Extremophiles
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NOTE

# Characterization of alcohol dehydrogenase from the haloalkaliphilic archaeon *Natronomonas pharaonis*

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**Abstract** Alcohol dehydrogenase (ADH; EC: 1.1.1.1) is a key enzyme in production and utilization of ethanol. In this study, the gene encoding for ADH of the haloalkaliphilic archaeon *Natronomonas pharaonis* (*Np*ADH), which has a 1,068-bp open reading frame that encodes a protein of 355 amino acids, was cloned into the pET28b vector and was expressed in *Escherichia coli*. Then, *Np*ADH was purified by Ni-NTA affinity chromatography. The recombinant enzyme showed a molecular mass of 41.3 kDa by SDS-PAGE. The enzyme was haloalkaliphilic and thermophilic, being most active at 5 M NaCl or 4 M KCl and 70°C, respectively. The optimal pH was 9.0. $Zn^{2+}$ significantly inhibited activity. The $K_m$ value for acetaldehyde was higher than that for ethanol. It was concluded that the physiological role of this enzyme is likely the catalysis of the oxidation of ethanol to acetaldehyde.

**Keywords** *Natronomonas pharaonis* · Haloalkaliphilic · Alcohol dehydrogenase · Molecular cloning

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## Introduction

Halophiles are microorganisms that are able to live in hypersaline environments. According to the widely used definition (Kushner 1978), halophiles can be classified into three groups: slight halophiles (many marine organisms which live in seawater containing about 0.5 M NaCl), moderate halophiles (optimal growth at 0.5–2.5 M NaCl), and extreme halophiles (optimal growth around 4 M NaCl). There are also borderline extreme halophiles that tolerate above approximately 2.5 M NaCl (Margesin and Schinner 2001). In the domain Archaea, we find aerobic halophiles (family *Halobacteriaceae*) as well as anaerobic halophilic methanogens (Kamekura 1998).

Proteins from the *Halobacteriaceae* are generally functional at high salt concentrations, in the range of 2–4 M NaCl or KCl, and they need high ionic strength to sustain their structure and function (Dym et al. 1995; Danson and Hough 1997; Demirjian et al. 2001). Halophilic proteins usually possess a much higher content of acidic amino acid residues than nonhalophilic homologs. The acidic residues can interact with the water molecules and salt ions, and they contribute to the stability and activity of halophilic proteins in concentrated salt solutions (Madern et al. 2000, 2004: Fukuchi et al. 2003; Irimia et al. 2003). The crystal structure of the halophilic malate dehydrogenase from *Haloarcula marismortui* has been extensively studied, and it was demonstrated that sodium and chloride ions are found at the periphery of the protein, interacting with the acidic residues, and are involved in the complex of salt bridge clusters located at monomer–monomer interfaces; the sodium ions can be replaced with potassium ions (Richard et al. 2000; Irimia et al. 2003).

Many halophilic Archaea oxidize glucose via modified Emden–Meyerhof and Entner–Doudoroff pathways

(Siebers and Schönheit 2005), by which the intermediate pyruvate is produced. Pyruvate serves as a precursor for the production of acetate and ethanol (Bräsen and Schönheit 2004). However, little is known about the metabolism of ethanol in the halophilic Archaea. The finding of a gene annotated as alcohol dehydrogenase (ADH) in the genome of *Natronomonas pharaonis* was the starting point for the current study. ADHs (EC: 1.1.1.1) are a family of oxidoreductases that catalyze the interconversion between alcohols and aldehydes, and are widely distributed in all the three domains of life (Reid and Fewson 1994), as well as in other members of *Halobacteriaceae* (*Halobacterium* sp. NRC-1, *Haloarcula marismortui*, and *Haloquadratum walsbyi*). Alcohol dehydrogenases can be distinguished based on their cofactor specificity, possible cofactors being (1) $NAD^+$ or $NADP^+$, (2) pyrrolo-quinoline quinone, haem or $F_{420}$, (3) FAD. The $NAD(P)^+$-dependent ADHs are mainly sub-divided into zinc-dependent ADHs, short-chain ADHs, and iron-activated ADHs, based on their catalytic properties and sequence information (Reid and Fewson 1994; Forrest and Gonzalez 2000).

*N. pharaonis*, one of the haloalkaliphilic Archaea, was isolated from highly saline soda lakes in Egypt, where it is exposed simultaneously to two forms of stress: hypersaline conditions and high pH (Falb et al. 2005). The cells grow optimally in the presence of 3.5 M NaCl and at pH 8.5. Relatively little information is available on the properties of the enzymes from this organism. In this work, we report the properties of ADH from *N. pharaonis*, the first purified ADH from the halophilic Archaea.

## Materials and methods

### Microbial strains and growth conditions

*Natronomonas pharaonis* CGMCC $1.1965^T$ (=DSM $2160^T$) was purchased from the China General Microbiological Culture Collection Center. *N. pharaonis* was grown at 40°C in a medium as described previously (Konstantinidis et al. 2007). As a host for DNA manipulations *Escherichia coli* DH5α was used. Protein expression was carried out in *E. coli* Rosetta.

### Construction of expression plasmids

The *Np*ADH gene (GenBank accession no.YP326290) was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase. The template DNA was prepared from *N. pharaonis* cells. Based on the *adh* gene of *N. pharaonis*, the sequences of forward and reverse synthetic primers which contained *Nde*I and *Xho*I recognition sites (underlined) were designed as 5′-TTTCATATGCGCGCT GTCGTCTTCGAGGAG-3′ and 5′-AATCTCGAGTCAG AACTCGTCGCAGACCG-3′, respectively. The expression vector was constructed using the plasmid pET28b (Novagen, Germany), which was digested with *Nde*I/*Xho*I (Takara, Japan). The PCR product was then ligated into the gap introducing an N-terminal histidine tag. The resulting recombinant plasmid was called pET-*Np*ADH.

### Protein expression and purification

pET-*Np*ADH was transformed into *E. coli* Rosetta. The transformed Rosetta cells were grown in 4 L of Luria–Bertani medium (containing 20 μg $mL^{-1}$ kanamycin) at 37°C. After an $OD_{600}$ of 0.6–0.8 was reached, 1 mM isopropyl-D-thiogalactopyranoside (IPTG) was added. Following an induction period of 3 h at 30°C, the cells were harvested by centrifugation (7,000×*g*, 5 min, 4°C), resuspended in cold sodium phosphate buffer (50 mM $NaH_2PO_4$, pH 8.0; 4 M NaCl; 10 mM imidazole), and finally broken up by sonication in ice (100 W, 1 s sonifying vs. 2 s pause, 500 cycles). After centrifugation (18,000×*g*, 20 min, 4°C), the supernatant was loaded onto a chromatography column filled with Ni-NTA agarose (Qiagen, Germany) and washed extensively with sodium phosphate buffer with increased imidazole concentration (≤100 mM) to remove unspecifically bound proteins. Subsequently the histidine-tagged protein was eluted in sodium phosphate buffer containing 250 mM imidazole. The purified protein was ultrafiltrated against buffer containing 50 mM Tris–HCl (pH 8.0) and 4 M NaCl, and was stored at −80°C until used. The ADH containing fractions were detected by SDS-PAGE, using 12% polyacrylamide gels, which were stained with Coomassie brilliant blue R250 (Laemmli 1970). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard.

### Enzymatic assays

Standard enzymatic assays were performed by monitoring the substrate-dependent absorbance change of NAD(H) at 340 nm using a Beckman Coulter DU 800 nucleic acid /protein analyzer as previously described (Ziegenhorn et al. 1976). The reaction was performed in 1 mL reaction mixture containing 50 mM Tris–HCl (pH 9.0), 4 M NaCl, 2 mM DTT, 0.2 mM NADH, 50 mM acetaldehyde and enzyme for the reduction of aldehyde; the assay mixture (1 mL) used for the oxidation of alcohol contained 50 mM Tris–HCl (pH 9.0), 4 M NaCl, 2 mM DTT, 0.4 mM $NAD^+$, 100 mM ethanol, and enzyme.

Sequence handling

DNA sequences and putative protein sequence homology comparisons were carried out using the BLAST tool at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei 1987), using software in MEGA program (Kumar et al. 1994).

## Results and discussion

The phylogenetic tree presented in Fig. 1 shows a comparison of the deduced amino acid sequence of *N. pharaonis* ADH and 39 other sequences identified as ADHs from the NCBI protein database. The phylogenetic relationship of these amino acid sequences indicates that *Np*ADH belongs to the family of zinc-dependent ADHs, which is one of the three NAD(P)-dependent subfamilies (Reid and Fewson 1994; Forrest and Gonzalez 2000).

Genome sequence analysis of *N. pharaonis* indicated that the *Np*ADH gene including start codon (ATG) and stop codon (TGA) is 1,068 bp in length. DNA sequencing showed that the *Np*ADH gene cloned in the plasmid exhibited 100% identity to the coding region published in the database. In order to generate large quantities of *Np*ADH, *E. coli* Rosetta carrying the plasmid pET-*Np*ADH was grown in Luria–Bertani medium, and production of the recombinant enzyme was induced with IPTG. The *Np*ADH was purified by Ni-NTA affinity chromatography methods. The expected 41.3 kDa fraction including one 6× histidine tag in the N-terminus was detected in 12% SDS-PAGE (Fig. 2). The yield of recombinant protein was 3.35 mg/L of culture.

The optimum pH for *Np*ADH activity was 9.0 and the enzyme was inactive below pH 7.0, while the pH optimum for *Np*ADH stability was rather broad, between 8.0 and 10.0 (Fig. 3a). The recombinant enzyme displayed optimal activity at 70°C, and its greatest stability was below 40°C (Fig. 3b). Consistent with other enzymes isolated from halophilic organisms, *Np*ADH was highly salt dependent. The activity of *Np*ADH increased with salt concentration to a maximum at 5 M NaCl or 4 M KCl. At salt concentrations below 2 M NaCl or 3 M KCl the stability was decreased (Fig. 3c).

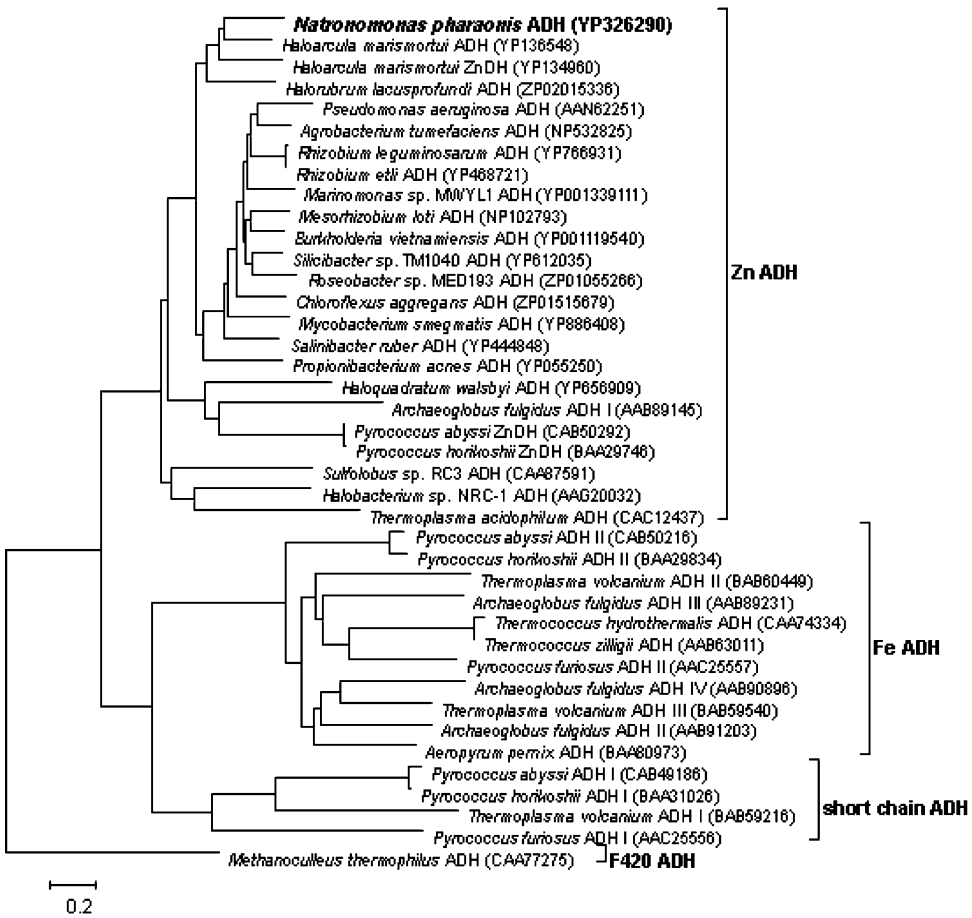


**Fig. 1** Phylogenetic tree derived from archaeal and bacterial ADH and related protein amino acid sequences extracted from NCBI database. The tree was constructed using the neighbour-joining method based on 1,000 resamplings. *N. pharaonis* ADH is shown in *bold*

The influences of several divalent cations ($CaCl_2$, $BaCl_2$, $MnCl_2$, $CoCl_2$, $MgCl_2$, and $ZnCl_2$) on *Np*ADH activity were investigated by adding the compounds at three concentrations of 0.5 mM, 2.0 mM, and 5.0 mM. The enzyme tolerated a range of divalent metal ions, except for zinc ions (89.1, 93.8, and 94.1% inhibition by 0.5, 2.0, and 5.0 mM).

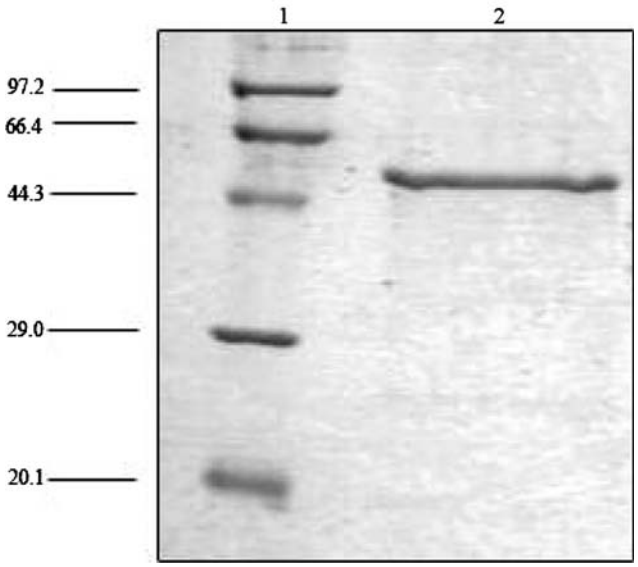


**Fig. 2** SDS-PAGE of purified recombinant *Np*ADH. *Lane 1* protein molecular mass standards, *Lane 2* purified recombinant *Np*ADH. Molecular mass standards are indicated in kDa

The purified enzyme catalyzed the oxidation of ethanol with $NAD^+$ and the reverse reaction of reducing acetaldehyde with NADH. The apparent kinetic constants, $K_m$, $V_{max}$, $k_{cat}$ and catalytic efficiency $k_{cat}/K_m$, for the cloned *Np*ADH with the substrates acetaldehyde and ethanol were determined at 40°C (the physiological temperature of organism) and at 70°C (the optimal temperature of enzyme activity) (Table 1). The four times higher $K_m$ value for acetaldehyde compared to that for ethanol at the physiological temperature indicated that the enzyme may play a role in oxidation of alcohols, rather than in reduction of

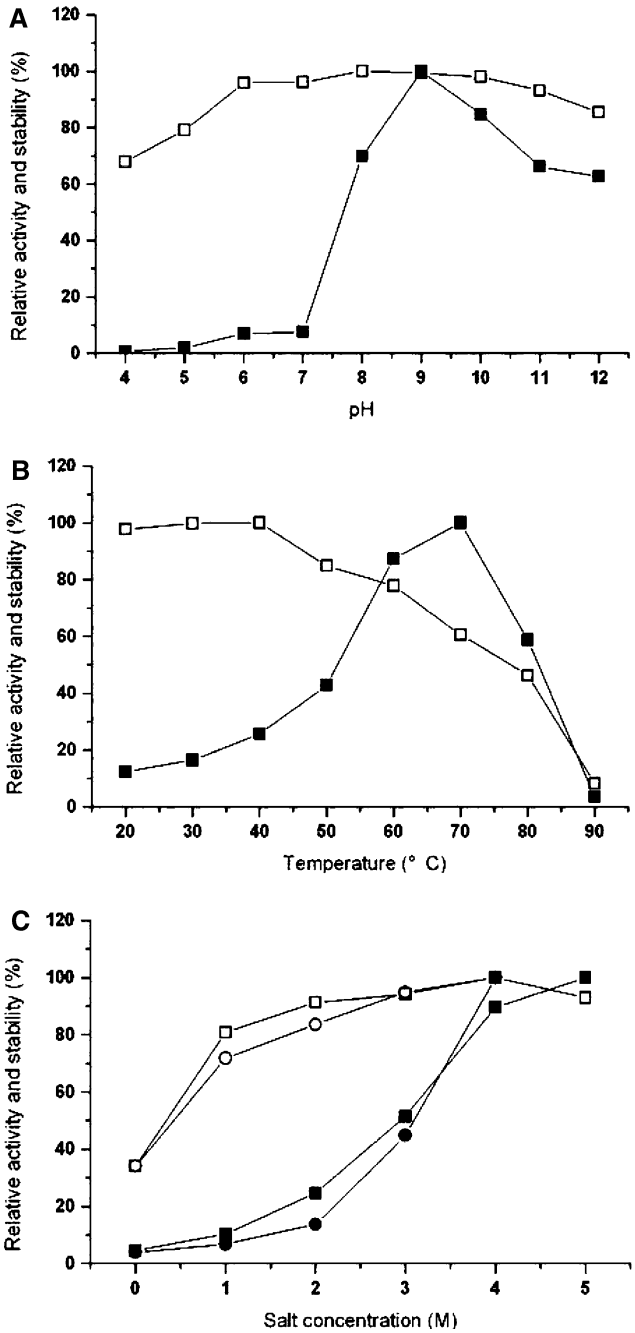


**Fig. 3** Effect of pH (**a**), temperature (**b**), and salt concentration (**c**) on NpADH activity and stability. **a** The pH dependence of NpADH activity (*filled square*) and stability (*open square*) were measured between pH 4 and 12, with three buffer systems, 50 mM $Na_2HPO_4$-citric acid (pH 4.0–5.0), 50 mM Tris–HCl (pH 6.0–9.0), and 50 mM glycine-NaOH (pH 10.0–12.0). All buffers contained 4 M NaCl. Activity assays were performed at various pH values at 70°C for 30 min. For stability assays, 50 µL portions of enzyme solution in buffers with different pH were preincubated at 30°C for 1 h. Then 950 µL of 4 M NaCl, 50 mM Tris–HCl buffer (pH 9.0) with substrates were added, if necessary the pH was adjusted to pH 9.0, and the remaining activity was assayed as described above. **b** The effect of temperature on NpADH activity (*filled square*) and stability (*open square*) were determined at temperatures from 20 to 90°C. Activity was measured for 30 min at different temperatures. For stability assays, the enzyme was preincubated for 1 h at the indicated temperature, and residual activity was then assayed at 70°C as described above. **c** To test the effects of salts on NpADH activity and stability, the salt concentrations in the assay were varied from 0 to 5 M NaCl (*filled square*, *open square*) and from 0 to 4 M KCl. (*filled circle*, *open circle*). Activity (*filled square*, *filled circle*) was determined at 70°C, pH 9.0 for 30 min. For stability measurements (*open square*, *open circle*), 50 µL portions of enzyme solution were preincubated at different concentrations at 30°C for 1 h. Then 950 µL substrates and salt solutions were added to adjust the final salt concentration to standard condition (4 M NaCl or KCl), and residual activity was assayed as described above

**Table 1** Kinetic parameters of purified recombinant alcohol dehydrogenase from *N. pharaonis*

Enzyme activity was measured with 50 mM Tris–HCl (pH 9.0) and 4 M NaCl

| Substrate | Temperature (°C) | $V_{max}$ (mM $s^{-1}$) | $K_m$ (M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|
| Acetaldehyde | 40 | $2.1 \times 10^{-5}$ | $5.9 \times 10^{-2}$ | 0.3 | 5.1 |
| Ethanol | 40 | $2.7 \times 10^{-5}$ | $1.3 \times 10^{-2}$ | 0.4 | 30.8 |
| Acetaldehyde | 70 | $3.6 \times 10^{-4}$ | $3.4 \times 10^{-1}$ | 5.4 | 15.9 |
| Ethanol | 70 | $5.5 \times 10^{-4}$ | $2.2 \times 10^{-2}$ | 8.2 | 372.4 |

aldehydes in vivo. The enzyme was highly specific for ethanol with $NAD^+$ as the coenzyme. With methanol, 2-propanol, 1-butanol, and 1-pentanol the rates were 1.1, 15.7, 0.3, and 0% of that with ethanol. The results suggested that the *Np*ADH was specific towards the transition between ethanol and acetaldehyde.

The present work shows that ADH from *N. pharaonis* is thermophilic and haloalkaliphilic. Studies to determine the optimum temperature reveal that the *Np*ADH has its maximum activity at 70°C, so that it can be classified as a thermoactive enzyme. There are more cases of halophilic enzymes with temperature optima far above the maximum growth temperature of the organism that produces them: *Halorubrum sodomense* excretes an amyloglucosidase that is optimally active at 70°C (Oren 1973), and *Halobacterium salinarum* ("*halobium*") has two glutamate dehydrogenases that are optimally active at 70°C (Bonete et al. 1986, 1987). *Np*ADH is active and stable at alkaline pH, and this agrees well with the intracellular pH of 9.0 or slightly higher generally observed in alkaliphiles (Padan et al. 1981, 2005). *Np*ADH depends on high salt concentrations for activity and stability, and shows a high percentage of excess acidic residues (18.3 %), similar to other halophilic enzymes that have a high content of aspartic acid and glutamic acid (Ng et al. 2000; Madern et al. 2000, 2004; Ebel et al. 2002; Fukuchi et al. 2003; Irimia et al. 2003). Three dimensional structures of halophilic proteins (Dym et al. 1995; Frolow et al. 1996; Pieper et al. 1998; Richard et al. 2000; Yamada et al. 2002; Bieger et al. 2003) imply that high densities of acids residues at the protein surface are involved in complex salt bridge networks with sodium or potassium and chloride ions compared with non-halophilic homologues. The amino acid sequence analysis indicates that *Np*ADH belongs to the zinc-binding dehydrogenase family. The catalytic zinc binding residues (Cys38, His64, Cys154) and structural zinc binding residues (Cys97, Cys100, Cys108) are conserved in *Np*ADH. We observed that $Zn^{2+}$ significantly inhibited enzyme activity of *Np*ADH at high salt concentration, while non-halophilic analogues depend on zinc ion for activity (Jörnvall et al. 1987; Ceccarelli et al. 2004). The nature of the interactions of *Np*ADH with zinc ions deserves further study.

As a conclusion, the heterologously expressed ADH from *N. pharaonis* appears thermophilic, alkaliphilic, and inactive at low salt concentration. The location of the *Np*ADH gene within the *N. pharaonis* genome does not provide any clues on the possible function of the enzyme within a known metabolic pathway. Thus far there is no indication for the importance of alcohols or aldehydes in the alkaline hypersaline lake environment, and the function of the enzyme in *N. pharaonis* remains unclear.

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