APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 4079–4082 Vol. 63, No. 10
0099-2240/97/$04.00+0

Purification and Characterization of a Novel NADP-Dependent Branched-Chain Alcohol Dehydrogenase from *Saccharomyces cerevisiae*

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Received 18 February 1997/Accepted 16 July 1997

An NADP-dependent branched-chain alcohol dehydrogenase was purified from *Saccharomyces cerevisiae* var. *uvarum* grown under anaerobic conditions. Its quaternary structure is monomeric, and it has a molecular mass of 37 kDa and a pI of 5.9. A possible role of the enzyme in flavor production during alcoholic fermentation is discussed.

A large number of different compounds contribute to the flavor of alcoholic beverages. Among these, aldehydes and alcohols have a high impact, caused by low flavor threshold values and high concentrations, respectively. Generally, aldehydes are considered off-flavors, but fortunately their presence is limited due to conversion by alcohol dehydrogenases (ADHs) of *Saccharomyces cerevisiae*. ADHs present in yeast belong to the group of nicotinamide dinucleotide-utilizing oxidoreductases (EC 1.1.1). In *S. cerevisiae*, so far four isoenzymes of ADH have been purified and characterized. All of the isoenzymes are NAD dependent, and their respective structural genes have been cloned and sequenced (1, 7, 9, 13, 14).

In alcoholic fermentation, the activity of the ADHs is not limited to acetaldehyde. Several so-called Strecker aldehydes, formed by transamination and subsequent decarboxylation of amino acids, are reduced to fusel alcohols. The final levels of these alcohols are determined by environmental factors, e.g., oxygen, temperature, substrate, and amino acids, as well as strain characteristics (11). During the production of alcohol-free beer, anaerobic conditions are used and the temperature is kept low, suppressing yeast growth and fermentation (16). ADH1 was found not to be involved in the reduction of branched-chain and higher aldehydes, since no correlation was found between the reduction of these compounds and the activity of this enzyme (2). Here, we report the purification and characterization of a novel NADP-dependent branched-chain ADH (bcADH), from a brewer's yeast strain of *Saccharomyces cerevisiae*, which is expressed under anaerobic growth conditions.

Expression of ADH activity. *S. cerevisiae* var. *uvarum* W34 was obtained from the Institute of Weihenstephan, Technical University of Munich, Munich, Germany. Cells were grown in MYGP (malt extract, 3 g liter^{-1}; yeast extract, 3 g liter^{-1}; mycological peptone, 5 g liter^{-1} and glucose, 10 g liter^{-1}) at 25°C on a rotary incubator (170 rpm), and ergosterol and Tween 80 were added under anaerobic conditions (17). In cell extract (CE) from strain W34 grown under anaerobic conditions, a significant increase was observed in NADPH-dependent aldehyde reductase activity, compared to that in CE obtained from cells grown under aerobic conditions (12). The CEs of late-exponential phase aerobic and anaerobic cells were therefore separated on a fast-performance liquid chromatography (FPLC) Mono-Q column (HR-5/5), by using a linear gradient of 0 to 200 mM NaCl in 20 mM BisTris (bis[2-hydroxyethyl]imino-Tris, pH 7)–0.1 mM $MgCl_2$–2 mM dithiothreitol (DTT) (Fig. 1). Measuring the reduction of 3-methylbutanal with CE of aerobically grown cells, we found one activity peak with each coenzyme (Fig. 1A). In cells grown under anaerobic conditions, however, two activity peaks with each coenzyme were found; the first eluted at approximately 50 mM NaCl, and the second eluted at 100 mM NaCl (Fig. 1B).

The reduction of hexanal was also tested (Fig. 1C and D). For each cofactor, one peak was found in the CEs of aerobic cells (Fig. 1C), and in the CEs of anaerobic cells for each coenzyme two peaks appeared (Fig. 1D). In contrast to the activity with 3-methylbutanal, the activity peaks of NADH-driven reduction with hexanal did not coincide with peaks of NADPH-driven reduction. Similar results were obtained with acetaldehyde (data not shown).

These results suggest that different types of ADH were present in the CE of the yeast, depending on the growth conditions. Apparently, the increased aldehyde reductase activity in CEs of anaerobically grown cells was related to the appearance of the additional peak in the Mono-Q chromatogram showing 3-methylbutanal reductase activity. Subsequently, we aimed to purify this enzyme since it may play a role in flavor formation under anaerobic conditions, i.e., during alcoholic fermentation.

Enzyme purification. Cells were broken by four passages through a French press (600 kg cm^{-2}). After cell debris was removed by centrifugation (30,000 × *g*, 40 min), the ADH present in the second activity peak of anaerobic CE (at 100 mM NaCl) was purified in a three-step procedure (Table 1). During purification, samples were analyzed for enzyme activity by measuring the decrease in NAD(P)H fluorescence. Already the first step (Q-Sepharose column calibrated with 20 mM BisTris [pH 7], 0.1 mM $MgCl_2$, 2 mM DTT) resulted in a high purification factor, owing to the relatively high concentration of NaCl at which this ADH eluted. Subsequently, $(NH_4)_2SO_4$ was added to a final concentration of 1 M and the sample was applied to a phenyl Sepharose column (2.6 cm by 10 cm;

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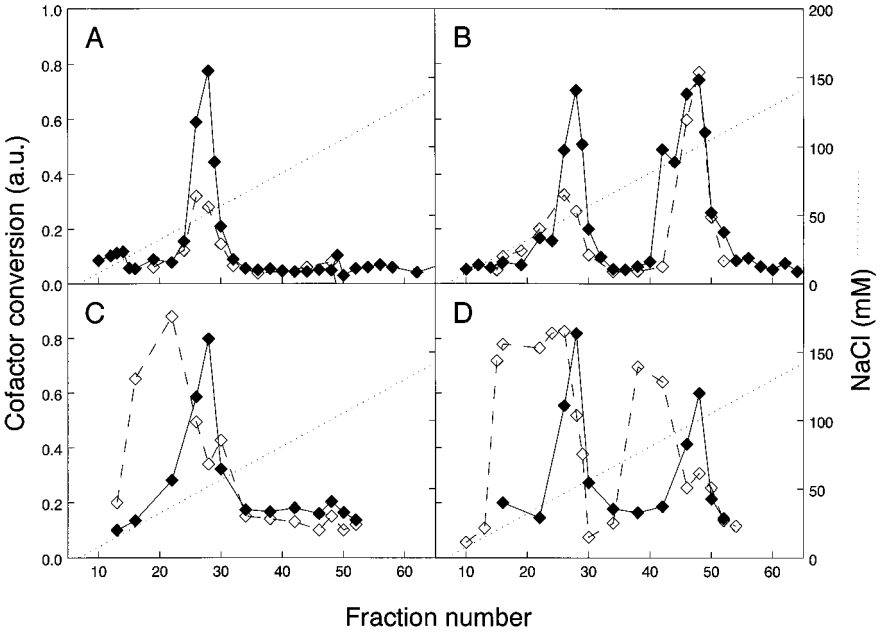

FIG. 1. Reduction of 3-methylbutanal (A and B) or hexanal (C and D) with fractions (1 ml) of CE from *S. cerevisiae* W34 obtained after ion-exchange chromatography on an FPLC Mono-Q column (HR-5/5). CEs were obtained from cells grown under aerobic (A and C) or anaerobic (B and D) conditions. Oxidation of NADPH (◆) or NADH (◇) was analyzed fluorimetrically. a.u., arbitrary units.

Pharmacia, Uppsala, Sweden). Elution occurred in BisTris buffer without salt. After dialysis against 20 mM BisTris (pH 8.0), 0.5 mM DTT, and 5 mM $MgCl_2$, the sample was applied to a procion red dye affinity column (Red Agarose; Amicon Inc., Beverly, Mass.), which was selected based on its capacity to bind to a wide range of NADP-dependent enzymes. The enzyme was eluted with 0.2 mM $NADP^+$ in the same buffer, and cofactor was removed by three successive dilution and ultrafiltration steps. With this column, a high degree of purification was obtained (Table 1). No rationale was found for the low yield of this step, since reductase activity was absent in fractions obtained during or shortly after loading of the column and after washing the affinity column with a high concentration of salt (1 M NaCl). Nevertheless, the high purification fold indicates that the total amount of enzyme present in the CE was low; it is estimated to be approximately 0.03%. The ratio of the activity with NADH to that with NADPH on 3-methylbutanal remained more or less constant during the purification (Table 1), which suggests that one specific type of ADH was being purified.

TABLE 1. Purification of ADH expressed under anaerobic conditions in *S. cerevisiae* W34

Purification step	Total protein (mg)	Total activity (U)[a,b]	Sp act (U/mg of protein)[b]	Yield (%)[b]	Fold purification[b]
CE	10,100	159 (366)	0.016 (0.036)	100 (100)	1 (1)
Q-Sepharose	147	150 (226)	1.02 (1.54)	94 (62)	65 (43)
Phenyl Sepharose	60	112 (133)	1.86 (2.21)	70 (36)	118 (61)
Red agarose	0.46	28 (43)	61.6 (93.9)	17 (12)	3,920 (2,593)

[a] Reduction of 3-methylbutanal was measured at pH 6.7 with NADPH as the coenzyme.

[b] Numbers in parentheses are based on the activities with the coenzyme NADH.

Structural properties. Estimation of the molecular mass of the purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) as well as by gel filtration on an FPLC Superose-12 column (HR-10/30) (data not shown) revealed an apparent molecular mass of 37 kDa. Therefore, it is presumed that no association of subunits occurs, in contrast to other yeast isoenzymes, which function as di- or tetramers (1, 6, 13). Isoelectric focusing of the purified enzyme revealed a single band with a pI of 5.9. In order to determine the N-terminal amino acid sequence, 300 pmol of purified protein was blotted onto a polyvinylidene difluoride membrane. However, the results of the Edman degradation were negative, presumably due to blocking of the N terminus.

Coenzyme specificity. To determine the coenzyme specificity in more detail, Michaelis constants for both NADH and NADPH were determined in the reduction with 3-methylbutanal. Kinetic parameters were determined with a model U-3000 spectrometer (Hitachi Ltd., Tokyo, Japan). The affinity of

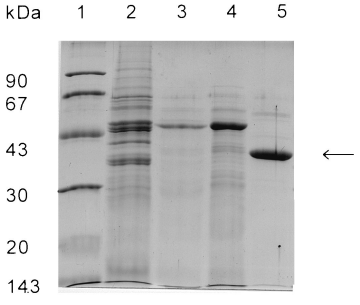

FIG. 2. Analysis of enzyme purification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: 1, marker; 2, CE; 3, Q-Sepharose pool; 4, phenyl Sepharose pool; 5, red agarose pool. Each lane contains approximately 3 μg of protein, except lane 2, to which approximately 10 μg was loaded.

TABLE 2. Kinetic parameters for purified bcADH from *S. cerevisiae* W34[a]

Substrate	NADPH				NADH			
	K'_m (mM)	K'_i (mM)	k'_{cat} (s^{-1})	k'_{cat}/K'_m ($M^{-1}s^{-1}$)	K'_m (mM)	K'_i (mM))	k'_{cat} (s^{-1})	k'_{cat}/K'_m ($M^{-1}s^{-1}$)
Acetaldehyde	158	ND[b]	5.6	36	ND	ND	ND	ND
Propanal	38.9	46.6	55	1.4×10^3	27	ND	84.0	306
Butanal	2.76	16.6	57	2.1×10^4	23.1	ND	47.3	2.1×10^3
2-Methylbutanal	1.85	2.74	113	6.1×10^4	17.7	4.61	92.3	5.2×10^3
3-Methylbutanal	0.21	7.14	74.9	3.6×10^5	1.89	253	91.8	4.9×10^4
Pentanal	0.16	3.90	56.6	3.6×10^5	3.01	13.9	81.6	2.7×10^4
Hexanal	0.18	0.79	71.1	4.0×10^5	0.83	2.80	50.1	6.1×10^4
Heptanal	0.27	0.88	72.9	2.7×10^5	4.25	1.17	101	2.4×10^4
Hexanol	1.22	ND	2.55	2.1×10^3	—[c]	—	—	—
t-2-Hexenol	1.69	ND	14.0	8.3×10^3	—	—	—	—

[a] Apparent values (K'_m, K'_i, and k'_{cat}) for different substrates were determined with 0.15 mM NADPH or 0.15 mM NADH.
[b] ND, not detectable.
[c] —, not performed.

the enzyme was highest for the phosphorylated coenzyme (K'_m = 20 μM), whereas that for NADH was approximately 80-fold lower (K'_m = 1.7 mM). In addition, reduction experiments were performed in the presence of both reduced and oxidized coenzymes. Hardly any effect on the oxidation of NADPH (0.15 mM) was observed when an excess of NAD^+ or $NADP^+$ was added. However, similar concentrations of NAD^+ or $NADP^+$ showed a severe effect on a reduction assay with NADH (0.15 mM); a 6.7-fold excess of $NADP^+$ completely inhibited this reaction, suggesting competition for the same binding site.

Substrate specificity. Substrate specificity of the purified ADH was elucidated by measuring the rates of aldehyde reduction and alcohol oxidation. Aldehydes or alcohols were solubilized in buffer by sonication, by a method adapted from that of Wales and Fewson (18). Reduction assays were performed at 30°C in a buffer containing 30 mM (each) MES (morpholineethanesulfonic acid), MOPS (morpholinepropanesulfonic acid), and Tris (MMT) (pH 6.7; ionic strength [*I*] = 0.03 M), 0.15 mM NAD(P)H, and enzyme. Oxidation of alcohols was performed in 20 mM CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid, pH 10), and 1 mM $NAD(P)^+$. Reactions were started by addition of 10 mM aliphatic substrate.

Kinetic measurements revealed substrate inhibition at high concentrations of aldehydes. In order to calculate kinetic parameters, a modified Michaelis-Menten equation was used as described by Dixon and Webb (5): $v = V'_{max}/(1 + [K'_m/S] + [S/K'_i])$, where v is the initial rate (per second) at substrate concentration S (millimolar), K'_m is the apparent Michaelis-Menten constant, K'_i is the apparent substrate inhibition constant, and V'_{max} is the apparent reaction rate at saturating substrate concentration. Application of this equation on a two-substrate reaction is allowed at saturating coenzyme concentrations. When fitting the equation to the observed data, excellent correlation was found (generally $r^2 > 0.99$).

Table 2 shows that both K'_m and K'_i decreased with increasing chain length. The catalytic efficiency (k'_{cat}/K'_m, where k'_{cat} is the catalytic rate constant) was highest with hexanal, whereas with 3-methylbutanal, the highest ratio of K'_m/K'_i was observed, indicating that this is the better substrate. Kinetic analysis of alcohol oxidation (hexanol and *t*-2-hexenol) revealed a significantly lower catalytic efficiency of the enzyme. The enzyme was also highly active towards aromatic aldehydes such as benzaldehyde, *p*-anisaldehyde, and furaldehyde, which had 20 to 50% of the activity observed with 3-methylbutanal. No reduction was observed with ketones, pyruvate, and glucose, and with vicinal diketones such as diacetyl and 2,3-pentanedione only a very low activity was observed, approximately 5% of the activity with 3-methylbutanal (data not shown).

Effect of pH and ionic strength. Ionic strength had a significant influence on enzyme activity. Increasing the ionic strength of the standard assay (I = 0.03 M) by adding NaCl, KCl, or Na_2SO_4 increased the rate of reduction with NADPH, whereas the activity with NADH was severely inhibited. Concentrations of 200 mM KCl or NaCl increased activity with NADPH five- to sixfold and decreased NADH activity by the same order of magnitude.

Reducing activity was tested at a wide pH range. As ionic strength influences activity, conditions with high (I = 0.1 M) as well as low (I = 0.01 M) ionic strengths were used (Fig. 3). At a high ionic strength, NADPH-dependent activity was high over a broad pH range (6.5 to approx. 8.5) and at a lower ionic strength, it was decreased over the entire pH interval; at the latter strength an optimum pH of 8.5 was observed (Fig. 3A). The reverse effect was observed with NADH (Fig. 3B). Here, activity was highest at a low ionic strength, with an optimum between pH 6 and 7. Since the pK_a of the 2′-phosphate of NADPH is 6.1 in solution (4), deprotonation of this phosphate

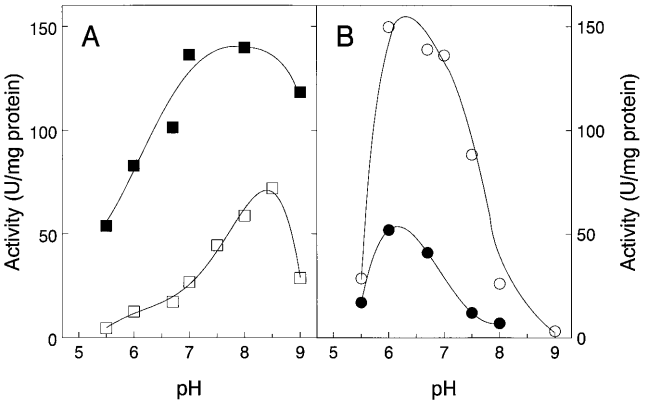

FIG. 3. Relative activity of purified ADH at various pH values. Reduction of 3-methylbutanal at a low ionic strength, I = 0.01 M (□, ○), and at a high ionic strength, I = 0.1 M (■, ●). Reductions with NADPH (A) and with NADH (B) are shown. Experiments were performed in 10 mM (each) MES, MOPS, and Tris. KCl was used to increase the ionic strength.

group may facilitate binding, explaining the relatively high pH optimum observed for the reaction with NADPH.

Enzyme properties. The results show that the purified NADP-dependent ADH has a preference for long and branched-chain substrates with up to seven carbon atoms. The increase in catalytic efficiency with substrates of increasing chain length is opposite to results reported for ADH1 to ADH4 (3, 9, 10, 19, 20). The bcADH is also different from the constitutively expressed ADH described by Wales and Fewson (18), since the M_r (37 versus 46 kDa), coenzyme specificity (NADH-to-NAD^+ conversion was reported not to occur), optimum pH, and regulation of expression differ. In addition, we observed a remarkable influence of ionic strength on the enzyme activity that had not been reported previously. Substrate and coenzyme preferences, combined with properties such as low pI and monomeric structure, indicate that we purified a novel NADP-dependent bcADH.

Physiological function. During sugar fermentation in rich media, fusel alcohol concentrations generally reach values well above the flavor threshold value (11). At 30°C, the estimated production rate of 3-methylbutanol during fermentation reaches values up to 0.6 μmol min^{-1} g^{-1} (dry weight) (12). With a soluble protein concentration of about 200 mg g^{-1} (dry weight) (i.e., half of the total protein), the minimal specific activity for the NADP-dependent ADH must be at least 3 mU mg of $protein^{-1}$. Since we measured 16 mU mg of $protein^{-1}$, the bcADH could easily account for the entire production of 3-methylbutanol during fermentation.

The physiological role of the bcADH is not known. A possible function may be that of maintaining the $NADP^+$-NADPH balance. No transhydrogenases have been found in *S. cerevisiae*, and the organism uses different strategies to maintain a proper redox balance (8). Under aerobic conditions, NADH and NADPH can be oxidized by mitochondrial respiration. Under anaerobic conditions, the proper NAD^+-NADH balance is maintained by glycerol production at the expense of ATP. Overproduction of NADPH by the pentose phosphate pathway (PPP) can only be compensated for by NADPH-linked reductions (15). The purified enzyme may therefore play an important role in the regeneration of $NADP^+$. Estimating the flux through the PPP during respiratory fermentation of glucose on a rich medium, Gancedo and Serrano (8) calculated a flux of 2.6 μmol of hexose monomers g^{-1} min^{-1}, resulting in approximately 15 μmol of NADPH g^{-1} min^{-1}. Thus, a small overproduction in the PPP already ensures sufficient NADPH for the reduction of branched-chain aldehydes.

Summarizing, a novel NADP-dependent bcADH which is expressed under anaerobic conditions was purified from *S. cerevisiae*. The enzyme may have a significant influence on flavor formation during alcoholic fermentation.

We thank Birgit Hasenack, Ben van den Broek, and Ellen Post for assistance during enzyme purification.

REFERENCES

1. **Brändén, C., H. Jörnvall, H. Eklund, and B. Furugren.** 1975. Alcohol dehydrogenases, p. 103–190. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. XI. Academic Press, London, United Kingdom.
2. **Collin, S., M. Montesinos, E. Meersman, W. Swinkels, and J. P. Dufour.** 1991. Yeast dehydrogenase activities in relation to carbonyl compounds removal from wort and beer. Proc. Eur. Brew. Conv. **23:**409–416.
3. **Dalziel, K.** 1975. Kinetics and mechanisms of nicotinamide-nucleotide-linked dehydrogenases, p. 1–60. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. XI Academic Press, London, United Kingdom.
4. **Dawson, R. M. C., D. C. Elliot, W. H. Elliot, and K. M. Jones.** 1986. Data for biochemical research, 3rd ed. Oxford University Press, London, United Kingdom.
5. **Dixon, M., and E. C. Webb.** 1979. Enzymes, 3rd ed., p. 126–138. Longman, London, United Kingdom.
6. **Drewke, C., and M. Ciriacy.** 1988. Overexpression, purification and properties of alcohol dehydrogenase IV from *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **950:**54–60.
7. **Feldmann, H., et al.** 1994. Complete DNA sequence of yeast chromosome II. EMBO J. **13:**5795–5809.
8. **Gancedo, C., and R. Serrano.** 1989. Energy-yielding metabolism, p. 205–259. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts 2nd ed., vol. 3. Metabolism and physiology of yeasts. Academic Press, London, United Kingdom.
9. **Ganzhorn, A. J., D. W. Green, A. D. Hershey, R. M. Gould, and B. V. Plapp.** 1987. Kinetic characterization of yeast alcohol dehydrogenases. Amino acid residue 294 and substrate specificity. J. Biol. Chem. **262:**3754–3761.
10. **Green, D. W., H.-W. Sun, and B. V. Plapp.** 1993. Inversion of substrate specificity of yeast alcohol dehydrogenase. J. Biol. Chem. **268:**7792–7798.
11. **Hammond, J. R. M.** 1993. Brewer's yeast, p. 7–67. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, 2nd ed., vol. 5. Yeast technology. Academic Press, London, United Kingdom.
12. **Post, E., and M. F. M. van Iersel.** Unpublished data.
13. **Reid, M. F., and C. A. Fewson.** 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. **20:**13–56.
14. **Sund, H., and H. Theorell.** 1963. Alcohol dehydrogenases, p. 25–83. *In* P. D. Boyer, H. Lardy, and K. Myrbäck (ed.), The enzymes, 2nd ed., vol. 7. Academic Press, London, United Kingdom.
15. **Van Dijken, J. P., and W. A. Scheffers.** 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. **32:**199–224.
16. **Van Iersel, M. F. M., E. Meersman, W. Swinkels, T. Abee, and F. M. Rombouts.** 1995. Continuous production of non-alcohol beer by immobilized yeast at low temperature. J. Ind. Microbiol. **14:**495–501.
17. **Visser, W., W. A. Scheffers, W. H. Batenburg-van der Vegte, and J. P. van Dijken.** 1990. Oxygen requirements of yeasts. Appl. Environ. Microbiol. **56:**3785–3792.
18. **Wales, M. R., and C. A. Fewson.** 1994. NADP-dependent alcohol dehydrogenases in bacteria and yeast—purification and partial characterization of the enzymes from *Acinetobacter calcoaceticus* and *Saccharomyces cerevisiae*. Microbiology **140:**173–183.
19. **Weinhold, E. G., and S. A. Benner.** 1995. Engineering yeast alcohol dehydrogenase. Replacing Trp54 by Leu broadens substrate specificity. Prot. Eng. **8:**457–461.
20. **Williamson, V. M., and C. E. Paquin.** 1987. Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. Mol. Gen. Genet. **209:**374–381.