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# Malolactic enzyme from *Oenococcus oeni*

## Heterologous expression in *Escherichia coli* and biochemical characterization

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**Keywords:** malolactic enzyme, *Oenococcus oeni*, *Escherichia coli*, heterologous expression

Malolactic enzymes (MLE) are known to directly convert L-malic acid into L-lactic acid with a catalytical requirement of nicotinamide adenine dinucleotide ($NAD^+$) and $Mn^{2+}$; however, the reaction mechanism is still unclear. To study a MLE, the structural gene from *Oenococcus oeni* strain DSM 20255 was heterologously expressed in *Escherichia coli*, yielding 22.9 kU $l^{-1}$ fermentation broth. After affinity chromatography and removal of apparently inactive protein by precipitation, purified recombinant MLE had a specific activity of 280 U $mg^{-1}$ protein with a recovery of approximately 61%. The enzyme appears to be a homodimer with a molecular mass of 128 kDa consisting of two 64 kDa subunits. Characterization of the recombinant enzyme showed optimum activity at pH 6.0 and 45°C, and $K_m$, $V_{max}$ and $k_{cat}$ values of 4.9 mM, 427 U $mg^{-1}$ and 456 $sec^{-1}$ for L-malic acid, 91.4 μM, 295 U $mg^{-1}$ and 315 $sec^{-1}$ for $NAD^+$ and 4.6 μM, 229 U $mg^{-1}$ and 244 $sec^{-1}$ for $Mn^{2+}$, respectively. The recombinant MLE retained 95% of its activity after 3 mo at room temperature and 7 mo at 4°C. When using pyruvic acid as substrate, the enzyme showed the conversion of pyruvic acid with detectable L-lactate dehydrogenase (L-LDH) activity and oxidation of NADH. This interesting observation might explain that MLE catalyzes a redox reaction and hence, the requirements for $NAD^+$ and $Mn^{2+}$ during the conversion of L-malic to L-lactic acid.

### Introduction

Lactic acid bacteria (LAB) are used in many industrial processes and their application in winemaking was already reported by Pasteur in 1858.[1] Since this time, the negative perception of the role of LAB as food spoilage agents has changed positively due to desirable sensory impacts such as those arising from deacidification of wine.[2] Nowadays the partial reduction of wine acidity, caused by conversion of L-malic to L-lactic acid, is known as malolactic fermentation (MLF). This fermentation occurs after the alcoholic fermentation and results in increased microbial stability.[3] The LAB isolated from grapes, must or wine belong to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*.[4] The strain, which is best adapted to the harsh conditions in wine, is *O. oeni,* reclassified from *Leuconostoc oenos*.[5] *O. oeni* can survive at pH values below 3.5 and ethanol concentrations above 10% (v/v) as well as moderately high $SO_2$ levels (50 mg/l).[6] For these reasons and to improve the control of MLF it is common for *O. oeni* to be directly inoculated into wine, typically as a commercial freeze-dried culture.[7] Unfortunately, the process is often delayed and even failure to induce MLF is not unusual, therefore alternative technologies, that enable more rapid and reliable MLF, are required.[8] Furthermore, undesired species can produce spoilage such as mousy taint, bitterness, geranium note, volatile acidity, oily and slimy-texture and overt buttery characters.[9]

In recent years it has become clear that the transformation of L-malic acid into L-lactic acid is not a true fermentation, but rather the enzymatic decarboxylation of malic acid, which could be catalyzed by three possible pathways **(Fig. 1).** First observations indicated a two-step reaction of malic enzyme (ME, EC 1.1.1.38-oxaloacetate-decarboxylating, 1.1.1.39-decarboxylating and 1.1.1.40-oxaloacetate-decarboxylating using $NADP^+$) and L-lactate dehydrogenase (L-LDH, EC 1.1.1.27).[10] Thereafter a three-step reaction including L-malate dehydrogenase (L-MDH, EC 1.1.1.37), oxaloacetate decarboxylase (OADC, EC 4.1.1.3) and L-LDH was also discussed[11] or even a possible complex of two or three enzymes was presumed.[12-15] Caspritz and Radler[16] finally proved that the responsible enzyme, referred to as the malolactic enzyme (MLE, not EC classified), consists of two identical subunits and directly converts L-malic into L-lactic acid. This reaction is performed in the presence of catalytic concentrations of $NAD^+$ and $Mn^{2+}$ but the mechanism of the MLE remains unclear because no reduction of $NAD^+$ or detection of free reaction intermediates were reported.[17]

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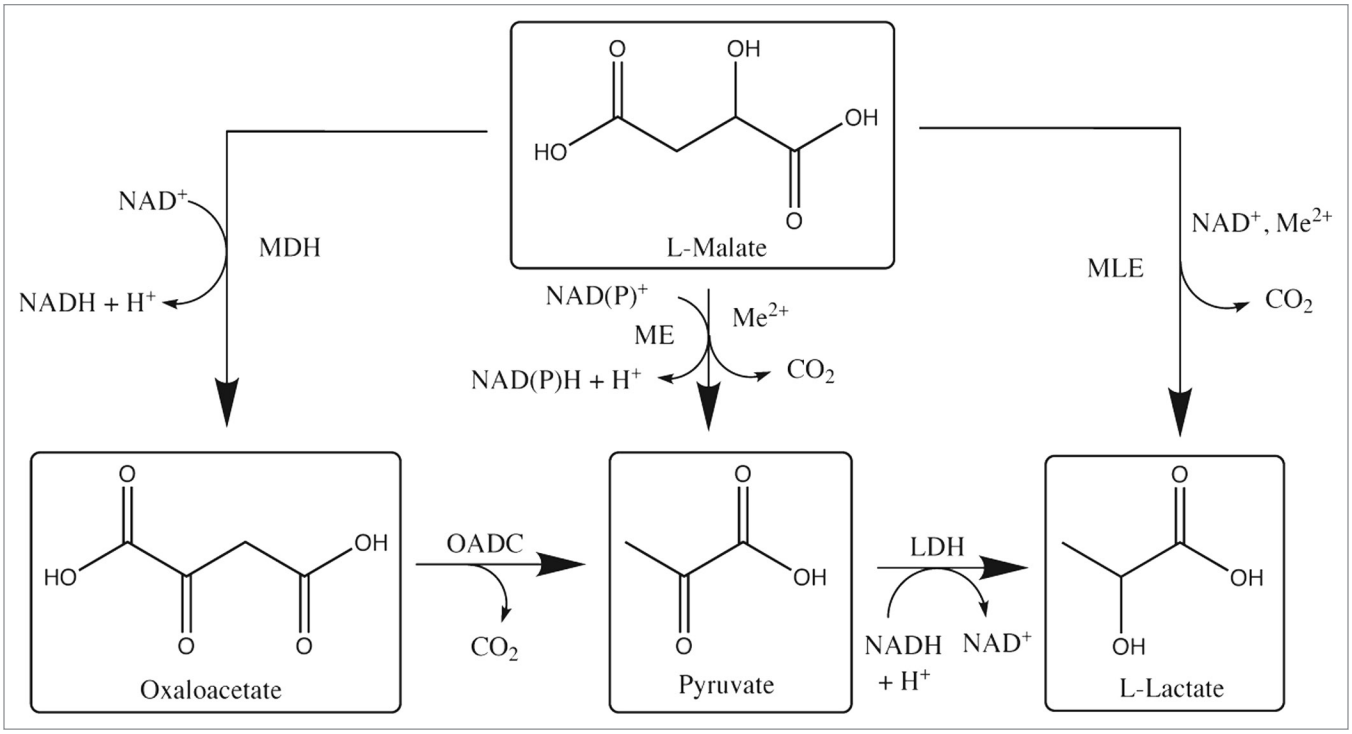


**Figure 1.** Possible pathways for the conversion of L-malic acid to L-lactic acid by different enzymes. MDH, malate dehydrogenase; ME, malic enzyme; MLE, malolactic enzyme; OADC, oxaloacetate decarboxylase; LDH, lactate dehydrogenase.

To date the *mle* genes from several organisms have been cloned and transformed into *E. coli* and resulted in low enzyme activity. Furthermore, heterologous expressions in *Saccharomyces cerevisiae,* allowing simultaneous alcoholic and malolactic fermentation, were reported to be more successful although the expression levels were not very high, as summarized by Schümann et al.[18] This study focused on high level heterologous expression of the MLE in *E. coli* with subsequent purification and biochemical characterization of the recombinant MLE.

## Results and Discussion

**Cloning, expression and purification of recombinant enzyme.** The *mle* gene from *O. oeni* (Accession number GQ924754) was expressed in *E. coli* BL21 (DE3) under control of the strong T7 promoter. The resulting protein carries a N-terminal 10-His-Tag encoded by the vector. The expressed protein consists of 562 amino acid residues with a calculated molecular mass of 61.7 kDa. The recombinant MLE showed a subunit size of approximately 64 kDa on SDS-PAGE (**Fig. 2**) and a molecular weight of 128 kDa which was determined by gel filtration using Sephacryl-S300 column. It is confirmed that active MLE is a homo-dimeric enzyme and it is in agreement with our work published previously.[18]

The expression resulted in approximately 22.9 kU of the recombinant MLE per liter fermentation broth with a specific activity of 14.9 U/mg. The enzyme was purified with a single-step purification using an IMAC column which gave 10-fold increase in enzyme purity and an overall yield of more than 60% (**Table 1**). The specific activity of the enzyme after this purification step was 145 U/mg of protein. The purified enzyme was then stored at 4°C and after the precipitate that was formed during cold storage being removed, the specific activity increased almost 2-fold further to 280 U/mg of protein. When using the natural sources, it often results in low enzyme yields.[19] Many attempts to express the MLE in *E. coli* were described previously, however very low expression levels of recombinant enzymes were obtained.[18] This study demonstrates high level expression of MLE in *E. coli* with subsequent easy purification. High level of expression of MLE enables efficient production of this enzyme.

**Characterization of the recombinant enzyme.** Among the tested buffers it was found that the enzyme showed highest activity with 100 mM HEPES (pH 6.0) (**Fig. 3**), therefore this buffer was used for the standard assay. The recombinant MLE was able to convert 4.2 mM L-malic to 4.2 mM L-lactic acid in the presence of 0.5 mM $NAD^+$ and 0.1 mM $Mn^{2+}$ in 5 min with no other acids being detectable by HPLC. The pH and temperature optima of the recombinant MLE were determined in 100 mM HEPES buffer. The enzyme showed highest activity at pH 6.0 (**Fig. 4A**) and the temperature optimum was determined to be 45°C when using HEPES buffer at pH 6.0 (**Fig. 4B**). This is in agreement with the MLE from *O. oeni* expressed in *Lactobacillus plantarum* described in our previous work.[18] The steady-state kinetic constants were determined for the conversion of L-malic acid. Kinetic analysis of recombinant MLE with increasing concentrations of L-malic acid as the substrate showed Michaelis-Menten kinetics with the following parameters obtained by nonlinear regression using SigmaPlot (SPSS Inc.): $K_m$ = 5.3 ±

0.33 mM, $V_{max}$ = 219 ± 6.87 μmol/min mg protein and $k_{cat}$ = 234 ± 7.33 $sec^{-1}$. The kinetic parameters, $K_m$, $V_{max}$ and $k_{cat}$, were also determined for the cofactors $NAD^+$ and $Mn^{2+}$: 0.082 ± 0.009 mM, 213 ± 3.14 μmol/min mg protein and 227 ± 3.35 $sec^{-1}$ for $NAD^+$; 0.0054 ± 0.001 mM, 175 ± 9.75 μmol/min mg protein and 187 ± 10.40 $sec^{-1}$ for $Mn^{2+}$, respectively.

**Stability of the MLE.** It was shown that the recombinant MLE was most stable in HEPES buffer without any added reagents and retained more than 95% of its activity after 100 d at room temperature (**Fig. 5**). The recombinant MLE is also very stable at 4°C as it retained 95% of its activity after 7 mo (data not shown).

The addition of sodium chloride and potassium chloride to HEPES buffer resulted in significant loss of initial enzyme activity and the enzyme is also less stable during storage compared with the HEPES buffer without any added salt. Furthermore, the enzyme was significantly less stable in $KH_2PO_4$ and $NaH_2PO_4$ compared with HEPES buffer. It is not clear why the enzyme is inhibited by different ions, but instability in phosphate buffer was previously reported.[20] HEPES buffer without any added reagents was found to be the best storage buffer for recombinant MLE.

The effect of freezing temperature (-30°C) on the stability of enzyme activity was also determined. It was found that only approximately 5% activity lost after the fifth freeze-thaw cycle (data not shown). This opens up optimal storage conditions for MLE which might be of interest for industrial applications.

**Reactions of the MLE with different substrates.** Besides using malic acid as the substrate, lactic, oxaloacetic and pyruvic acids were also tested as substrates for MLE in the presence of either $NAD^+$ or NADH. Photometric determination revealed the production or consumption of NADH when malic acid or pyruvic acid were used as the substrates (**Table 2**), respectively, using 10-fold more MLE compared with the standard assay. When using malic acid as the substrate, the production of NADH in the ME activity assay was found to be higher compared with that in the L-MDH assay in which the only difference was the presence of $Mn^{2+}$ as ME requires this divalent metal ion as cofactor. No L-MDH activity was obtained with oxaloacetic acid as substrate. When using pyruvic acid as the substrate, NADH consumption in L-LDH activity assay conditions was higher in the presence of 0.1 mM $Mn^{2+}$, resulting in stoichiometrically conversion of pyruvic into L-lactic acid, with a specific activity of 4.5 U/mg protein. The reverse reaction was performed in the presence of alanine transaminase (equilibrium of the reaction being toward L-lactic acid) but no L-LDH activity was obtained. The detection of L-LDH activity in our study is questionable. It is likely that the MLE alone is responsible for the conversion of $NAD^+$ and it uses the intermediates that are not free but bound in an enzyme-substrate complex to convert L-malic acid. It is possible that the MLE forms oxaloacetic and pyruvic acid as intermediates during the reaction where $NAD^+$ is continuously regenerated. To the best of our knowledge, no recombinant MLE was purified apart from our recombinant enzymes expressed in *L. plantarum*[18] and in *E. coli* in this study. Also, the mechanism of the MLE was not yet studied in detail.

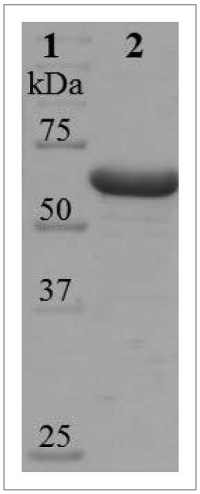


**Figure 2.** Determination of the MLE subunit molecular weight by SDS-PAGE with Coomassie blue staining. Lane 1: Precision Plus Protein Standard (Bio-Rad). Lane 2: purified recombinant MLE.

**Table 1.** Purification of the recombinant malolactic enzyme

| Purification step | Total activity (U) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|
| Crude extract | 34,410 | 14.9 | 1 | 100 |
| Affinity chromatography (IMAC) | 21,930 | 145.0 | 9.7 | 63.7 |
| Cold storage* | 21,150 | 281.2 | 18.9 | 61.5 |

The MLE was produced from 1.5 L fermentation broth. Values reported are the mean of two independent measurements. *After removing precipitated (inactive) protein by centrifugation.

## Materials and Methods

**Chemicals and enzymes.** All chemicals were purchased from Sigma-Aldrich or Roth. Fructose was obtained from VWR and imidazole from AppliChem. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (NEB) while *PfuUltra* II Fusion HS DNA Polymerase was obtained from Stratagene.

**Bacterial strains, plasmids and media.** The strain used in this study, *Oenococcus oeni* DSM 20255, was purchased from the German Collection of Microorganisms and Cell Cultures, *Escherichia coli* OneShot TOP10 cells were from Invitrogen and expression strain *E. coli* BL21 (DE3) was from Novagen. The plasmids used in this study were pCR-Blunt II-TOPO (Invitrogen) and pET16b (Novagen). *O. oeni* cells were grown at 25°C in de Man-Rogosa-Sharp (MRS) broth.[21] *E. coli* transformants were grown in Luria-Bertani (LB) medium[22] or in Terrific Broth (TB) medium[23] at 37°C with addition of 50 μg/ml ampicillin. Agar plates were made of LB media including 15 g/l agar.

**Construction of MLE expression vector.** Genomic DNA from *O. oeni* was extracted using GenElute Bacterial Genomic

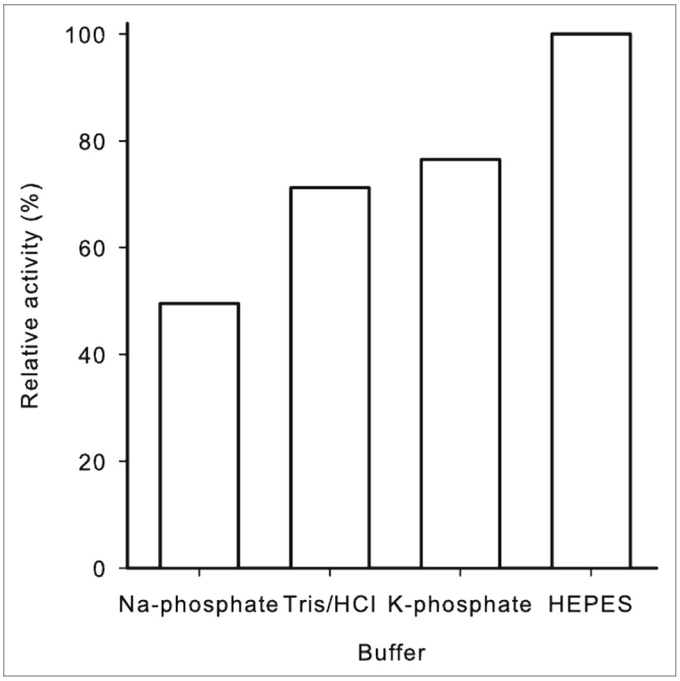


**Figure 3.** Activity of the recombinant MLE expressed in *E. coli* in different buffers. The assay mixtures consisted of 15 mM L-malic acid, 0.1 mM $Mn^{2+}$ and 0.5 mM $NAD^+$ in 100 mM each buffer at pH 6.0. Values reported are the mean of two independent measurements.

DNA kit (Sigma-Aldrich). The oligonucleotides 5'-GAG GAG AAA ATA TGA CAG ATC C and 5'-GCA TTC ATT AGT ATT TCG GAT CCC used for PCR amplification of the *O. oeni* malolactic (*mle*) gene, were designed based on the sequence from Labarre et al.[24] The PCR-amplified product was subcloned into the vector pCR-Blunt II-TOPO (Invitrogen) and the resulting plasmid pCSmle1 was transformed into chemically competent *E. coli* TOP10 cells. Upstream and downstream primers (forward: 5'-GGT CGT CAT ATG ACA GAT CCA GTA AGT ATT TTA and reverse: 5'-CGG ATC CTC GAG TTA GTA TTT CGG ATC CCA C) were designed to amplify the fragment containing *mle* gene from pCSmle1. These primers created a restriction site, *Nde*I and *Xho*I (underlined in the sequences), respectively, at each end of the gene fragment. The PCR-amplified product was digested with *Nde*I and *Xho*I and inserted into the respective sites of the expression vector pET16b (Novagen). The resulting over-expression vector, pCS16mle, was transformed into chemical competent *E. coli* BL21 (DE3) cells and the construct was verified by sequencing (AGOWA Genomics).

**Expression and purification of recombinant enzyme.** Expression was performed in 6 baffled shaking flasks each containing 250 ml TB medium. *E. coli* BL21 (DE3) carrying pCS16mle was grown at 37°C in TB medium containing 50 μg/ml ampicillin for 10 h at 140 rpm. Induction was performed by adding lactose to a final concentration of 0.5% (w/v) and the cultures were incubated further for 16 h at 25°C and 100 rpm. The induced cells were harvested by centrifugation (4,000 × g, 10 min, 4°C) and resuspended in buffer A (100 mM HEPES, 100 mM KCl and 20 mM imidazole at pH 6.0). Cell disruption was performed on ice by ultrasonication (Bandelin Sonopuls HD60), and debris was removed by ultracentrifugation (25,000 g for 30 min at 4°C) to obtain the cell-free extract. The crude extract was loaded on an immobilized metal affinity chromatography column (profinity IMAC column, 15 ml, Bio-Rad Laboratories) that was pre-equilibrated with buffer A. The protein was eluted with buffer B (100 mM HEPES, 100 mM KCl and 500 mM imidazole, pH 6.0). Active fractions were pooled, desalted, concentrated and finally resuspended in storage buffer (100 mM HEPES, 0.5 mM $NAD^+$ and 0.1 mM $Mn^{2+}$, pH 6.0).

**Standard assay for MLE activity.** Activity of the MLE was determined by measuring the decreasing amount of malic acid and increasing amount of lactic acid in the assay. The reaction mixture contained 100 mM HEPES, 0.5 mM $NAD^+$, 0.1 mM $Mn^{2+}$ and 15 mM L-malic acid (pH 6.0), and was incubated at 45°C using an Eppendorf thermomixer. The reaction was started with the addition of 20 μl enzyme and stopped after 5 min reaction time by heating at 70°C for 1 min to inactivate the enzyme. Subsequent measurement of organic acids using high performance liquid chromatography (HPLC) using a Dionex System was performed as described previously.[18] The enzyme activity (U) is expressed as micromoles of L-malic acid converted per minute at 45°C.

**pH and temperature dependence of activity.** The influence of pH and temperature on the activity of the recombinant malolactic enzyme was studied under standard assay conditions. HEPES buffer and L-malic acid solution were adjusted to pH between 5.0 and 7.0 and the assays were performed in the temperature range from 20°C to 60°C.

**Determination of protein, molecular weight and kinetic measurements.** The protein concentration was determined using the method of Bradford[25] with bovine serum albumin as standard. Protein samples were analyzed by sodium dodecyl sulfate PAGE (SDS-PAGE)[26] using Protein Standard Precision Plus (Bio-Rad) and Low Molecular Weight (LMW, GE Healthcare). Coomassie blue staining was used for the visualization of the protein bands. The apparent size of the MLE was further estimated by gel filtration using a Sephacryl-S300 column (190 ml, GE Healthcare) equilibrated with 100 mM HEPES and 100 mM KCl (pH 6.0), and the molecular weight marker kit for gel filtration (Sigma-Aldrich).

All steady-state kinetic measurements were obtained at 45°C using 100 mM HEPES buffer (pH 6.0) with varying concentrations as followed: 3–11 mM for L-malic acid with 0.5 mM $NAD^+$ and 0.1 mM $Mn^{2+}$; 50–300 μM for $NAD^+$ with 15 mM L-malic acid and 0.1 mM $Mn^{2+}$; and 5–25 μM for $Mn^{2+}$ with15 mM L-malic acid and 0.5 mM $NAD^+$. Malic and lactic acid were analyzed by HPLC as described previously,[18] for the calculation of initial reaction velocities. All measurements were determined in triplicate. The kinetic parameters $K_m$ and $V_{max}$ were calculated by nonlinear regression and the observed data were fitted to the Henri-Michaelis-Menten equation using Sigma Plot (SPSS Inc.). The $k_{cat}$ values were subsequently calculated on the basis of theoretical $V_{max}$ values.

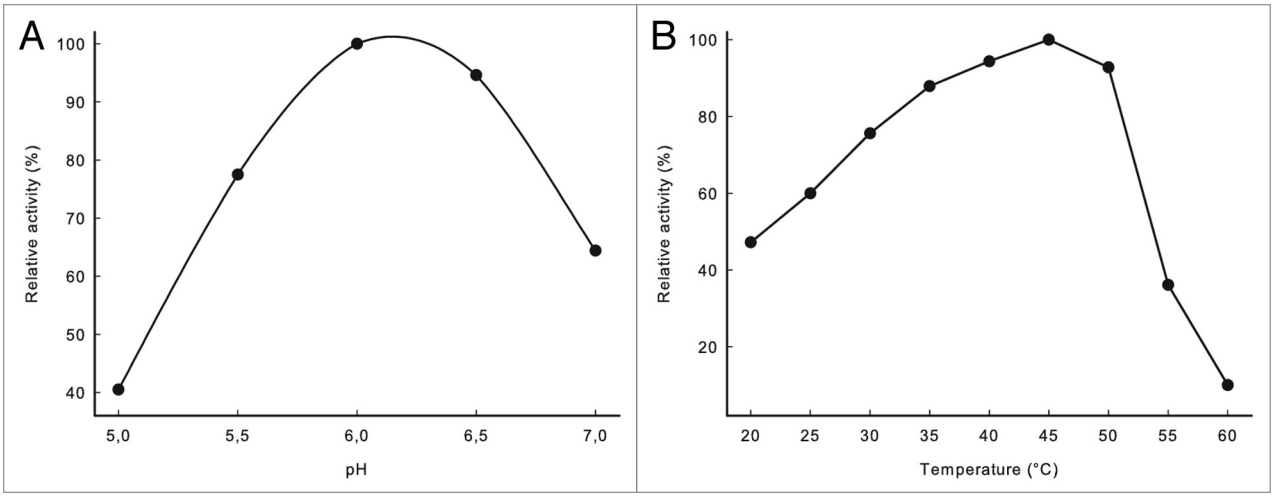


**Figure 4.** pH optimum (**A**) and temperature optimum (**B**) of recombinant MLE produced in *E. coli*. The enzyme activity was measured in 100 mM HEPES buffer containing 0.1 mM $Mn^{2+}$ and 0.5 mM $NAD^+$. Values reported are the mean of two independent experiments.

**Stability measurements.** The stability of the MLE was tested in six different buffers: 100 mM HEPES or 100 mM $KH_2PO_4$ or 100 mM $NaH_2PO_4$ (pH 6.0) containing 0.5 mM $NAD^+$, 0.1 mM $Mn^{2+}$ and either 100 mM KCl or 100 mM NaCl. Enzyme activity in 100 mM HEPES (pH 6.0) containing 0.5 mM $NAD^+$ and 0.1 mM $Mn^{2+}$ storage buffer was used as a control. The enzyme preparations in different buffers were kept at room temperature over 100 d and at certain time intervals, samples were withdrawn and the residual activity was measured under standard assay conditions. The stability of the enzyme at 4°C and under freezing temperature (-30°C) was also checked. The enzyme preparation was frozen at -30°C in storage buffer and thawed at room temperature one hour before performing the enzyme assays. All measurements were performed in duplicate.

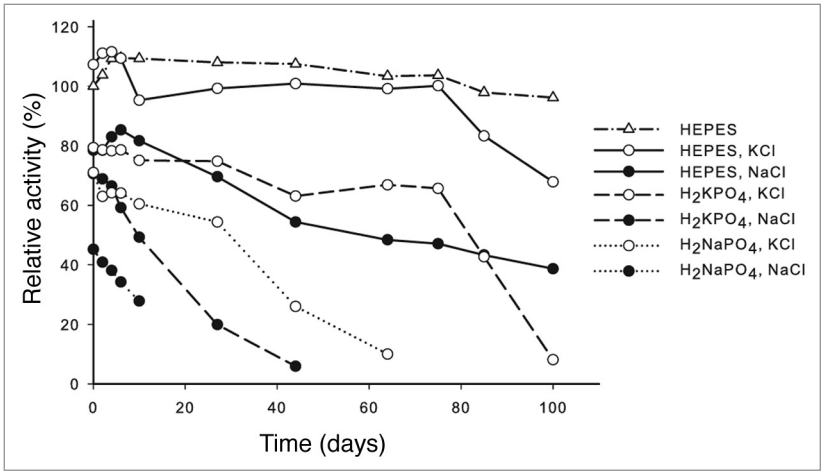


**Figure 5.** Stability of the recombinant malolactic enzyme produced in *E. coli* at room temperature in different storage buffers. Seven different buffers were tested: 100 mM HEPES containing 0.5 mM $NAD^+$ and 0.1 mM $Mn^{2+}$ (storage buffer); 100 mM HEPES or 100 mM $KH_2PO_4$ or 100 mM $NaH_2PO_4$ (pH 6.0) containing 0.5 mM $NAD^+$, 0.1 mM $Mn^{2+}$ and either 100 mM KCl or 100 mM NaCl.

**Activity assays with L-lactic-, L-malic-, oxaloacetic- and pyruvic acid as substrates.** Activity assays with L-lactic-, L-malic-, oxaloacetic- and pyruvic acid as substrates were performed to determine lactate dehydrogenase (L-LDH), malate dehydrogenase (L-MDH) and malic enzyme (ME) activities (see also **Fig. 1**) of the recombinant MLE in this study and also to investigate possible redox reaction ($NAD^+$/NADH) catalyzed by this enzyme. Changes in the amounts of NADH were observed by using a Beckman DU 800 spectrophotometer at 340 nm and organic acids were quantified by HPLC. The assay mixtures consisted of 10 mM substrate (L-lactic-, L-malic-, oxaloacetic- or pyruvic acid), 8 U of MLE with either 0.5 mM NADH or 0.5 mM $NAD^+$ in 100 mM HEPES buffer (pH 6.0). The assays with these substrates were performed both in the presence or absence of $Mn^{2+}$ (0.1 mM). In the assay for L-LDH using pyruvic acid as the substrate, L-LDH from rabbit muscle (Sigma-Aldrich) was used as a control. In the assay for L-LDH in the reverse reaction converting L-lacte to pyruvate, L-lactic acid was used as substrate and the assay was performed in the presence of 8 U/ml alanine transaminase (ALT, EC 2.6.1.2, Roche) and 25 mM L-glutamic acid (pH 6.0). L-MDH from *Thermus flavus* (Sigma-Aldrich) was used as the control in the assay for L-MDH using oxaloacetic acid as the substrate. The assay for ME using L-malic acid as the substrate was started with the addition of 0.5 mM $NADP^+$. The reaction rates were measured at 45°C for 5 min and specific enzyme activity (U/mg protein) is reported as microles of NADH consumed or produced per minute and per miligram of protein. All measurements were performed in duplicate.

**Table 2.** Side activities of the recombinant malolactic enzyme

| Substrate (10 mM) | Cofactor | $Mn^{2+}$ (mM) | Specific activity (U/mg protein) | Activity assay for |
|---|---|---|---|---|
| L-malic acid | 0.5 mM $NAD^+$ | - | 0.8 | L-MDH |
| L-malic acid | 0.5 mM $NAD^+$ | 0.1 | 1.2 | ME |
| Oxaloacetic acid | 0.5 mM NADH | - | ND | L-MDH |
| Oxaloacetic acid | 0.5 mM NADH | 0.1 | ND | (L-MDH) |
| L-Lactic acid | 0.5 mM $NAD^+$ | - | ND | L-LDH |
| L-Lactic acid | 0.5 mM $NAD^+$ | 0.1 | ND | (L-LDH) |
| Pyruvic acid | 0.5 mM NADH | - | 1.2 | L-LDH |
| Pyruvic acid | 0.5 mM NADH | 0.1 | 4.5 | (L-LDH) |

The enzyme activities for malic enzyme (ME), L-malate dehydrogenase (L-MDH) and L-lactate dehydrogenase (L-LDH) were determined. Values reported are the mean of two independent experiments. ND, not detectable.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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