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Characterization of alcohol dehydrogenase 1 of the thermotolerant methylotrophic yeast *Hansenula polymorpha*

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Abstract The thermotolerant methylotrophic yeast *Hansenula polymorpha* has recently been gaining interest as a promising host for bioethanol production due to its ability to ferment xylose, glucose, and cellobiose at elevated temperatures up to 48 °C. In this study, we identified and characterized alcohol dehydrogenase 1 of *H. polymorpha* (HpADH1). HpADH1 seems to be a cytoplasmic protein since no N-terminal mitochondrial targeting extension was detected. Compared to the ADHs of other yeasts, recombinant HpADH1 overexpressed in *Escherichia coli* exhibited much higher catalytic efficiency for ethanol oxidation along with similar levels of acetaldehyde reduction. HpADH1 showed broad substrate specificity for alcohol oxidation but had an apparent preference for medium chain length alcohols. Both ADH isozyme pattern analysis and ADH activity assay indicated that ADH1 is the major ADH in *H. polymorpha* DL-1. Moreover, an *HpADH1*-deleted mutant strain produced less ethanol in glucose or glycerol media compared to wild-type. Interestingly, when the *ADH1* mutant was complemented with an *HpADH1* expression cassette, the resulting strain produced significantly increased amounts of ethanol compared to wild-type, up to 36.7 $g l^{-1}$. Taken together, our results suggest that optimization of ADH1 expression would be an ideal method for developing *H. polymorpha* into an efficient bioethanol production strain.

Keywords Alcohol dehydrogenase·ADH1 · *Hansenula polymorpha* · Ethanol production · Glycerol fermentation

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Introduction

The thermotolerant methylotrophic yeast *Hansenula polymorpha* is an attractive model organism for various fundamental studies, e.g., the genetic control of enzymes involved in methanol metabolism, peroxisome function and biogenesis, nitrate assimilation, and resistance to heavy metals and oxidative stress (Gellissen 2002; Martin et al. 2008; Park et al. 2007). *H. polymorpha* has been widely applied as host organism for the production of foreign proteins (Kang et al. 2002; Oh et al. 2008). In addition, this yeast has been shown to metabolize and ferment ethanol from glucose, xylose, cellobiose, starch, and xylan substrates (Ryabova et al. 2003; Voronovsky et al. 2009), which makes it an ideal candidate for lignocellulosic biomass-based ethanol fermentation.

Yeast alcohol dehydrogenase (ADH) is an oxidoreductase enzyme that catalyzes the final metabolic step in ethanol fermentation, the reduction of acetaldehyde to ethanol along with the concomitant oxidation of NADH or NADPH, as well as the reverse reaction. Sequences of ADH genes are well-conserved among several yeasts, but regulation, physiological function, and gene copy number are different between species. Until now, seven *ADH* genes (*ADH1* to *ADH7*) have been identified from both *Saccharomyce cerevisiae* and *Pichia stipitis* and submitted to GenBank, whereas four genes (*ADH1* to *ADH4*) have been reported in *Kluyveromyces lactis*. Among them, cytosolic ADH1 and ADH2 of *S. cerevisiae* have been studied in detail since they play crucial roles in alcoholic fermentation, specifically, in the production and use of ethanol, respectively (Denis et al. 1983; Lutstorf and Megnet 1968). In contrast, *P. stipitis* ADH1 (PsADH1) appears to have both fermentative and respiratory functions (Cho and Jeffries 1998; Passoth et al. 1998). This evidence supports divergent adaptation between Crabtree negative and positive species.

H. polymorpha is a Crabtree negative yeast, even though neither the genetic nor physiological characteristics of ADH have been published for this species. Understanding the ADH system of *H. polymorpha* not only will provide basic knowledge, but can also contribute to increase ethanol fermentation. In the present study, the *HpADH1* gene was identified and characterized based on the phenotype of its deletion mutant, kinetic parameters of in vitro enzyme reaction, and expression using different carbon sources. Further, ethanol production from glucose and glycerol was investigated.

Materials and methods

Strains and growth conditions

H. polymorpha DL1-LdU (*leu2 Δura3::lacZ*) and DL1-L (*leu2*) strains, derivatives of DL-1 (ATCC26012) strain (Kang et al. 2002) were used, respectively, as the parent strain to construct the *HpADH1* disruption mutant and as a reference strain for comparing growth and ADH activity between the *HpADH1* disruption mutant and complemented strain. The *H. polymorpha* cells were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose). When necessary, YPE, YPG, or YPX media containing 2% ethanol, glycerol, or xylose instead of glucose, respectively, were used. For selection of recombinant strains by auxotrophic markers, cells were incubated on a plate of synthetic complete (SC) medium (0.67% yeast nitrogen base [YNB] without amino acids, 2% glucose, 0.77 $g l^{-1}$ drop-out supplement without uracil and/or leucine [Clontech]) at 37 °C. For fermentation experiments, modified fermentation medium (0.05% yeast extract, 0.34% $(NH_4)_2SO_4$, 0.77 $g l^{-1}$-Ura drop-out supplement [Clontech], 40 $mg l^{-1}$ uracil) supplemented with 10% glucose or glycerol was used.

Escherichia coli DH5α and BL21 (DE3) strains were used as a host for vector propagation and overexpression of recombinant protein, respectively. *E. coli* was cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 30 $\mu g ml^{-1}$ of kanamycin or 100 $\mu g ml^{-1}$ of ampicillin.

Construction and functional complementation of *HpADH1* mutant strain

The *HpADH1* gene was disrupted by replacement with a *lacZ–Ura3–lacZ* deletion cassette via two-step PCR and in vivo recombination (Fig. 1a), as previously described (Kim et al. 2006). The *ADH1* disruption mutant was screened on SC medium lacking uracil and stabilized by alternative subculture on selective and non-selective media.

The full-length *HpADH1* gene was amplified from the genomic DNA of *H. polymorpha* DL1-L by PCR using the primers HpADH1F and HpADH1R (Table 1). The PCR fragments were digested with *Hind*III and *Spe*I and ligated to *Hind*III–*Spe*I digested pDLG-LK vector, resulting in pDLG-ADH1 (Fig. 4a). The pDLG-ADH1 vector contains the P_{GAPDH} promoter for constitutive expression of HpADH1, Hp*LEU2* as an auxotrophic marker, and *Hansenula* autonomous replication 36 sequence (HARS36) enhancing multiple tandem integration of the plasmid into genome (Sohn et al. 1999).The pDLG-ADH1 was transformed into *H. polymorpha* Δ*ADH1* cells and the complemented strain was screened on SC medium lacking leucine and uracil and confirmed by PCR using primer pairs of pDLGseqF and pDLGseqR (Table 1).

To compare phenotypes, wild-type, *ADH1*-disrupted, and complemented strains were first cultured in YPD liquid medium overnight at 37 °C, after which the inoculums were prepared by centrifugation and washing with sterile distilled water. Cells were then re-inoculated in duplicate tubes containing YPD and YPE media at concentrations of OD_{660}= 0.2, followed by incubation at 37 °C with shaking at 180 rpm. Samples were taken at different time intervals for OD_{660} measurement. The experiments were repeated two times.

Comparison of ADH isozyme pattern

H. polymorpha DL1-L, *ΔADH1*, and pDLG-ADH1/*ΔADH1* cells grown in YPD and YPE media at 37 °C for 24 h, were harvested by centrifugation. The cell pellets were resuspended in lysis buffer (50 mM Tris–Cl [pH 8.0], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and broken by glass beads. After centrifugation, clear supernatant was collected and used as cell-free extract. Equal amounts of cell-free extracts (15 μg) were loaded

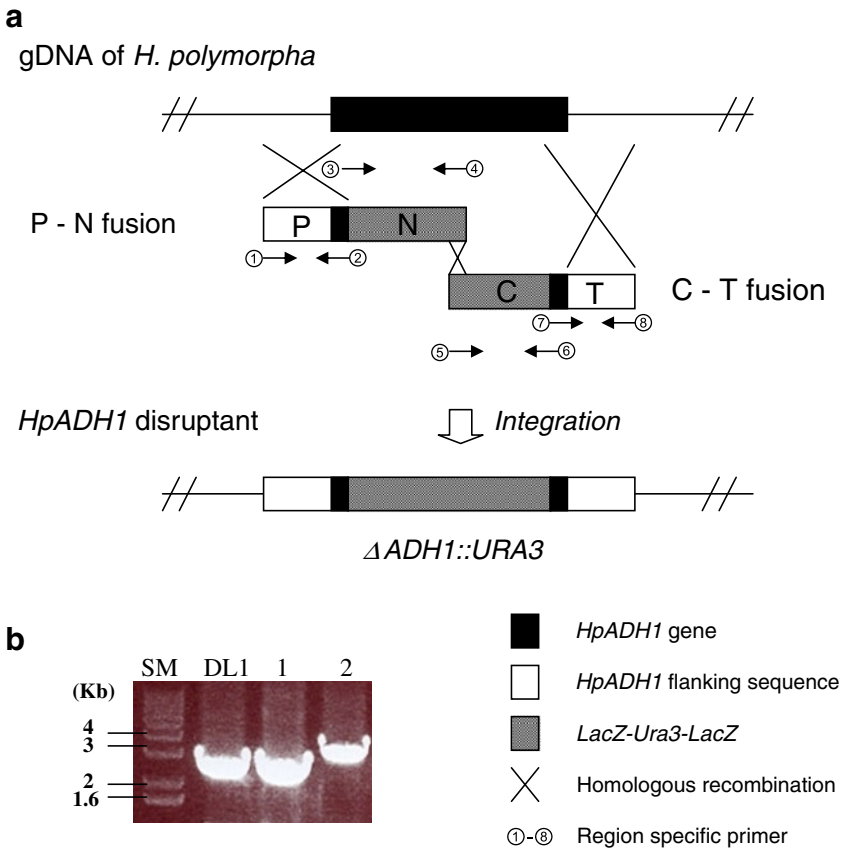

Fig. 1 Gene disruption scheme of *HpADH1*. **a** Two DNA fragments, P and T, containing the promoter and terminator regions of *HpADH1* were PCR amplified from the genomic DNA of *H. polymorpha* using primer pairs ①–② and ⑦–⑧, respectively. Primers ② and ⑦ were designed to contain flanking sequences of HpURA3-lacZ blaster. The N-term and C-term fragments of HpURA3-lacZ blaster N and C, which overlap each other with 100 bp, were PCR amplified from the pLacUR3 plasmid using the primer pairs ③–④ and ⑤–⑥, respectively. Fused DNA fragments, P-N and C-T, were prepared by fusion PCR of fragments P and N and C and T, respectively, followed by PCR amplification. Resulting fused DNA fragments were transformed simultaneously into *H. polymorpha* DL-LdU to obtain Δ*ADH1*::*ura3* mutant by in vivo homologous recombination-mediated target gene replacement. **b** PCR validation of *ADH1* gene disruption. A presence of 3.5-Kb PCR product instead of 2.8-Kb product indicated ADH1 gene deletion (Δ*ADH1*::*ura3*). *SM*, size marker, *DL1* wild-type strain, 1 wild-type strain, *2* Δ*ADH1* mutant

onto two 6% PAGE gels and separated at 80 V at low temperature. One gel was stained for ADH activity as previously described (Fejér et al. 1979), while the other gel was stained for total protein using Coomassie brilliant blue R250. Samples from three independent experiments were examined.

Expression and purification of recombinant 6×His-tagged HpADH1 protein

The *HpADH1* gene was PCR amplified using the genomic DNA of *H. polymorpha* DL-1 strain as the template and the primers pETADH1F and pETADH1R (Table 1). PCR products were digested with *Hind*III and *Nde*I and ligated to the *Hind*III–*Nde*I digested pET28a^{+} expression vector, resulting in pET28a-HpADH1.

An overnight culture of *E. coli* BL21 (DE3) cells transformed with pET28a-HpADH1 was inoculated in LB medium supplemented with 30 μg ml^{-1} of kanamycin and cultured at 37 °C for 3 h with shaking at 180 rpm. Protein expression was induced by the addition of 1 mM IPTG, and the cells were incubated at 16 °C for 24 h with shaking at 180 rpm. The recombinant 6×His-tagged HpADH1 was then purified by using Ni-NTA agarose resin (Qiagen) under native conditions following the manufacturer's instructions.

Determination of alcohol dehydrogenase activity

The dehydrogenase activity of the recombinant ADH1 protein (2 μg) was measured by following the reduction of NAD^{+} at OD_{340} using 100 mM ethanol as a substrate (Postma et al. 1989). The reductase activity was measured by recording the decrease in OD_{340} due to NADH oxidation in the presence of 100 mM acetaldehyde as previously described (Verduyn et al. 1988). Activity units are defined as the amount of enzyme producing or consuming 1 μmol of NADH per min. K_m and V_{max} were obtained by varying

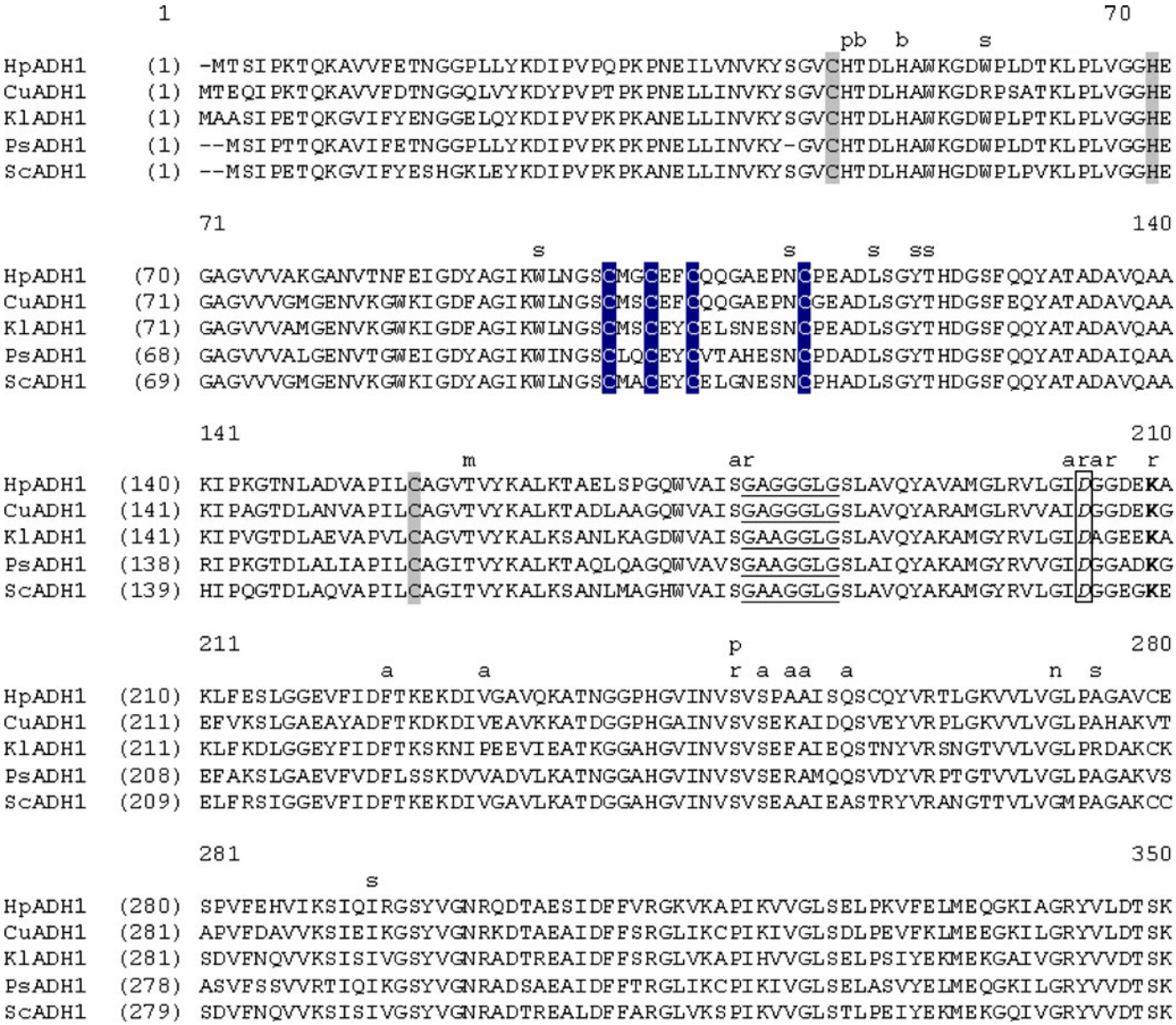

Fig. 2 Alignment of the amino acid sequences of HpADH1 with other yeast cytosolic ADHs (*Sc*=*S. cerevisiae*, *Kl*=*K. lactis*, *Ps*=*P. stipitis*, *Cu*=*C. utilis*) using the AlignX program (Informax, USA). Residues involved in enzyme function are headed by *lower case letters*: adenine binding pocket (*a*), adenosine ribose binding (*r*), pyrophosphate binding (*p*), nicotinamide ribose (*n*), nicotinamide (*m*), substrate binding pocket (*s*), and acid-base system (*b*) (Jornvall et al. 1978). *Boxed letters* mark Asp residues, which determine specificity for NAD (Sun and Plapp 1992). *Underlined residues* indicate NAD (P^+)-binding moieties (Park et al. 2006). *Bold letters* represents Lys residue that is conserved among NAD(H)-dependent ADHs. *Grey* and *black highlighted residues* correspond to catalytic and structural Zn^{2+} binding residues, respectively (Kim and Howard 2002)

substrate concentrations from 0.1 to 20 mM for ethanol and 1 to 100 mM for acetaldehyde. The data were plotted and calculated using a one-site ligand binding equation of Sigma plot 10.0 software.

Substrate specificity of HpADH1 was investigated using various alcohols and aldehydes as substrates at 100 mM. Each reaction was performed at least three times and contained a standard error of less than 10%. Total ADH activity of cell-free extracts of *H. polymorpha* cells was determined in terms of ethanol dehydrogenase activity.

Transcription analysis of *HpADH1*

H. polymorpha DL1-L cells were grown in YPD, YPG, YPX, and YPE media at 37 °C with shaking at 180 rpm until log phase. The cells were collected by centrifugation at 4,000 rpm at 4 °C. Then, cell pellets were rapidly frozen in liquid N_2 and kept at −70 °C until RNA extraction. Total RNA was extracted by hot phenol method followed by purification using a RNeasy column kit (Qiagen) as previously described (Lyne et al. 2003). RNA quantity and

Table 1 Primers used in this study

Name	Sequence (5′->3′)
HpADH1F	CGAGCGAAGCTTATGACTTCCATTCCAAAGACTCAAAAGGCC
HpADH1R	CGAGCTACTAGTCTATTTGGAAGTGTCAAGAACGTATCTTCC
Del ADH1 NF①	GTCCTTGATTTTCCGTTTGAGTACCTCG
Del ADH1 NR②	AGCTCGGTACCCGGGGATCCCTCATTTGGCTTTGGTTGTGGAACAGG
Del ADH1 CF⑤	GCACATCCCCCTTTCGCCAGGCTCCAATCAAGGTTGTTGGCCTTTCTG
Del ADH1 CR⑥	TCAGTAGCTTGTGTTTTTCTGCCGTAGTG
LacZ_NF③	TCCCCGGGTACCGAGCT
LacZ_NR④	CACCGGTAGCTAATGATCCC
LacZ_CF⑦	CGAACATCCAAGTGGGCCGA
LacZ_CR⑧	CTGGCGAAAGGGGGATGTGC
pETADH1F	CGAGCGCATATGACTTCCATTCCAAAGACTCAAAAGGCC
pETADH1R	CGAGCTAAGCTTCTATTTGGAAGTGTCAAGAACGTATCTTCC
pDLGseqF	GTGATGAAAATCAGGTGGCGCACGAC
pDLGseqR	GTCATTTTACGAACCGTTGACCCCGC
HpGAPDH_F	TAGAAACAAAATGACCGCAA
HpGAPDH_R	GTAATCAGGAGCAATGAATG

AAGCTT: *Hin*dIII site, ACTAGT: *Spe*I site, CATATG: *Nde*I site. ①–⑧: Region-specific primers described in Fig. 1a

quality were determined by measuring OD_{260} and the ratio of OD_{260}/OD_{280}, respectively. Semi-quantitative RT-PCR was performed using poly T primer and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. After cDNA synthesis, expression of the *HpADH1* gene was analyzed by PCR using the primers HpADH1F and HpADH1R. The expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was analyzed as an internal standard by PCR using the primers HpGAPDH_F and HpGAPDH_R.

Ethanol production

Ethanol fermentation was assessed in both the *ADH1* deletion and complementation strains by comparison to DL1-L. The cells were grown in a 250-ml flask containing 100 ml of modified fermentation medium supplemented with 10% glucose or 10% glycerol as a sole carbon source. Cultivation was performed at 37 °C under respiro-fermentative conditions (shaking at 100 rpm) for 5 or 6 days. *Hansenula polymorpha* biomass was calculated from the OD_{660} as indicated (Kang et al. 2001), in which $OD_{660}=1$ is equivalent to 0.3 gl^{-1} of dry cell weight. Ethanol was quantified using an EnzyChrom™ ethanol assay kit (ECET-100, BioAssay Systems) according to the manufacturer's instructions.

Results

Identification of the putative *HpADH1* gene of *H. polymorpha*

In searching for putative alcohol dehydrogenase genes, close inspection of the whole genome sequence of the *H. polymorpha* DL-1 strain revealed at least seven putative ADH genes. Among them, an open reading frame of 1,047 nucleotides coding a polypeptide of 349 amino acids was designated as *HpADH1*, since the predicted amino acid sequence of this polypeptide showed strong similarity to ADH1 of *S. cerevisiae*. The nucleotide sequence of *HpADH1* gene derived from *H. polymorpha* DL-1 was submitted to GenBank under Accession No. HM105499.

HpADH1 seems to be a cytoplasmic protein based on the absence of an N-terminal mitochondrial targeting extension. Comparison of the polypeptides encoded by *HpADH1* to the ADH polypeptides of *S. cerevisiae*, *P. stipitis*, *K. lactis*, *Candida albicans*, *P. pastoris*, and *Candida utilis* confirmed considerably high amino acid sequence similarities ranging from 83% to 89%. The amino acid sequence of HpADH1 was 75% and 76% identical to *S. cerevisiae* ADH1 and ADH2, respectively. The molecular weight of HpADH1 was calculated to be 36,671 Da, which is in the same range of other yeast ADH subunits such as ScADHs (Russell et al. 1983) and KlADHs (Bucciarelli et al. 2009).

The multiple amino acid sequence alignment of HpADH1 with cytosolic ADHs from other yeasts revealed several conserved motifs (Fig. 2). Binding residues for the natural cofactor Zn^{2+} were also found. These residues are known to be essential for enzyme catalytic activity and structure (Eklund et al. 1976). Moreover, similar to other ADHs, the HpADH1 sequences showed highly conserved GA(G/A)GGLG motifs for NAD(P^{+})-binding, adenosine ribose binding, pyrophosphate binding, nicotinamide binding, substrate binding pocket, an acid-base system, and a Lys residue specific to NAD. This suggests that HpADH1 is an NAD-dependent Zn^{2+}-binding alcohol dehydrogenase.

Biochemical characterization of recombinant HpADH1

In order to investigate the biochemical properties of HpADH1, 6×His-tagged HpADH1 was overexpressed and purified from *E. coli*. The kinetic constants of HpADH1 for ethanol and acetaldehyde were determined by varying substrate concentrations with constant amounts of cofactor (NAD^+ or NADH) (Table 2). The K_m of HpADH1 for ethanol was about eightfold lower than that for acetaldehyde. Meanwhile, the turnover numbers (K_{cat}) and catalytic efficiencies (K_{cat}/K_m) for either substrate were in the same range. Compared to ADH1 and ADH2 of other yeasts, the K_m for acetaldehyde of HpADH1 was similar to those of ScADH1, KlADH1, and KlADH2, but was about 21-fold higher than that of ScADH2. Conversely, the catalytic efficiency of HpADH1 for ethanol was apparently higher than those of ScADH1, ScADH2, KlADH1, and KlADH2, due to a lower K_m and higher K_{cat}. This peculiar feature reflects diversity among ADHs and might be functionally useful for the microorganism itself.

The substrate specificities of HpADH1 were also investigated using various alcohols and aldehydes (Table 3). HpADH1 showed very high alcohol oxidation activity for medium-chain alcohols (C_2–C_5) and no ADH activity for methanol (C_1). However, the oxidation activity was slightly decreased for long-chain alcohols (C_8). For secondary alcohols, HpADH1 showed high relative activity for 2-propanol compared to that for ethanol and low relative activity for 2-pentanol or 2-octanol. Interestingly, HpADH1 was able to oxidize 2-methoxy ethanol, as well as 1,2-butanediol, even though the relative activities were comparably low. Thus, HpADH1 seems to have broad substrate specificity for alcohol oxidation. In addition, HpADH1 was able to reduce acetaldehyde very efficiently, but not formaldehyde or acetone.

Table 3 Substrate specificities of HpADH1

Substrate	Relative activity[a,b]
Primary alcohols	
Methanol	0
Ethanol	100
1-Propanol	161
1-Butanol	188
1-Pentanol	358
1-Octanol	89
Secondary alcohols	
2-Propanol	190
2-Pentanol	20
2-Octanol	78
Alcohol derivatives	
2-Methoxy ethanol	34
1,2-Butanediol	14
Aldehydes and ketone	
Acetaldehyde	1,346
Formaldehyde	8
Acetone	0

[a] Relative activity is regarded as ethanol oxidation activity equals to 100

[b] Standard error in each substrate is less than 10%

ADH isozyme analysis of *H. polymorpha* wild-type and *HpADH1* disrupted mutant

In order to understand the physiological role of HpADH1, electrophoretic patterns of the ADH isozymes of *H. polymorpha* DL1-L and a disruption mutant Δ*HpADH1* were compared. With regards to NAD^+-dependent ethanol oxidation, we observed at least four ADH isozymes expressed from the DL1-L strain cultured in glucose or ethanol media (Fig. 3a). One isozyme expressed in the wild-type strain was absent in the extracts of *ΔHpADH1*,

Table 2 Comparison of enzymatic properties between HpADH1 and those of ADH isozymes from *S. cerevisiae* and *K. lactis*

	Ethanol			Acetaldehyde		
	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m ($min^{-1}mM^{-1}$)	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m ($min^{-1}mM^{-1}$)
HpADH1[a]	0.25	2.1×10^5	8.4×10^5	1.94	2.0×10^5	1.0×10^5
ScADH1[b]	17	2.0×10^4	1.2×10^3	1.1	1.0×10^5	9.3×10^4
ScADH2[b]	0.81	7.8×10^3	9.6×10^3	0.09	6.2×10^4	6.9×10^5
KlADH1[c]	27	2.5×10^5	9.3×10^3	1.2	3.6×10^5	3.0×10^5
KlADH2[c]	23	2.8×10^4	1.2×10^3	1.7	3.3×10^4	2.0×10^4

Kinetic parameters of HpADH1 for ethanol and acetaldehyde were determined in the presence of 0.15 mM of [NAD^+] or [NADH]

[a] Standard error range up to 10%

[b] Data calculated from Ganzhorn et al. (1987)

[c] Data obtained from Bozzi et al. (1997)

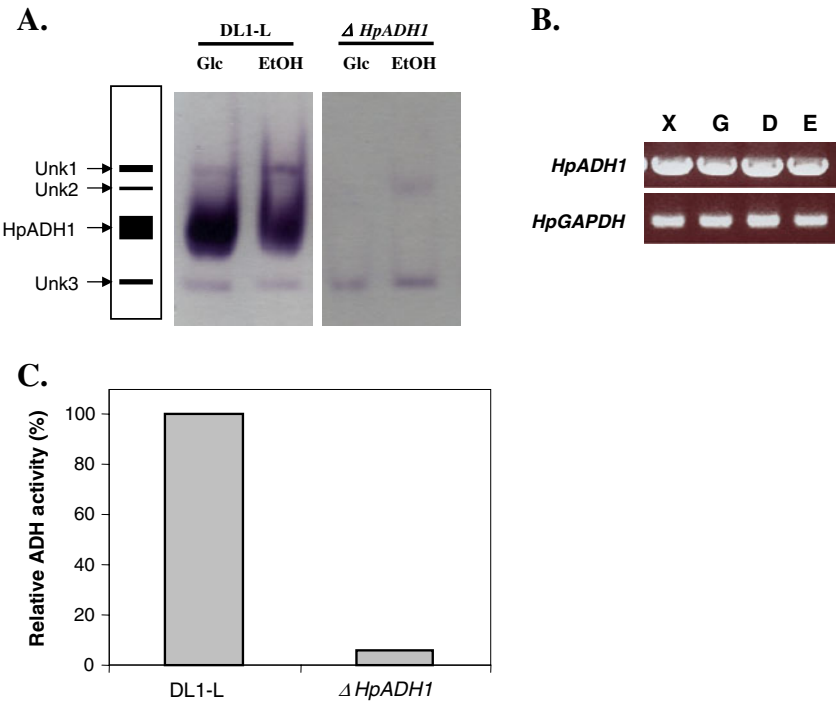

Fig. 3 Comparison of ADH expression between the *H. polymorpha* DL1-L and Δ*HpADH1* strains. **a** Isozyme patterns. Cell extracts of wild-type and Δ*HpADH1* mutant strains cultured in YPD or YPE media were separated by SDS-PAGE and stained for ADH activity. **b** RT-PCR analysis of *HpADH1* transcripts obtained from *H. polymorpha* DL1-L cells growing on YP medium containing 2% of indicated carbon source (*X* xylose, *G* glycerol, *D* glucose, *E* ethanol). A pair of primers specific to *GAPDH* was used as an internal standard. **c** Ethanol oxidation activities of protein extracts from YPD-grown wild-type and Δ*HpADH1* mutant cells

suggesting it belongs to HpADH1. HpADH1 was the most highly expressed ADH isozyme in wild-type DL1-L, indicating that it is the major ADH in the DL1-L strain under our experimental conditions. To test this, we compared ADH activities between the crude extracts of wild-type and *ΔHpADH1* mutant. *ΔHpADH1* mutant had only 6% total ADH activity compared to wild-type strain DL1-L (Fig. 3c). Furthermore, HpADH1 was constitutively expressed in both glucose and ethanol media. For other ADH isozymes, the first isozyme, namely, Unk1, was observed especially in ethanol-grown cell. The Unk2, which was located below the Unk1, can be detected in DL1-L cell growing on glucose medium. In all conditions, the last isozyme Unk3 presented at the bottom of the gel.

To further investigate the effects of different carbon sources, the expression of *HpADH1* in *H. polymorpha* DL1-L cells cultured on media containing glucose, xylose, glycerol, or ethanol as a sole carbon source was analyzed by RT-PCR using a pair of primers specific to the *HpADH1* gene (Fig. 3b). For all respirative and fermentative carbon sources tested, it was found that the *HpADH1* transcripts were uniformly expressed in accordance with the above observation. Thus, our results indicate that *HpADH1* is constitutively expressed regardless of the carbon source used.

Functional complementation of Δ*HpADH1* using pDLG-ADH1 vector

To confirm that the phenotype of *ΔHpADH1* was due to disruption of *HpADH1*, functional complementation was conducted by reintroducing the *HpADH1* gene cloned under the P_{GAPDH} promoter as the recombinant plasmid pDLG-ADH1 (Fig. 4a). The cell-free extract of pDLG-ADH1/*ΔHpADH1* strain cultured in YPD medium was measured for ADH activity. Interestingly, ADH activity of the complemented strain was about threefold higher than that of wild-type (Fig. 4b). This may suggest that the P_{GAPDH} promoter was stronger than the native promoter of the *HpADH1* gene. Even though we did not investigate this in detail, it is also possible that multiple copies of the pDLG-ADH1 plasmid were integrated into the genome.

We investigated the effect of *HpADH1* complementation on cell growth in YPD and YPE liquid media in comparison to wild-type and the deletion mutant strain (Fig. 5). In glucose medium, the growth of *ΔHpADH1* cells was significantly reduced compared to that of wild-type DL1-L cells. This phenotype was recovered by pDLG-ADH1/*ΔHpADH1* complementation. In contrast to glucose medium, all strains grew at similar rates in ethanol medium.

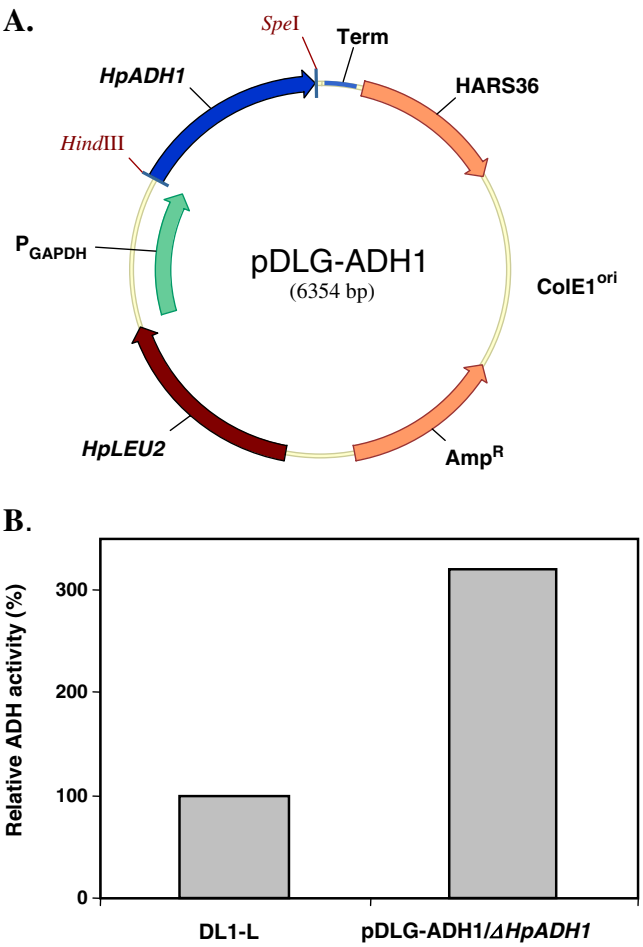

Fig. 4 Functional complementation of *ΔHpADH1* mutation. Construction of pDLG-ADH1 vector (**a**) and comparison of ADH activities of wild-type and pDLG-ADH1/*ΔHpADH1* complemented strains (**b**). The cells were grown on YPD medium until mid-log phase. Cell-free extracts were examined in vitro ADH activity as described in the "Materials and methods" section

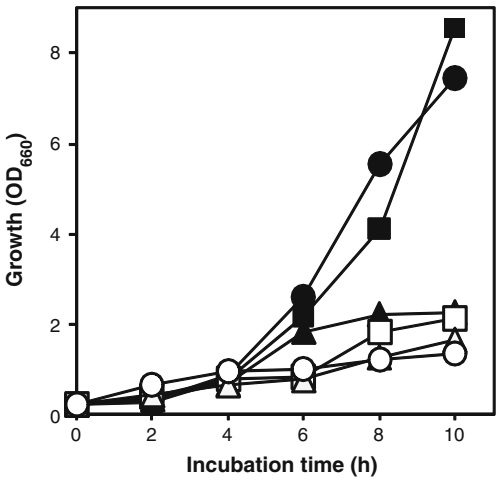

Fig. 5 Effects of ADH1 on growth of *H. polymorpha*. Cells were incubated in YPD (*closed symbols*) and YPE (*open symbols*) media at 37 °C with shaking at 180 RPM. *Squares*, *triangles*, and *circles* represent growth of DL1-L, *ΔADH1*, and pDLG-ADH1/*ΔHpADH1* complemented strains, respectively

Ethanol production from glucose and glycerol

To assess the effect of *HpADH1* on ethanol production, *H. polymorpha* DL1-L, *ΔHpADH1*, and pDLG-ADH1/*ΔHpADH1* strains were examined for the production of ethanol from glucose (Fig. 6a, b) and glycerol (Fig. 6c, d) under respiro-fermentative conditions. In glucose medium, the growth of *ΔHpADH1* was slightly retarded compared to that of *H. polymorpha* DL1-L wild-type strain, especially at the early stages of cultivation. The cell biomass of *ΔHpADH1*, however, was nearly identical to that of DL1-L on days 4–5. Growth retardation in glucose medium was not observed in the pDLG-ADH1/*ΔHpADH1* complemented strain. Conversely, the pDLG-ADH1/*ΔHpADH1* cells grew better than wild-type DL1-L cells at the beginning of fermentation, reaching maximum biomass in just 2 days. The effect of *ADH1* deletion became more evident for ethanol production. The *ΔHpADH1* mutant strain produced less than one-fourth the amount of ethanol compared to DL1-L strain. The pDLG-ADH1/*ΔHpADH1* strain accumulated the highest level of ethanol, up to 36.7 gl^{-1} at day 4. In glycerol medium, all strains showed similar growth patterns. Similar to glucose medium, only a small amount of ethanol was produced by the *ΔHpADH1* strain, whereas the ethanol productions of the DL1-L and pDLG-ADH1/*ΔHpADH1* strains were comparable.

Discussion

Alcohol fermentation by *H. polymorpha* from simple sugars such as glucose can achieve ethanol yields of up to 13 gl^{-1} at 37–40 °C (Ryabova et al. 2003). However, it has been reported that other carbon sources inhibit ethanol production by *H. polymorpha* for unknown reasons. Use of *H. polymorpha* for the production of ethanol from xylose has also been studied. However, the fermentation yield is relatively low (approximately 3 gl^{-1}). In yeast, alcohol fermentation begins with decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylase (PDC), followed by the reduction of acetaldehyde to ethanol by alcohol dehydrogenase (ADH) (Nevoigt and Stahl 1997). Overexpression of pyruvate decarboxylase results in a threefold increase in ethanol production from xylose, even though the yield is still low compared to fermentation of glucose (Ishchuk et al. 2008). In *S. cerevisiae*, two cytosolic ADH enzymes play different metabolic roles in alcohol fermentation. ADH1 (encoded by *ADH1* gene) is responsible for ethanol formation from acetaldehyde on glucose medium, whereas ADH2 (encoded by *ADH2* gene) catalyzes the opposite reaction and is repressed by glucose (Bennetzen

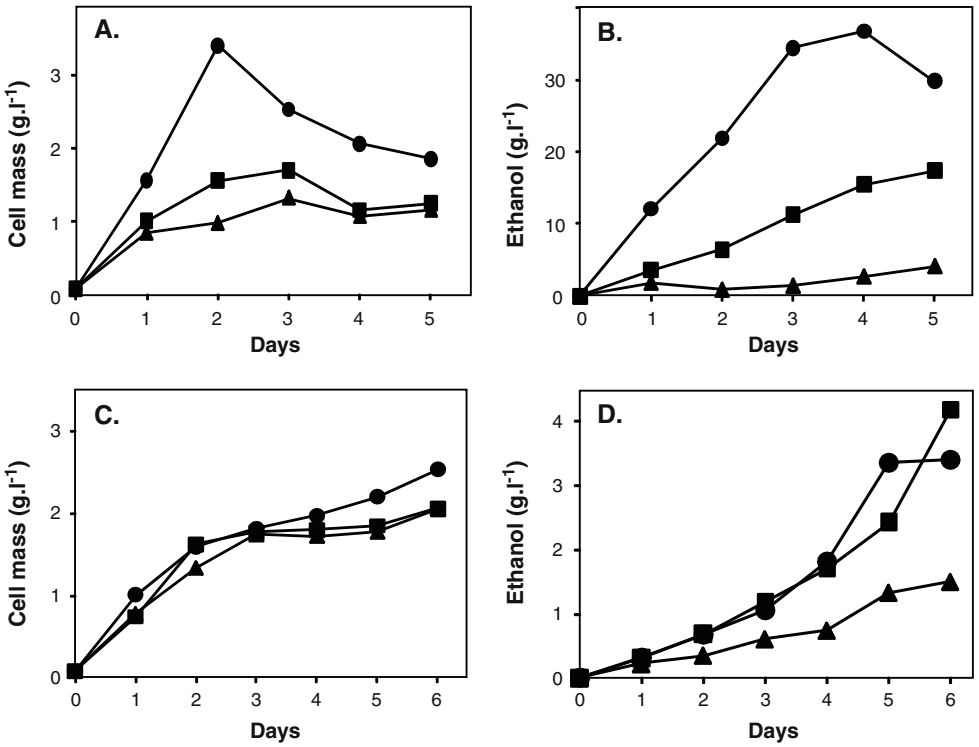

Fig. 6 Effects of deletion and complementation of *HpADH1* on cell growth and ethanol production. Cells were cultured in modified fermentation medium supplemented with drop-out supplement along with 10% glucose or 10% glycerol at 37 °C with shaking at 100 rpm. **a**, **b** Cell growth and ethanol production in medium with 10% glucose, respectively. **c**, **d** Cell growth and ethanol production in medium with 10% glycerol, respectively. The *squares*, *triangles*, and *circles* correspond to DL1-L, *ΔHpADH1*, and pDLG-ADH1/*ΔHpADH1* strains, respectively

and Hall 1982). Thus, high levels of PDC and ADH1 should be required for improved ethanol production.

Here, we have reported that *H. polymorpha ADH1* shows high amino acid sequence similarity to other cytosolic ADHs from different yeast species, such as ScADH1 and ScADH2 (de Smidt et al. 2008; Leskovac et al. 2002). To investigate the physiological role of HpADH1, a *ΔHpADH1* mutant was constructed and its growth examined on glucose and ethanol. Indeed, this enzyme seemed to play a significant role in glucose metabolism based on the observation that *ΔHpADH1* was unable to utilize glucose efficiently. Moreover, the mutant had a defect in ethanol production from glucose compared with DL1-L. Although its mRNA was detected in wild-type cultured in the ethanol medium, deletion of the *HpADH1* gene had no effect on ethanol assimilation. Therefore, HpADH1 may have acted with another isozyme in ethanol oxidation in which it was less efficient or with an isozyme with dominant activity. Similar results have been found in *P. stipitis*. Disruption of *PsADH1* results in a lower growth rate under fermentative conditions, whereas a double disruption mutant of *PsADH1* and *PsADH2* was unable to consume ethanol (Cho and Jeffries 1998). In the *H. polymorpha* DL1 genome sequence, we found at least seven putative ADH isozymes. Although their functions have not been clarified yet, one of them may be an analogue to PsADH2. Further characterization of these genes will allow us to understand the ADH system in this species. It is noteworthy that the ADH isozyme pattern of *H. polymorpha* DL1-L in this study corresponds to the isozyme pattern of methanol-grown *H. polymorpha* CBS4732 (Verduyn et al. 1988), while also being slightly different from that of xylose-grown *H. polymorpha* NCYC495 (Ishchuk et al. 2008). In the latter case, there is one additional ADH isozyme, most likely in response to another carbon source such as xylose. Thus, it is necessary to employ comparative genomic studies to fully understand the composition and regulation of ADHs in different strains of *H. polymorpha*.

Furthermore, ADH isozyme analysis of the DL1-L strain revealed an HpADH1 band uniformly abundant in both glucose and ethanol media. Cell-free extract of *ΔHpADH1* had only 6% residual ADH activity. Thus, it is unlikely that any ADH isozyme fully took the place of HpADH1. Therefore, this gene was classified as an ADH family class I gene based on nomenclature and its physiological similarity to ScADH1 and PsADH1. Further biochemical studies on HpADH1 revealed that its K_m for acetaldehyde is similar to that of ScADH1, while its K_m for ethanol is nearly the same as that of ScADH2. Moreover, the catalytic efficiencies for both ethanol oxidation and acetaldehyde reduction were comparable. These data might relate to the constitutive expression of the gene. In addition, HpADH1 preferentially acted on linear primary alcohols rather than branched chain alcohols. For example, high oxidative activities were found using butanol and pentanol as substrates. HpADH1 also displays some activity using long-chain alcohols like octanol but no activity using methanol. Our results are similar to the substrate specificity of native ADH prepared from methanol-grown cells of *H. polymorpha* CBS4732 (Verduyn et al.

1988). In that case, only a low K_m ADH isozyme for ethanol was presented. Compared to ScADH1, HpADH1 apparently showed broader substrate specificity. This is consistent with the presence of Lys-270 (ScADH1 numbering) in place of Met-270, which reportedly restricts accessibility of the enzyme to the substrate (Ganzhorn et al. 1987; Green et al. 1993).

The increasing demand of ethanol-containing fuels has resulted in substantial growth of the ethanol-manufacturing industry. In response, engineering of *H. polymorpha* to produce ethanol from glucose and xylose has previously been performed (Ishchuk et al. 2008; Ryabova et al. 2003). In this study, a pDLG-ADH1/*ΔHpADH1* strain without any further optimization substantially improved ethanol production from glucose and glycerol. To meet increasing energy demands, glycerol fermentation to ethanol has become more feasible since it is a co-product of biodiesel production. Glycerol fermentation has been previously investigated in *Enterobacter aerogenes* HU-101 (Ito et al. 2005) and *E. coli* (Dharmadi et al. 2006). Alcohol fermentation by yeast is still favorable since it is more tolerant to ethanol and resistant to virus infection (Jeffries and Jin 2000). Ethanol yield from glycerol by *H. polymorpha* DL1-L, as demonstrated in this study, was about 4 $g l^{-1}$, which is comparable with that previously reported by *E. coli* (4 $g l^{-1}$) (Murarka et al. 2008). The process, however, requires many optimizations and improvements prior to the commercial application. The low level of ethanol formed in glycerol fermentation might be because of production of other metabolites such as acetate, lactate, and butyrate (Paulo da Silva et al. 2009). Further study about all by-products formed during glycerol fermentation in *H. polymorpha* should be performed. *H. polymorpha* is therefore a promising biofuel cell factory for use in industrial operations due to its intrinsic resistance to various harsh environmental conditions.

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