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Expression, Purification and Functional Characterization of Two Recombinant Malate Dehydrogenases from *Mortierella isabellina*[1]

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Abstract—To study the characteristics of two malate dehydrogenases (MDHs), their coding genes *MIMDH1* and *MIMDH2* were cloned and expressed in *Escherichia coli* BL21 cells. The molecular weights of both recombinant enzymes partially purified using Ni-NTA affinity chromatography were 32 kDa, and the specific activities of purified MIMDH1 and MIMDH2 were 329.3 and 241.3 U/mg protein, respectively. The optimal temperatures for MIMDH1 and MIMDH2 activities were 55 and 30°C, respectively, with 70% MIMDH1 activity and ≥70% MIMDH2 activity remaining after 30 min incubation at 45°C. Addition of 2 mM Zn^{2+} enhanced MIMDH1 activity, whereas the addition of other metal ions resulted in different degrees of inhibition. The inhibitory effect of Co^{2+} was most pronounced on MIMDH1, reaching 82.8% inhibition. Addition of 2 mM Ba^{2+} or Mn^{2+} increased MIMDH2 activity, the addition of other metal ions resulted in different degrees of inhibition. Furthermore, MIMDH1 was stable at pH range of 7.5–8.5, with optimal activity observed at pH of 8.0. MIMDH2 showed similar stability at the same pH range, but was optimal at pH of 7.5. The V_{max} and K_M values for recombinant MIMDH1 and MIMDH2 catalyzing the reduction of oxaloacetate to malate were 17.66 μmol $mg^{-1}min^{-1}$ and 0.541 mM, 15.59 μmol $mg^{-1}min^{-1}$ and 0.683 mM, respectively.

Keywords: Mortierella isabellina, malate dehydrogenase, recombinant enzyme, purification, V_{max}, K_M

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Malate dehydrogenase (EC 1.1.1.37) (MDH) catalyzes the interconversion of malate and oxaloacetate using the NAD/NADH coenzyme system in the tricarboxylic acid (TCA) cycle. Several MDH have been identified, exhibiting differences in their subcellular localization and specificity for the coenzyme NAD^+ or NADP. There are two major forms of MDH, cytosolic MDH (cyMDH) and mitochondrial MDH (mMDH), in most eukaryotic cells [1]. cyMDH catalyzes the conversion of oxaloacetate to malate, which forms pyruvate following catalysis by malic enzymes to generate NADPH. The resultant NADPH is then used for polyunsaturated fatty acid (PUFA) biosynthesis [2, 3]. Additionally, Mühlroth et al. [3] described TCA-cycle genes as exhibiting high degrees of co-expression with fatty acid metabolism-related genes. mMDH converts oxaloacetate into pyruvate in the mitochondrion or cytosol, followed by pyruvate being used for lipid synthesis [4, 5]. MDHs are involved in stress resistance in plants. A gene encoding an MDH from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* [Lour] Olsson) transformed into *Arabidopsis* increased the tolerance to aluminum stress [6]. Furthermore, overexpression of nodule-enhanced MDH from alfalfa nodules and cytosolic MDH from *Arabidopsis thaliana* in transgenic alfalfa and tobacco enhanced citrate, oxalate, malate, succinate, acetate or malate synthesis and aluminum tolerance [7, 8]. However, little is known concerning MDH functions in microbes.

Mortierella isabellina can produce large amounts of high-value PUFAs. Microorganisms usually increase PUFA content to adapt to low environmental temperatures by increasing membrane fluidity [9–11]. Our previous studies showed that *M. isabellina* M6-22 is capable of growth at ≥5°C; however, the cold-adaption mechanisms in this strain remain unknown. Our previous transcriptome analysis also showed that the mRNA expression levels of some genes potentially coding for MDH increased significantly at low temperatures, indicating that these genes in this microorganism may be associated with the adaptability to cold stress [12]. Malate dehydrogenase activity is connected with lipogenesis [2], and lipids are involved not only in resistance to low temperatures but also in respiration of cells after their damages after freezing [13]. Thus, the study of malate dehydrogenase activity is actual. The aim of the study was to clone the genes

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encoding MDH from filamentous fungus *M. isabellina* M6-22 and to characterize the recombinant MDHs, including kinetic parameters and physicochemical properties.

MATERIALS AND METHODS

Strains and culture conditions. *M. isabellina* M6-22 was kindly presented by Prof. Mingchun Li at the Nankai University in Tianjin, China. The fungus was cultured at 28°C in liquid medium containing (g/L): glucose—2.0, yeast extract—10.0, KH_2PO_4—2.0 and $MgSO_4$—1.0 (pH 6.0). *Escherichia coli* Trans1-T1 (TransGen. Biotech., China) was used for gene cloning. Vector pET-32a (+) and *E. coli* BL21 (DE3) (Invitrogen, USA) were used for recombinant protein expression.

***MIMDH1* and *MIMDH1* cloning.** Total RNA was isolated from *M. isabellina* M6-22 using TRIzol (Thermo Fisher Scientific, USA) according to the manufacture's instruction and DNase I (Promega, USA) was used to remove residual genomic DNA contaminants in the RNA. cDNA was synthesized using M-MLV reverse transcriptase (Thermo Fisher Scientific, USA).

MIMDH1 and *MIMDH1* fragments were obtained by PCR using the cDNA as the template. The primers used for the amplification were MIMDHF1 (5'-CGCGGATCCATGGTCAAAGTTACAGTTTGCGG-3') and MIMDHR1 (5'-CCGCTCGAGCAACTTGGCATCAGTGATGAAAG-3'), MIMDHF2 (5'-CGCGGATCCATGTTTGCCGCTACCCGTG-3') and MIMDHR2 (5'-CCGCTCGAGAGCCTTGACGAAAGACTTTCC-3'). Both fragments were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced (Sangon Biotech, China). After verification, both fragments were digested with restriction enzymes *Bam*H I and *Xho* I and subcloned into the *Bam*H I and *Xho* I sites of the pET-32a (+) expression vector to generate pET32a*MIMDH1*and pET32a*MIMDH2*, respectively. The insertion of both genes was also verified by enzyme analysis and sequencing.

Heterologous expression and protein purification. The pET32a*MIMDH1* and pET32a*MIMDH2* vectors were transformed into *E. coli* BL21 cells for expression. Positive clones were incubated at 37°C in Luria-Bertani liquid medium (Sangon Biotech, China) to an OD_{600} of 0.8, followed by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside at 16°C with shaking at 80 rpm for an additional 12 h. Cells were collected, resuspended in 50 mM K-phosphate buffer (pH 7.5), sonicated repeatedly 4 s with an interval of 3 s for up to 8 min on ice (25% power, Sonic Vibra-cell Model VCX 750, USA), and centrifuged at 10000 *g* for 15 min at 4°C. After filtration through a 0.22 μm filter (Millipore, USA), the resultant supernatant was used for recombinant protein purification using Ni-NTA affinity chromatography (HiTrap chelating HP column, 1 mL; GE Healthcare, UK) by elution with 150 mM imidazole for MIMDH1 and 200 mM imidazole for MIMDH2. SDS-PAGE was used to determine the purity of the recombinant proteins.

Activity analysis of recombinant MIMDH1 and MIMDH2. Malate dehydrogenase activity was examined by measurement of the NADH-consumption rate according to absorbance at 340 nm and based on methods described by Pines et al. [14] and Maloney et al. [15], with some modifications. The assay was performed in 50 mM K-phosphate buffer (pH 7.5) containing 140 μM NADH, 0.75 mM oxaloacetate, and 20 μL enzyme solution at a final volume of 3 mL. MIMDH1 and MIMDH2 enzyme activity units refer to the amount of enzyme required to oxidize 1 μM NADH per min at 28 and 30°C, respectively. To determine the maximum reaction rate, the OD_{340} was measured every 4 min for up to 32 min, followed by stopping the reaction.

Effects of temperature, pH, and metal ions on enzyme activity and stability. The optimal temperature was determined by assaying enzyme activity under standard assay conditions at different temperatures (15–75°C). Thermostability was determined by measuring the residual activity following pre-incubation of the enzyme at 30, 40, 50, and 60°C for 60 min. Assays were performed in triplicate.

To determine the pH for optimal enzyme activity, MIMDH1 and MIMDH2 activities were assayed at a pH range from 6.0 to 10.0. Buffers (50 mM) were prepared at various pH levels, and to determine pH stability, the recombinant enzymes underwent a 10-h preincubation in the appropriate buffers at the same ionic concentrations and different pH values at 4°C. Samples were taken at regular time intervals, and activity assays were performed according to the standard assay protocol.

Effects of different detergents and metal ions on recombinant enzyme stability were determined in 50 mM phosphate buffer (pH 7.5). The reactions contained Ba^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, or EDTA taken in 2 mM concentration. The remaining activity was determined under standard conditions. The activity of MIMDH1 and MIMDH2 in the absence of metal ions or EDTA was used as the 100% standard. Assays were performed in triplicate.

To evaluate the effect of malate and pyruvate on MIMDH1 and MIMDH2 activities, 0.375, 0.75, or 1.5 mM malate and pyruvate were added to the reaction buffer to determine their inhibitory effect on enzyme activity. Assays were performed in triplicate.

RESULTS AND DISCUSSION

Determination of the *MIMDH1* and *MIMDH2* sequences. MDHs exist in a wide variety of prokaryotic and eukaryotic organisms, representing one of the

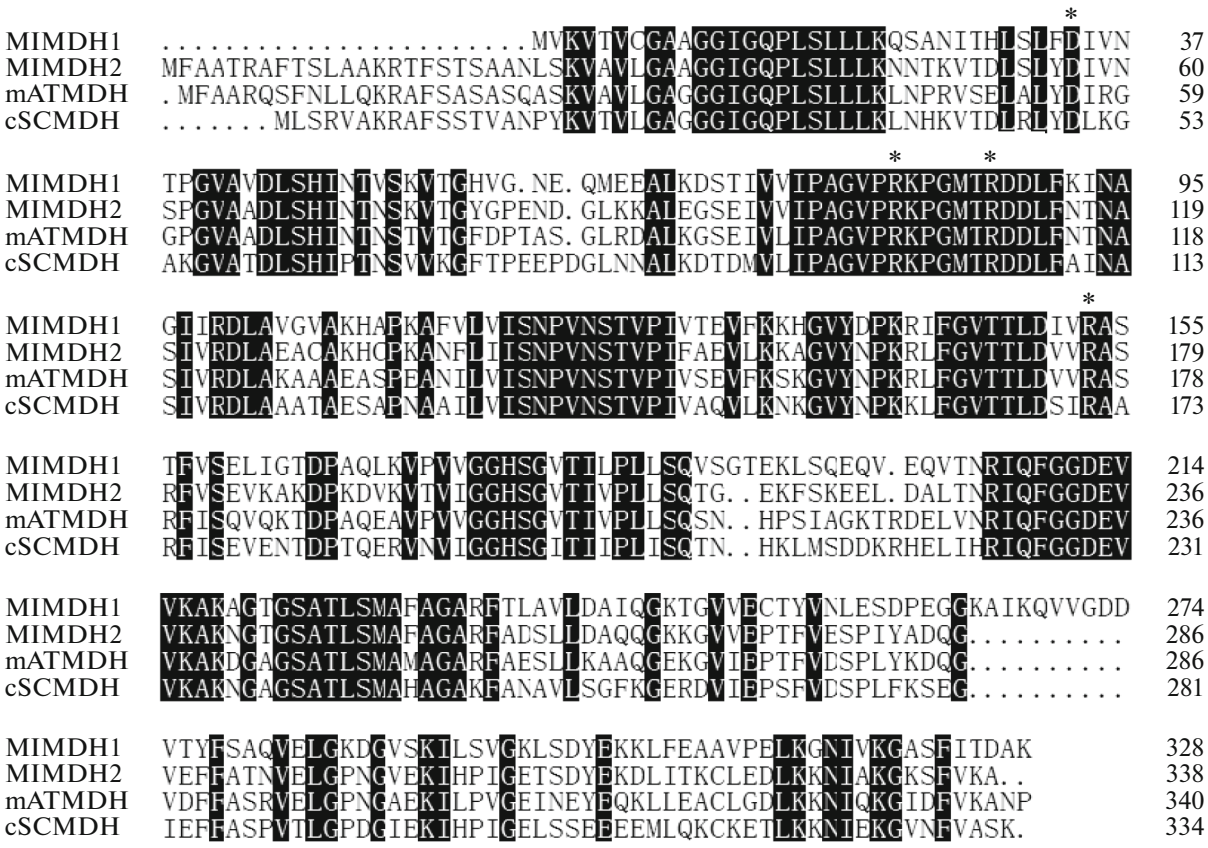

Fig. 1. Sequence alignment of deduced amino acids of the MIMDH1 and MIMDH2 with those of MDHs from *Aspergillus terreus* NIH2624 (mATMDH, GenBank accession no. EAU32902) and cytosolic MDH from *Saccharomyces cerevisiae* S288c (cSCMDH, GenBank accession no. NP_012838). Black background indicates the identical amino acid residues, and the conserved active Asp residue and 3 Arg residues are marked with an asterisk (*).

key enzymes involved in glucose metabolism through catalyzing reversible conversion of malate and oxaloacetate. NAD-dependent MDHs are present in the cytoplasm and mitochondria and play important roles in a variety of physiological cellular activities, including glucose metabolism and mitochondrial energy metabolism [16]. MDHs are involved in several stress responses, including those associated with aluminum, cadmium, copper, and oxidative stress [6–8, 17–20]. Our previous studies have shown that the mRNA-expression level of some MDH genes in *M. isabellina* M6-22 increased significantly at low temperatures [12]. There are several MDH isoforms that are classified as mitochondrial, peroxisomal, plastidial, and cytosolic in nature [1]. Cytosolic MDHs and mitochondrial MDHs represent 2 major isoforms, with their roles dependent on their subcellular localization and metabolic activities. According to our transcriptome data, we detected 3 MDH isoforms.

To characterize MDHs in *M. isabellina*, *MIMDH1* and *MIMDH2* were amplified using cDNA as a template and specific primers designed according to the sequences obtained through transcriptome sequencing. The *MIMDH1* and *MIMDH2* fragments were 990 bp and 1017 bp, encoding 329 and 338 amino acids, respectively. The *MIMDH1* and *MIMDH2* sequences have been submitted to GenBank with accession numbers KX863353 and KX424916, respectively.

Sequence analysis indicated that the nucleotide and deduced amino acid sequences of MIMDH1 and MIMDH2 shared 61.7% and 58.6% identity, respectively. The amino acid sequence of MIMDH1 shares higher homology with the cyMDHs from fungi. It shares 77 and 76% identity with cyMDHs from *Choanephora cucurbitarum*, *Phycomyces blakesleeanus* NRRL 1555(−), and *Lichtheimia ramose* [21]. The amino acid sequence encoded by *MIMDH1* exhibited a high degree of similarity to the sequences of cyMDHs from various microorganisms. This sequence contained a "GAAGQI" binding motif, which also exists in the cyMDHs of microorganisms and includes a domain containing 9 α-helices, 20 β-sheets, and 2 Cys residues (Fig. 1). Similarly, the amino acid sequences of cyMDHs from *C. cucurbitarum*, *P. blakesleeanus* NRRL 1555(−), and *L. ramose* harbor domains containing 11 α-helices and 18 β-sheets, 6 α-helices and 20 β-sheets, and 12 α-helixes and 14 β-sheets, and

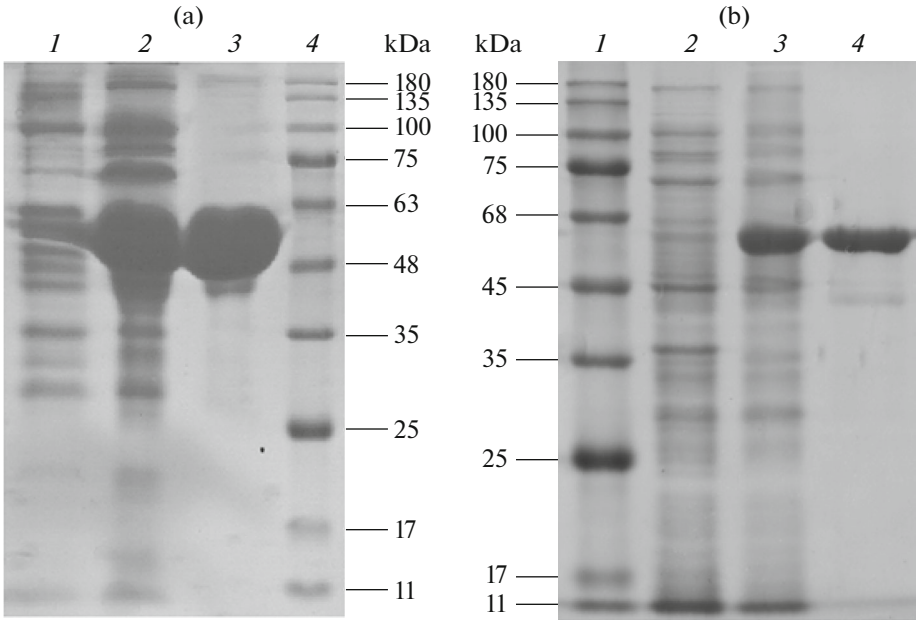

Fig. 2. Purification of the recombinant proteins MIMDH1 (a) and MIMDH2 (b). a: *1*—*E. coli* BL21 containing pET32a(+); *2*—*E. coli* BL21 containing pET32a(+)*MIMDH1*; *3*—purified MIMDH1; *4*—Protein standards. b: *1*— protein standards; *2*—BL21 containing pET32a(+); *3*—BL21 containing pET32a(+)*MIMDH2*; *4*—purified MIMDH2.

more than 2 Cys residues, respectively. These results indicated that *MIMDH1* might encode a cyMDH.

Figure 1 also showes MIMDH2 has highly conserved active residues in MDHs, such as Asp57, Arg105, Arg111, and Arg177, involved in substrate binding, catalysis and coenzyme-binding [22]. However, sequeces analysis indicated that the deduced amino acid sequence of MIMDH2 shares higher homology with the mitochondrial MDHs from fungi. It shares 80.0, 75.7, and 70.6% identity with the mitochondrial MDHs from *Choanephora cucurbitarum*, *Bifiguratus adelaidae*, and *Aspergillus terreus*. Additionally, the MIMDH2 sequence contains N-terminal leader sequences commonly found in other mitochondrial enzymes. These leader sequences do not share common features, but contain several conserved protease-cleavage sites [23], which are also present in MIMDH2, such as Arg15, Phe17, Ser18 and Ser20.

Gene expression and protein purification. To characterize the functions of the *MIMDH1*and *MIMDH1* products, both genes were inserted into the prokaryotic expression vector pET32a(+) to generate recombinant expression plasmids pET32a(+)*MIMDH1* and pET32a(+)*MIMDH2*. The sequences were verified by sequence analysis, and the resulting plasmids were transferred into *E. coli* BL21 cells for expression of the enzymes as His-tagged fusion proteins. Recombinant MIMDH1 and MIMDH2 were expressed following IPTG induction and purified by Ni-NTA affinity chromatography involving elution with 150 mM and 200 mM imidazole, respectively. The recombinant proteins MIMDH1 and MIMDH2 were partially purified as determined by Coomassie brilliant blue staining of SDS-polyacrylamide gels. The molecular weights of both purified recombinant enzymes were ~50 kDa, including the respective His-tag (Fig. 2). The molecular weights were about 32 kDa without the tags, which was consistent with results previously reported by Maloney et al. that the monomeric mass of native mMDH from *Talaromyces emersonii* is 35 kDa [15].

Characteristics of recombinant MIMDH1 and MIMDH2. The optimal temperature associated with MIMDH1 activity was revealed at 55°C (Fig. 3a), with 70% of enzyme activity remaining after 30 min incubation at 45°C. After incubation at 50°C for 60 min, the enzyme retained 50% activity. Enzyme activity decreased significantly following incubation at 60°C for 15 min, with no enzyme activity detected after 30 min. Most of the activity was undetectable after 30 min incubation at 80°C and was completely undetectable after incubation at 100°C for 30 min (Fig. 4a). The optimal temperature associated with MIMDH2 was determined at 30°C (Fig. 3b), with >70% of enzyme activity remaining following incubation at 40°C for 30 min. MIMDH2 activity was almost completely undetectable after 30 min incubation at 80°C and completely undetectable after incubation at 100°C for 30 min (Fig. 4b).

MIMDH1 showed the highest activity at pH 8.0, with decreases in activity at pH levels above and below this pH value (Fig. 5a). At pH range from 7.5 to 8.5, MIMDH1 exhibited stable activity at ~90% that observed at pH 8.0 (Fig. 5c). Additionally, the optimal

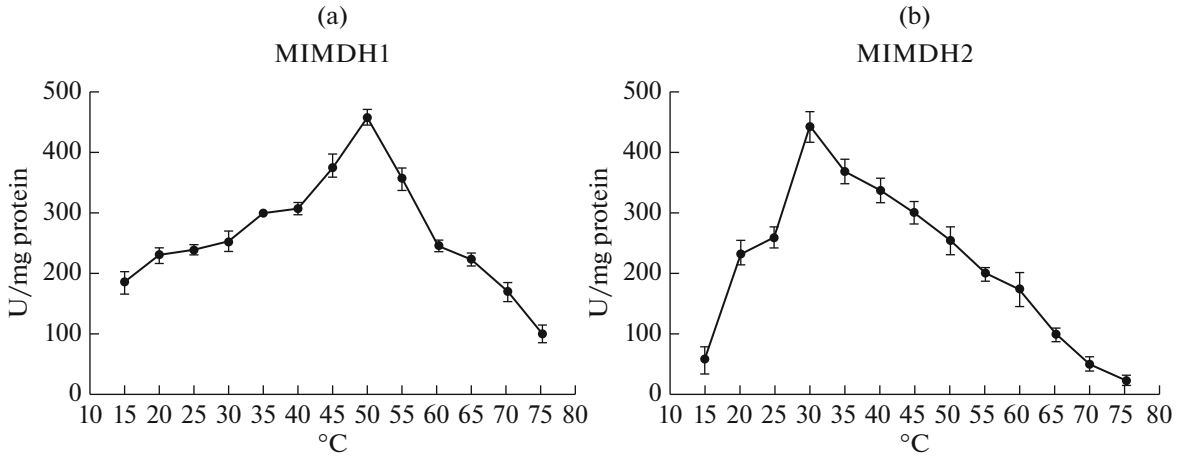

Fig. 3. The optimum temperature of the recombinant MIMDH1 (a) and MIMDH2 (b) activities.

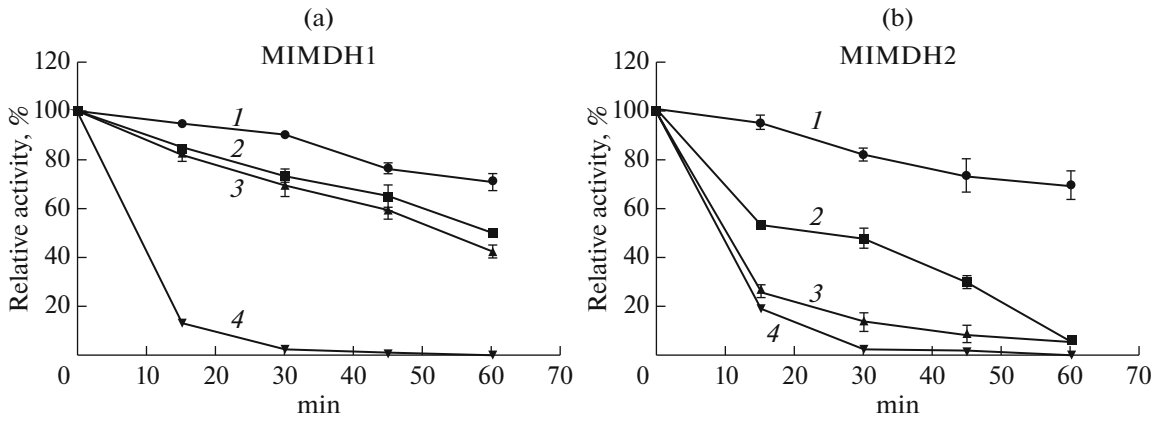

Fig. 4. The thermostability of the recombinant MIMDH1 (a) and MIMDH2 (b) activities. The thermostability was determined by measuring the residual activity following incubation of the enzyme at 30 (*1*), 40 (*2*), 50 (*3*) and 60°C (*4*) for 60 min. Assays were carried out in triplicate.

pH for MIMDH2 was 7.5 (Fig. 5b), with stable activity maintained at pH range of 7.5–8.0 (Fig. 5d). These results are comparable with those for native mMDH from *Talaromyces emersonii* exhibiting the highest activity at pH 7.5 and 52°C [15].

We analyzed the impact of different metal ions or surfactants on MIMDH1 and MIMDH2 activities. The effect of different metal ions or surfactants taken in 2 mM concentration on MIMDH1 or MIMDH2 activity was determined under optimal reaction temperature and pH conditions. We observed enhanced MIMDH1 activity in the presence of 2 mM Zn^{2+}. However, the addition of other metal ions showed different degrees of inhibition of enzyme activity (Table 1). The inhibitory effect was most pronounced in the presence of Co^{2+}, resulting in an 82.8% loss in activity. For MIMDH2, 2 mM Ba^{2+} or 2 mM Mn^{2+} increased enzyme activity, whereas the addition of other metal ions exhibited different degrees of inhibition. Additionally, 2 mM EDTA had no significant effect on MIMDH1 and MIMDH2 activities.

Measurement of enzyme kinetics. The enzyme kinetics of MIMDH1 and MIMDH2 were measured under optimal pH and temperature conditions. The specific activities of purified MIMDH1 and MIMDH2

Table 1. Effect of divalent cations and surfactants on activity of MIMDH1 and MIMDH2

Cations and surfactants, 2 mM	Relative activity, %	
	MIMDH1	MIMDH2
Control	100	100
EDTA	98.1	96.2
Ba^{2+}	58.2	107.3
Ca^{2+}	42.8	52.9
Co^{2+}	17.2	58.2
Cu^{2+}	51.0	67.9
Mg^{2+}	63.6	80.5
Mn^{2+}	88.9	109.2
Zn^{2+}	109.5	96.2

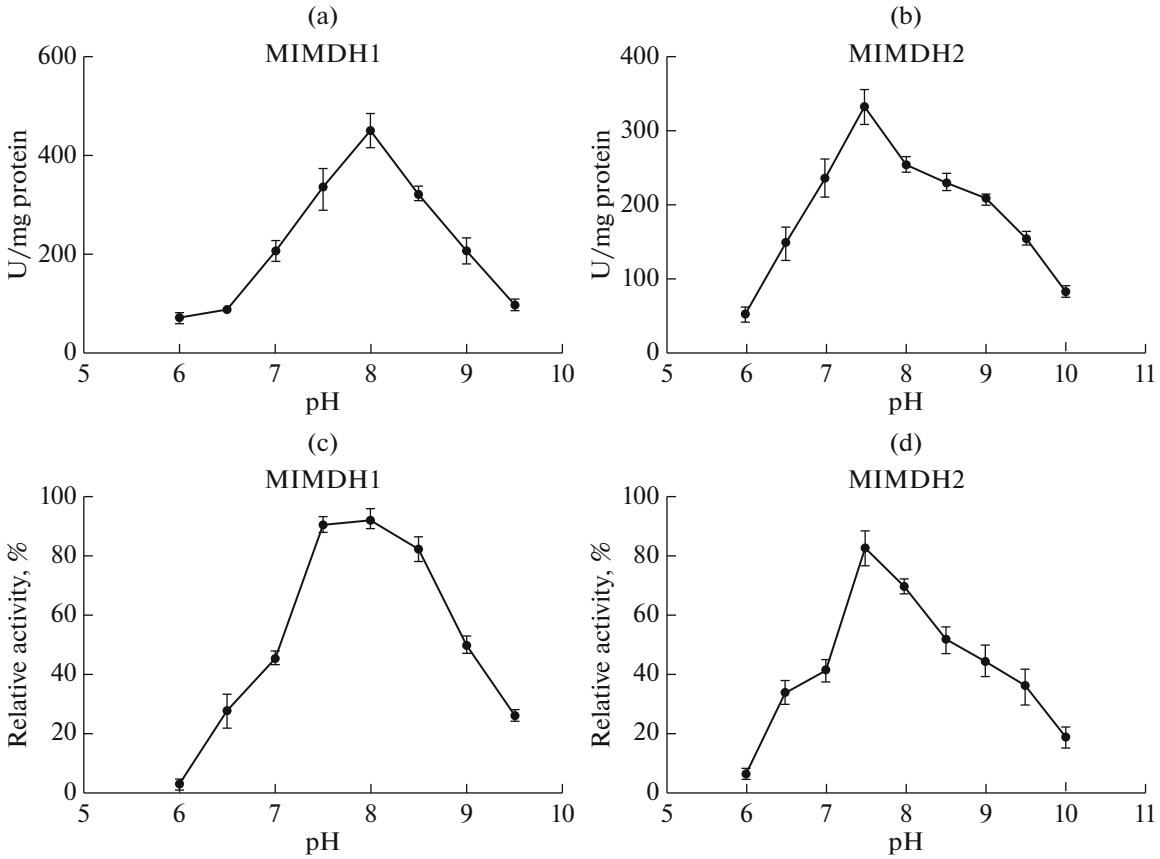

Fig. 5. Effect of pH on the activity of MIMDH1 (a and c) and MIMDH2 (b and d). (a, b)—pH-optima, (c, d)—pH stability. Assays were carried out in triplicate.

were 329.28 and 241.33 U/mg protein, respectively, which was much higher than those reported mMDH homologs from *Penicillium oxalicum* (48.7 U/mg protein), *S. cerevisiae* (66.7 U/mg protein), and *E. coli* (112.5 U/mg protein) [22, 24, 25].

K_M and V_{max} values were measured using a Lineweaver–Burk plot. The V_{max} and K_M values for recombinant MIMDH1 and MIMDH2 catalyzing the reduction of oxaloacetate to malate were 17.66 μmol $mg^{-1}min^{-1}$ and 0.541 mM and 15.59 μmol $mg^{-1}min^{-1}$ and 0.683 mM, respectively.

* * *

MIMDH1 and *MIMDH2* coding MDHs were cloned and expressed in *E. coli*. The recombinant proteins were partially purified and the molecular weight was calculated about 32 kDa. The enzymatic activities of the purified MIMDH1 and MIMDH2 were 329.28 and 241.33 U/mg protein, respectively. The optimum temperatures for MIMDH1 and MIMDH2 were 55 and 30°C, respectively. Addition of 2 mM Zn^{2+} increased the MIMDH1 activity and addition of 2 mM Ba^{2+} or Mn^{2+} increased the MIMDH2 activity, whereas addition of other metal ions caused different degrees of inhibition on MIMDH1 and MIMDH2. The optimum pH of MIMDH1 was 8.0. The optimum pH of MIMDH2 was 7.5. The V_{max} and K_M values for recombinant MIMDH1 and MIMDH2 toward malate from oxaloacetate were 17.66 μmol mg^{-1} min^{-1} and 0.541 mM and 15.59 μmol mg^{-1} min^{-1} and 0.683 mM, respectively. These results provide knowledge to understand the roles of the MDHs in *M. isabellina* M6-22.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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