYPATAKILAIEVYRPLVPEAKG.VPG.EVELVARRVEEWFSAIGITEK...EDVGFSETDVNRLTELAMKTPSLNLLLLSM	382
Tli_ADH	AVKPDHGLGLAMLLAVVKHIYPATAEILAIEVYRPIAPEAKG.YPG.EAEFVAKRIEQWLFSAIGIKEK...TDVGFGEDDIDKLTKLAMETPGLDGLLAM	383
Tko_ADH2	GFYPEVHGLGLCILGYYLFEYILDHAYERLAIVGREVFVYVES.NDRKAAELAVKKLRDQSLFGVNKK...RELGVKEEDIPKMAETAYR..MMKGVIDV	386
Pfu_ADH	GFYPEVHGLGLCILGYYLFEYILDYAYEKLAIVGREVFVYVEP.NDRKAAELAIKKLRDQSLFGVNKK...RELGVKEEDIPEMARTAYR..MMKPVIEA	372
Zmo_ADH	GYV.NLHGLVCNAVLLHVLAYNASVAVAGRLKDVGVAMGLDIANLGDKEGAEATIQAVRDLAASIGIPANTELGAKKEDVPLLDHALK...DACALT	365
Sce_ADH	GFY.HLHGLVCNAVLLHVQEANMQCPKAKKRLDEIALHFGAS...QEDPEETIKALHVLNRTMNIIPRN...KELGVKTEDFEILAEHAMH...DACHLT	362
Consensus	phg p l	
Tba_ADH547	NEVEPSIEEIKELYKRAFYD...	378
Tan_ADH	NEVEPKPEEIRELYLRAFYGR...	379
Tth_ADH	NEVEPKPEEIKELYLKAYRGE...	378
Tko_ADH1	NEVEPKPEEIRELYLKALNAL...	378
Tba_ADH641	AEIPATKETVEAIYRDSLYPLSE	405
Tpa_ADH	AEVEATKETIEAIYRDSLYPLSK	406
Thy_ADH	AEVEATKERIAAIYRDSLYPIGR	405
Tsp_ADH	AEGEATRERIAAIYRDSLYPISR	405
Tli_ADH	AEVEANEDVIRAIYRDSLYPLSE	406
Tko_ADH2	TEGNLSVEDLEEYRRAY.....	404
Pfu_ADH	TEGDLKVEDLEEYRRAY.....	390
Zmo_ADH	NERQGDQKEVEELFLSAF.....	383
Sce_ADH	NEVQFTKEQVVAIIKKAYEY...	382
Consensus	p	

Figure

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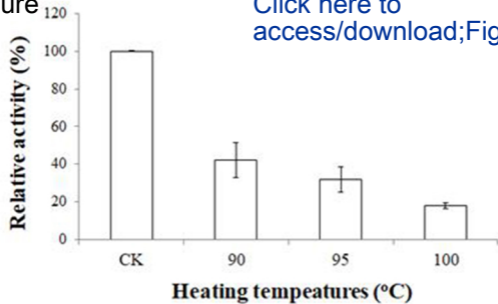


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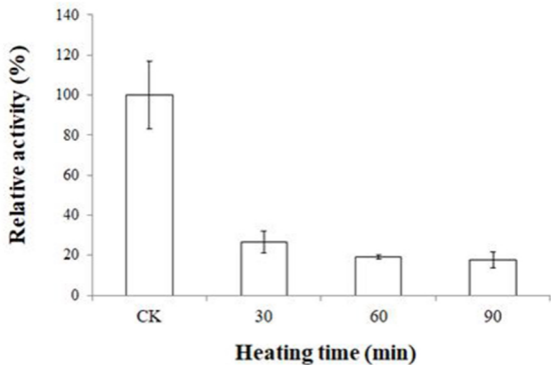


A Figure

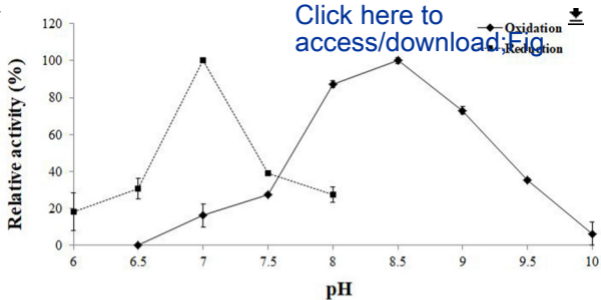
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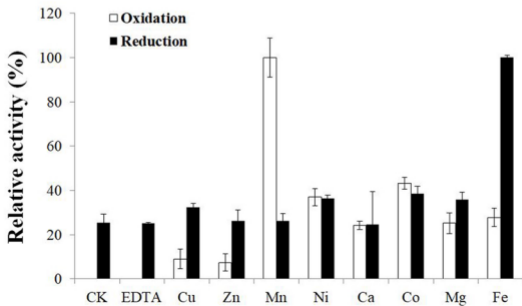
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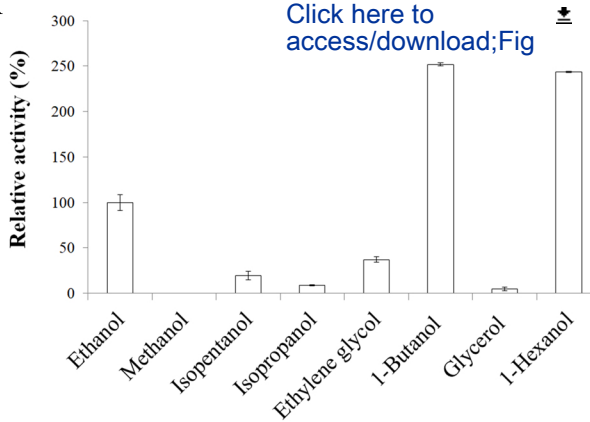
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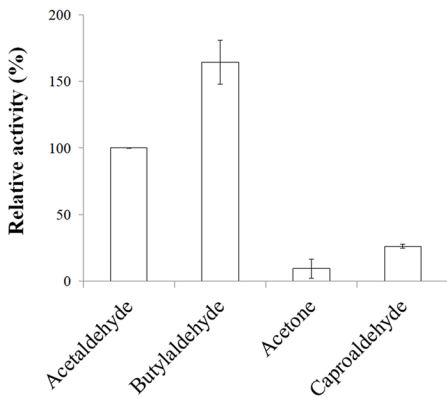
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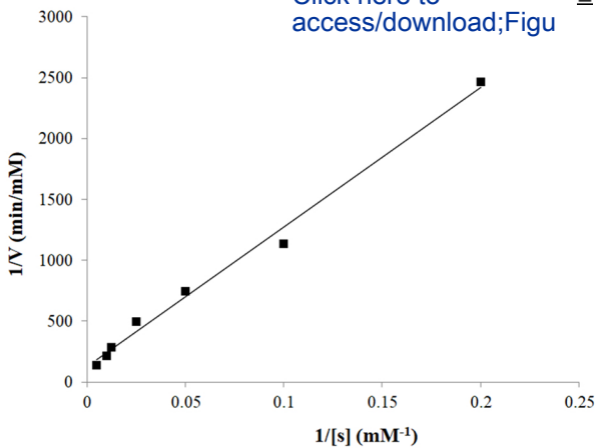
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