

# Induction, purification and characterization of malolactic enzyme from *Oenococcus oeni* SD-2a

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**Abstract** The aim of this study was to purify a malolactic enzyme (MLE) from *Oenococcus oeni* (*O. oeni*) strain and determine its properties in detail. *O. oeni* SD-2a was cultivated in the ATB broth supplemented with 7 g/L L-malic acid for harvesting the cells. After harvest, the cells were washed and disrupted for purification of MLE. MLE was purified from the supernatant of the disrupted cells through protamine sulfate precipitation, anion exchange chromatography and gel filtration chromatography. The purified MLE was identified using mass spectrometry. The MLE was purified by 43-fold with a yield of 0.42 % and possessed a specific activity of 419.2 U/mg. The purified enzyme with a nominal molecular mass of 59 kDa and a theoretical *pI* of 4.76 exhibited a maximum enzyme activity at 35 °C and pH 6.0, which retained over 50 % of its initial activity in the presence of 14 % (v/v) ethanol.  $Mn^{2+}$  was proven to be the most effective divalent cation to promote enzyme activity. Under the conditions of temperature 30 °C and pH 6.0, the  $K_m$  and  $V_{max}$  of MLE on L-malic acid were  $12.5 \times 10^{-3}$  M and 43.86  $\mu\text{mol}/(\text{min} \times \text{mg})$ , respectively. Moreover, the purified enzyme exhibited a higher stability with 0.1 M NaCl in addition and had a half-life of 30 days at 4 °C.

**Keywords** Malolactic enzyme · Induction · Purification · Characterization

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## Introduction

The complex process of winemaking involved alcoholic fermentation (AF) and malolactic fermentation (MLF). AF is carried out by yeast that transforms sugars into ethanol and carbon dioxide, while MLF is conducted by lactic acid bacterium (LAB) that converts malic acid to lactic acid and carbon dioxide [1]. MLF usually occurs during storage of young wines or several weeks after AF and normally develops naturally, which is a very slow and unpredictable process and often causing unsatisfactory result [2]. In this process, L-malic acid is converted to L-lactic acid and  $\text{CO}_2$ , which results in deacidification and microbial stability and flavor modification of the wine. L-lactic acid, due to its monocarboxylic nature, imparts a more elegant and round taste to the wine, as opposed to the astringent taste from L-malic acid [3, 4]. Conversion of L-malic acid to L-lactic acid can occur through three possible enzymatic pathways [5]. The pathway generally utilized by the wine LAB is the direct conversion of malic acid to lactic acid by the malolactic enzyme (MLE) which is active in the presence of  $\text{NAD}^+$  and  $\text{Mn}^{2+}$ , and no free intermediates are produced [6].

Up to date, MLF is not entirely controlled since the wine presents unfavorable conditions for the growth of microorganisms. Stuck or sluggish MLF and processing problems, microbial alteration or even spoilage of wine occur frequently in winemaking [7–9]. Even when the wine is inoculated with malolactic starter, there is no guarantee of the implementation of MLF [10–12]. To figure out such problems, MLF research, especially research concerning the MLE predominant for decarboxylation of L-malic acid, needs to be conducted.

Malolactic enzyme is the key enzyme for *O. oeni* to complete MLF and is an intracellular enzyme. It was possible to use MLE to conduct MLF with the aid of immobilized

**Table 1** Factors and levels of single-factorial experiments about effects of culture parameters on MLE biosynthesis

Factors	Levels				
L--malic acid addition (g/L)	3	5	7	9	20
pH	3.2	3.5	3.8	4.1	4.5
Ethanol concentration (% v/v)	4	8	10	12	14
Growth phase	Mid-lag phase	Early-stationary phase	Stationary phase	–	–

enzyme technology [16]. Using MLE to conduct MLF was not only feasible but also much more efficient than using lactic acid bacteria. However, the biochemical properties of the MLE from *Oenococcus oeni* were not well studied.

The overall aim of this study was to purify a MLE from *O. oeni* strain and determine its properties. The first objective was to investigate the effects of L-malic acid concentration, pH and ethanol content on MLE biosynthesis. The second objective was to describe an efficient purification method and some properties of a MLE from a strain of *O. oeni* SD-2a isolated from spontaneous MLF wine of Yantai, Shandong Province, China.

## Materials and methods

### Strain and culture medium

The strain used in this study was *O. oeni* SD-2a isolated from Chinese wine. The strain was cultivated in a modified ATB broth containing: glucose, 1 g/L; fructose, 5 g/L; L-malic acid, 7 g/L; yeast extract, 5 g/L; peptone, 10 g/L; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g/L; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.05 g/L; cysteine/HCl, 0.5 g/L; tomato juice, 250 mL/L; which was adjusted to pH 4.8 with NaOH.

### Cell extracts preparation

First, cells from a culture of *O. oeni* SD-2a grown at 25 °C in ATB broth up to the stationary phase ( $OD_{600} = 1.8$ ) were harvested by centrifugation (6,000g, 10 min) and suspended in 154 mM NaCl and washed twice. Second, the cells were resuspended in a buffer solution (pH 6.0) containing monopotassium and disodium phosphate (0.05 M), potassium chloride (0.1 M) and β-mercaptoethanol (0.1 %) to yield an  $OD_{600}$  of 1.0. Third, cells were chilled and maintained in an ice bath during the disruption in a Vibra-Cell™ sonifier (130 watts, Sonics & Materials Inc, USA). The ultrasonic disruption condition is 130 W for 20 min. Finally, the cell debris was removed by centrifugation (12,000g, 15 min) at 4 °C, and the supernatant was used as crude enzyme extract.

The procedure was partially modified to prepare the cells for the enzyme localization studies according to Michlmayr et al. [22].

### Determination of enzyme activity and protein content

The activity of MLE was assayed with L-malic acid (Aladdin Industrial Corporation, Shanghai, China) as substrate using the method described previously with some modification [20]. Assays were made in a final volume of 2.4 mL in plastic tubes. The reaction mixture contained 200 μmol of L-malic acid, 2 μmol of NAD<sup>+</sup>, 0.4 μmol of MnCl<sub>2</sub> and 2 mL of 0.2 M KH<sub>2</sub>PO<sub>4</sub>–Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0) unless otherwise stated. Assays were incubated at 30 °C for 20 min, and the reaction was stopped by the addition of 200 μL of H<sub>3</sub>PO<sub>4</sub> and stirred vigorously on a vortex mixer.

In each assay, blanks were prepared in which the enzyme extract had been substituted by buffer. All the enzyme activity assays were performed in replicate with values typically being within 5 % of one another. One unit of enzyme activity (U) was defined as the amount of protein that degraded 1 μmol of L-malic acid consumed per min.

The protein content of the crude extract and purified extract were determined using the Bradford method, using Bovine albumin as a standard and absorbance (595 nm) measured in micro-cuvette. Whereby the Bradford solution was prepared by ourselves.

### Effects of L-malic acid supplementation, pH, ethanol level and growth phase on MLE biosynthesis associated with *O. oeni* SD-2a growth in ATB broth

MLE synthesis associated with wine LAB growth was determined for *O. oeni* SD-2a in ATB broth. The composition of this medium was further modified to assess the effects of culture parameter. A single-factorial experiment was used to assess the effects of variables on MLE. The single-factorial experiments were conducted according to Table 1. Aliquots (50 mL) of modified ATB broth to satisfy different objective were dispensed into 100-mL sterile glass Schott bottles and inoculated (2 % v/v) with *O. oeni* SD-2a pre-cultured in ATB broth as described above. Duplicate inoculated media were incubated at 25 °C. Cultures grown in the ATB broth free of L-malic acid and alcohol, at the pH value of 4.8 served as control to evaluate the effects of growth parameter on MLE activity.

## Purification of malolactic enzyme

The cell-free crude enzyme was fractionally precipitated by protamine sulfate (0.15 mg of protamine sulfate was added per mg of protein) which was used to precipitate nucleic acid and inactive protein. The mixture was kept at 4 °C for 40 min, and then the precipitate was removed by centrifugation at 8,000g for 20 min at 4 °C. The supernatant was concentrated and desalted with a dialysis bag (cut off 7 kDa, Union Carbide Co., Houston, Texas, USA). The concentrated supernatant with MLE activity was purified using AKTA Purifier (GE Healthcare Life Science, Amashia, Sweden). The dialyzed fraction was filtered through 0.22- $\mu$ m cellulose membrane and loaded on Hitrap DEAE FF column (1 mL, GE Healthcare Life Science, Amashia, Sweden) equilibrated with the 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0). The column was washed with ten column volumes (CV) of the equilibration buffer, followed by elution with a segmentation linear gradient of NaCl (0–1 M) over an 80-fold column volume. The active fractions were pooled and concentrated. The concentrated fractions were then subjected to gel filtration chromatography on Superdex™ 200 column (1.0 cm  $\times$  30 cm, GE Healthcare Life Science, Amashia, Sweden) equilibrated and eluted with 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0). Then, obtained enzyme solution by above purification steps was applied to a HiPrep Sephacryl S-100 column (1.6 cm  $\times$  60 cm, GE Healthcare Life Science, Amashia, Sweden), which had been pre-equilibrated with 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0), and the elution was performed at a flow rate of 0.5 mL/min. Fractions containing the MLE activity were collected.

## Malolactic enzyme identification

The purity of MLE was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and further identification was conducted by mass spectrometry. SDS-PAGE was performed on a gel composed of 5 and 10 % polyacrylamide for concentration and separation, respectively. Protein bands were visualized in gels by being stained with Coomassie Brilliant Blue R250 or silver. High-resolution mass spectrometry was conducted by MALDI-TOF/TOF. The data obtained from MS were submitted to MASCOT for identification, and the search was performed against the NCBI database dealing with bacteria (Eubacteria). The protein mass tolerance was set to 0.5 Da for MS/MS. For protein identification, the MASCOT identity threshold corresponding to a *p* value inferior to 0.05 was considered as a prerequisite for protein validation with a high degree of confidence.

## Influence of temperature, pH, ethanol and divalent cations on MLE activity

The influence of temperature on MLE activity was studied using reaction mixture described above. For accurate temperature control, the 0.2 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffers (pH 6.0) were pre-incubated at 25, 30, 35, 40 and 45 °C.

To determine the pH influence, 0.2 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffers were prepared at different pH values (5.00, 5.25, 5.50, 5.75, 6.00, 6.25, 6.50, 6.75 and 7.00).

To determine the influence of the inclusion of ethanol and divalent cations, 0.2 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffers (pH 6.0) were prepared, to contain ethanol concentrations of 4, 8, 10, 12 and 14 % (v/v), or divalent cation ( $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$  or  $\text{Cu}^{2+}$ ), at concentration of 0.4  $\mu$ mol/L, respectively. At each assay, blanks were prepared as described above.

## Kinetic studies

The kinetic properties of the purified MLE were studied in 0.2 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0) at 30 °C. Particularly, apparent Michaelis–Menten constant ( $K_m$ ) and the maximal reaction rate ( $V_{max}$ ) for L-malic acid degradation were determined at the range of 7.63–18.87 mM, with a constant NAD and  $\text{Mn}^{2+}$  concentrations. The  $K_m$  and  $V_{max}$  were calculated according to the Lineweaver–Burk double-reciprocal plot.

## Determination of half-life

Half-life in this paper was defined as the time at which a residual activity of 50 % was experimentally observed. Half-life of the purified enzyme stored at 4 °C in 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0), and the same buffer supplemented with 0.1 M NaCl was obtained by determining the MLE activity every day, respectively.

## Results and discussion

### Enzyme localization

Results of the cellular localization of the enzyme in Fig. 1 revealed that the washed cells exhibited the highest MLE activity, while almost no enzyme activity was detected in the supernatant from the culture which indicated the enzyme was not secreted outside the cell. After sonication treatment, a reasonable decrease of enzymatic activity was observed in the supernatant as compared with that of the intact cells, while no activity was detected in the cell debris, suggesting that MLE could be released as soluble enzyme in the supernatant after sonication. Moreover, the decrement of activity (about 6 % of the intact cells activity)

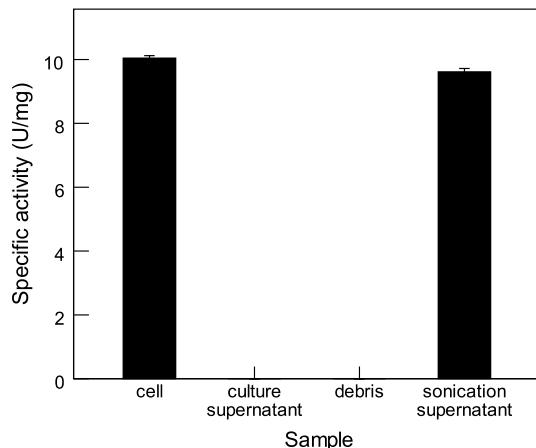
could be due to protein damage during the sonication although all procedures were conducted in an ice bath.

Malolactic enzyme biosynthesis associated with growth of *O. oeni* in ATB broth

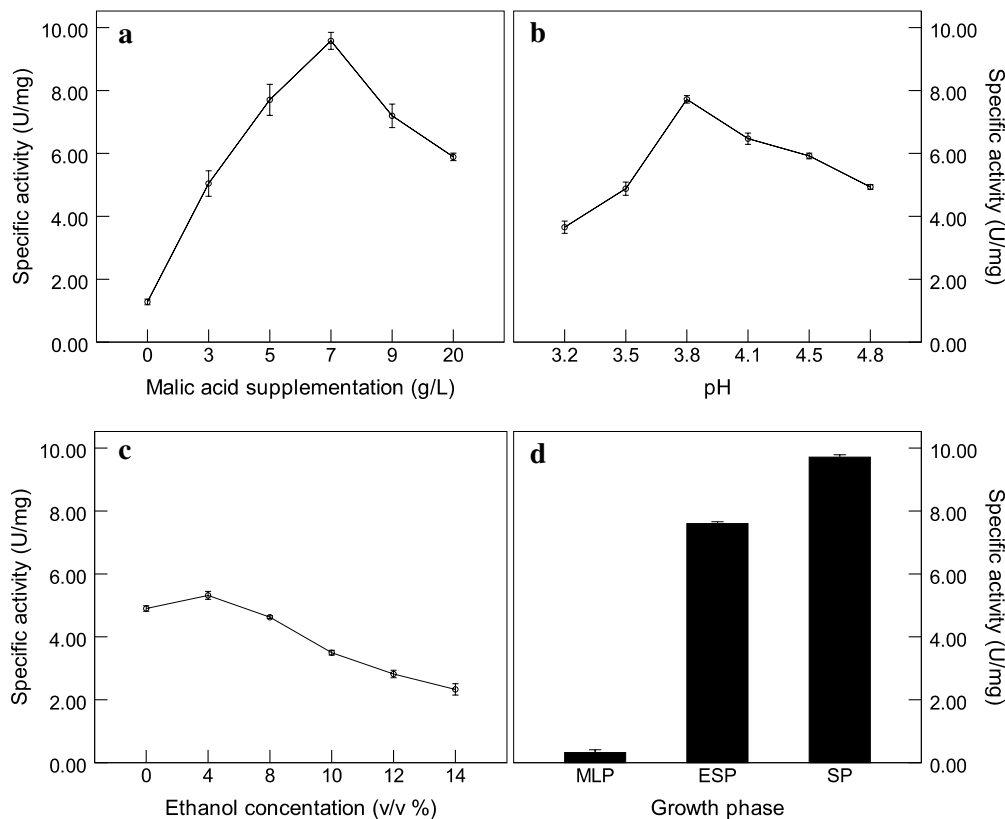
It has been known that L-malic acid, pH and ethanol concentration were the main factors for lactic acid bacteria to

induce biosynthesis of MLE, in order to obtain the optimum condition with the highest MLE activity for the purification of MLE, the effects of L-malic acid, ethanol and pH were studied. The effects of pH and ethanol were studied with an addition of 3 g/L L-malic acid. The results from the induction indicated that the non-inducing *O. oeni* SD-2a showed only slight MLE activity as expected. This was probably attributed to the production of L-malic acid through the metabolic of glucose or fructose.

The effect of L-malic acid concentration on induction of MLE is shown in Fig. 2a. For *O. oeni* SD-2a, the crude extract showed enzyme activity noticeably increased with the L-malic acid concentration increasing from 0 to 7 g/L and enzyme activity substantially decreased when the L-malic acid occurred in the medium at concentrations above 7 g/L. Hence, 7 g/L L-malic acid concentration was identified as the optimal condition for MLE induction, which could increase the enzyme activity considerably. Previous studies [1, 23, 24] also reported that malolactic enzymes could be induced by malic acid and showed the addition of L-malic acid resulted in increased malolactic activity for *Lactobacillus collinoides*. The difference between this study and previous studies was malic acid concentration, and previous studies used low malic acid concentration which usually below 3 g/L.



**Fig. 1** Cellular localization of MLE in *O. oeni* SD-2a



**Fig. 2** Effect of culture parameters on malolactic enzyme biosynthesis. **a** malic acid concentration, **b** pH, **c** ethanol concentration, **d** growth phase (MLP mid-lag phase, ESP early-stationary phase, SP stationary phase)

**Table 2** Purification of MLE in *O. oeni* SD-2a

Purification step	Total protein (mg/mL)	Total enzyme activity (U/mL)	Specific activity (U/mg)	Purification fold (fold)	Yield (%)
Crude extract	282.4	2,731	9.67	1	100
Protamine sulfate precipitation	253.2	2,674	10.56	1.09	97.91
DEAE-Sepharose FF	3.943	1,271	322.4	33.34	46.54
Superdex™ 200	2.839	951.9	335.3	34.67	34.86
Sephacryl S-100	0.02732	11.45	419.2	43.35	0.4194

The effect of culture pH on MLE activity of *O. oeni* SD-2a is shown in Fig. 2b. Based on Fig. 2b, at wine pH values (pH 3–4), *O. oeni* SD-2a had different MLE activity even with a slight pH change, with the highest activity being retained at pH 3.8. Whereas MLE activity was significantly decreased with pH increased in the range of 3.8–4.8. Despite low pH has been described as having negative effect on the growth and metabolic activity [25, 26], it has a positive effect on the MLE activity. The results were in good agreement with the previous studies [1, 17, 27]. The increase in MLE activity in acidic medium could be linked to the fact that MLF played a part in the regulation of the intracellular pH, providing the LAB a biological advantage under stress conditions.

The induction effect of ethanol on MLE was not worthy to be considered. When ethanol concentration was lower than 4 % (v/v), a slight increase in MLE activity was observed as ethanol concentration increased, while when ethanol concentration continued to increase, MLE activity decreased (Fig. 2c). Still half of the highest enzyme activity was detected in the presence of 12 % ethanol in the culture, and 30 % of the activity could still be preserved with 14 % (v/v) ethanol concentration and 3 g/L malic acid supplementation. It was good for wine aging and second fermentation. Our results coincided with early reports that MLF was hindered by high ethanol concentrations [16, 25].

To investigate the effect of growth phase on MLE activity, *O. oeni* SD-2a was cultured in a ATB broth with an addition of L-malic acid 7 g/L. Cells at different stages including mid-log phase, early-stationary phase and stationary phase were harvested by centrifugation. As shown in Fig. 2d, cells in stationary phase possessed notably higher MLE activity than that in other phase while cells in mid-log phase just demonstrated a very low MLE activity, suggesting that MLE production lagged behind cell production, the same situation was observed in real wine system inoculated with *O. oeni* activity [28]. Moreover, the stationary phase cultures retained a high cell density; it was conducive to perform experiment with stationary phase cultures.

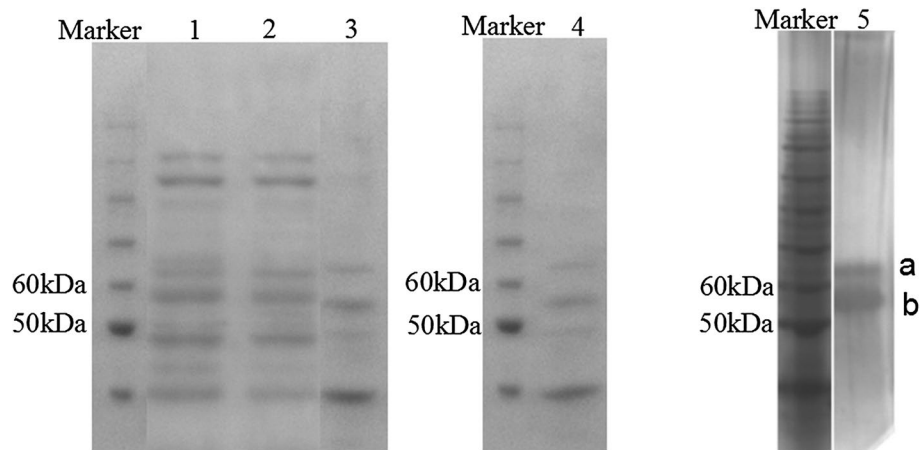
MLE was induced and influenced by a number of factors including L-malic acid concentration, pH, ethanol, temperature, SO<sub>2</sub>, metabolic products and interactions of yeast, as

well as phenolic compounds [17, 18]. In previous studies, the authors simply used a certain amount of L-malic acid to induce the production of MLE [7, 14]. In this study, we investigated the effects of L-malic acid concentration, pH, ethanol concentration and growth phase on the MLE activity. This study paved a foundation for MLE purification.

#### Purification and identification of the malolactic enzyme

MLE was purified from the supernatant of the protamine sulfate-treated crude enzyme extract, followed by ion exchange chromatography on DEAE-Sepharose FF column and two steps of gel filtration on both Superdex™ 200 column and Sephacryl S-100 column. The purification results of MLE are summarized in Table 2. As expected, specific MLE activity was increased and total enzyme activity was lost as more methods were used. Of which, DEAE-Sepharose FF and Sephacryl S-100 were the most two effective ways to purify this enzyme. Purified enzyme was obtained with its specific activity being increased by 43-fold and its specific activity being 419.2 U/mg, but its yield was only 0.42 %. The use of AKTA purifier system saved time and effort compared to conventional approach, and the purification method used in this study had a better repeatability since the chromatographic columns and conditions used were much more stable than previous studies [7, 14].

The purity of MLE was monitored by SDS-PAGE (Fig. 3). Silver staining was used to detect the band corresponding to the purified protein resulting from Sephacryl S-100 column gel filtration. As shown in Fig. 3, there were two bands with molecular masses of around 60 kDa. Further identity was conducted by mass spectrometry. Band a (Fig. 3) was identified as MLE (*O. oeni*) with a score of 1,078; exactly, 9 of the 13 peptides belonged to MLE have scores exceed the threshold (Table 3). As can be drawn from the MS results, the MLE purified from *O. oeni* SD-2a consists of 541 amino acids, exhibiting a nominal molecular mass of 59 kDa and a theoretical pI of 4.76. The amino acid sequence of MLE (*O. oeni*) and the 13 matched peptides of MLE are shown in Fig. 4. Protein sequence coverage of the purified MLE and the MLE of *O. oeni* was 32 %. The MLE has been purified from various species of LAB



**Fig. 3** SDS-PAGE profile of MLE purification. *Lane 1* crude extract; *lane 2* extracts after protamine sulfate precipitation; *lane 3* active fraction after DEAE-Sepharose FF purification; *lane 4* active fraction by gel filtration on Superdex™ 200 column; *lane 5* active fraction by Sephacryl S-100 gel filtration chromatography

**Table 3** Database search result of the purified MLE by MALDI-TOF/TOF mass spectrometry (a) and matched peptide sequence (b)

a					
Protein ID	Protein score	Protein mass (Da)	Isoelectric point	Coverage rate	Description
gil75755626	1078	59125	4.76	32 %	Malolactic enzyme of <i>O. oeni</i>
b					
Number	Peak_List_Id	Peptide	Score <sup>a</sup>		
1	105769	HFFLVDK	42		
2	105772	YYDFIDK	56		
3	105766	AVDDLKWEPK	69		
4	105771	TDPVSILNDPFINK	90		
5	105774	LTDQTYMSFGAGTAGMGIVK	158		
6	105763	LTDQTYMSFGAGTAGMGIVK	99		
7	105764	QLHEEMVEQGLSDEEAKK	133		
8	105776	VQALQEQVDQTYAQFQSK	142		
9	105779	DMSGYTERPIIFPISNPTK	87		
10	105775	DMSGYTERPIIFPISNPTK	49		
11	105778	GTAFTAEEREELGLNGLLPAK	39		
12	105773	QGLLFDDDPDLTPEQKPFAAK	114		
13	105777	FVNHAESLFPNLYLHWEDFGR	148		

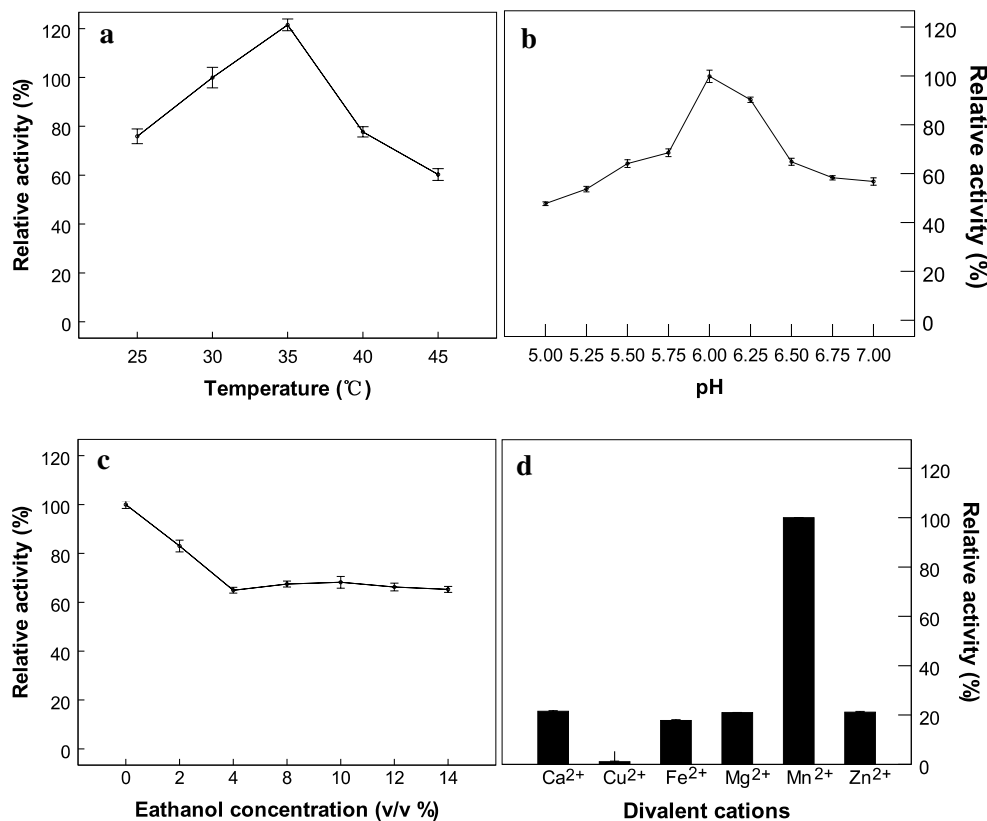
<sup>a</sup> score >60 indicates identity

including *Lactobacillus* spp., *O. oeni* and *Lactococcus lactis* [7, 13–16]. The molecular masses of the subunits of the purified MLEs range from 60 to 70 kDa [19, 20]. The active form of each protein is a dimer or a tetramer consisting of identical subunits [14, 21]. Band b was considered as lactaldehyde dehydrogenase (data not given), which could catalyze the formation of lactate from lactaldehyde with NAD serving as the oxidant. This enzyme was difficult to eliminate from MLE since their molecular mass and pI

were really similar. Various methods were applied to obtain a highly purified MLE, but the results were disappointing. However, it was exciting that this enzyme exhibited a higher enzyme activity compared with enzymes purified from *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and *Leuconostoc oenos* [7, 14, 15]. MLE in *O. oeni* SD-2a was probably combined with lactaldehyde dehydrogenase since there was no conflict in their function of catalyze the formation of lactate.

<u>MTDPVSILND</u>	<u>PFINKGTAFT</u>	<u>EAEREELGLN</u>	<u>GLLPAKVOAL</u>	<u>QEOVDOTYAO</u>
<u>FQSK</u> VSNLEK	RLFLMEIFNT	NHVLFYKLFS	QHVVEFMPIV	YDPTIADTIE
NYSELFVEPQ	GAAFLDINHP	ENIQSTLKNA	ANGRDIKLLV	VSDGEGILGI
GDWGVQGVDI	AVGKLMVYTV	AAGIDPSTVL	AVVIDAGTNN	EKLLKDPMYL
GNKFNVRVGD	<u>KYYDFIDK</u> FV	<u>NHAESLFP</u> NL	<u>YLHWEDFGR</u> S	NASNILNSYK
DKIATFNDDI	QGTGIVVLAG	VLGALKISDQ	<u>KLTDOTYMS</u> F	<u>GAGTAGMG</u> IV
<u>KOLHEEMVE</u> Q	<u>GLSDEEA</u> KKH	<u>FFLVDKQ</u> GLL	<u>FDDDPDL</u> TPE	<u>OKPFAA</u> KRSD
FNNANQLTNL	QAAVEAVHPT	ILVGTSTHPN	SFTEEIVKDM	<u>SGYTER</u> PIIF
<u>PISNPT</u> KLAE	AKAEDVLKWS	NGKALIGTGV	PVDDIEYEGN	AYQIQANNA
LIYPGLGFGA	IAAQSKLLTP	EMISAAHSL	GGIVDTTKVG	AAVLPPVSKL
ADFSRTVAVA	VAKKAVEQGL	NRQPIDDVEK	AVDDLKWEPK	Y

**Fig. 4** Amino acid sequence of MLE (*O. oeni*) and the 13 matched peptides. The sequences *highlighted with underline* represent matched amino acid sequences of MLE by MALDI-TOF/TOF mass spectrometry

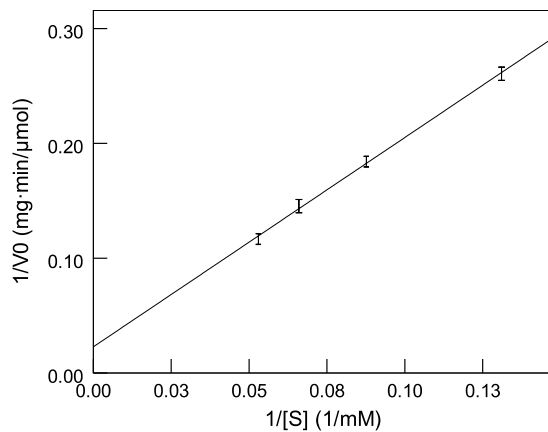


**Fig. 5** Properties of MLE. **a** effect of temperature on purified MLE activity, **b** effect of pH on purified MLE activity, **c** effect of ethanol concentration on purified MLE activity, **d** effect of divalent cations on purified MLE activity

#### Properties of the malolactic enzyme

The active fraction eluted from the Sephacryl S-100 column gel filtration was used as purified MLE to investigate the characterization of MLE; 100 % relative activity was based

on enzyme activities measured at buffer solution without ethanol and with Mn<sup>2+</sup> concentration of 0.4 μmol/L at pH 6.0 and 30 °C. The MLE activity was assayed over a temperature range of 25–45 °C. Our results indicated that the MLE activity increased remarkably by 1.6-fold when the temperature



**Fig. 6** Line weaver-Burk plot

was increased from 25 °C with the maximum activity at 35 °C (Fig. 5a). The activity decreased significantly when temperature continued to rise. As temperatures approached 45 °C, as much as 50 % of the enzyme activity was lost.

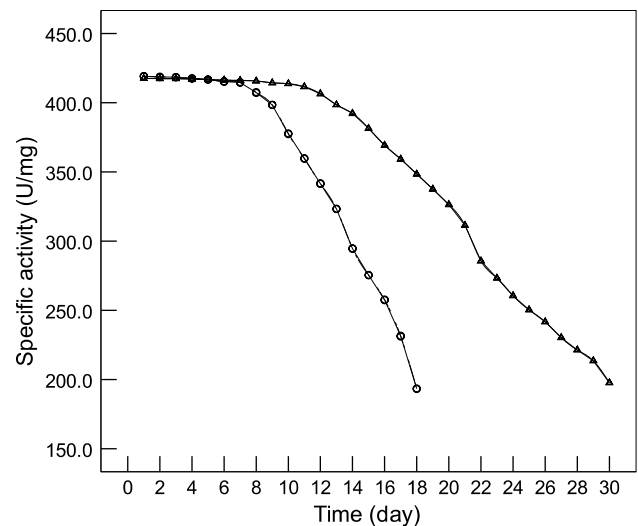
The effect of pH on the purified MLE activity was determined in 0.2 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer at the pH range of 5.0–7.0 (Fig. 5b). In this trial, the enzyme activity increased gradually over a range of pH 5.00–pH 5.75, and then the enzyme activity decreased as pH continued to increase. The maximum enzyme activity was observed at pH 6.0, it was a little higher than the reports that the optimum pH of MLE was approximate 5.8 [7, 15].

The effect of ethanol concentration on the purified MLE activity was studied by incubating the enzyme with ethanol at final concentration of 2, 4, 8, 10, 12 and 14 % (v/v), respectively (Fig. 5c). The results revealed that the enzyme activity declined rapidly when ethanol concentration increased from 0 to 4 %, while the enzyme activity was not significantly influenced by ethanol concentration when it increased from 4 to 14 % even if 10 % ethanol concentration found to cause a slight increase in the enzyme activity.

Effect of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$  on the purified MLE activity was examined (Fig. 5d). All the divalent cations caused a notable decrease of relative activity in comparison with  $\text{Mn}^{2+}$ . Divalent cations such as  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  inhibited the MLE activity by nearly 80 % and  $\text{Cu}^{2+}$  caused almost 99 % activity loss. Compared with previous study [15], MLE purified from *O. oeni* SD-2a exhibited a higher cation cofactor specificity.

#### Kinetic studies

The Line weaver-Burk double-reciprocal plot is shown in Fig. 6. The  $K_m$  and  $V_{max}$  of the purified enzyme for L-malic acid were calculated from the Line weaver-Burk plot to be  $12.51 \times 10^{-3}$  M and 43.86  $\mu\text{mol}/(\text{min} \times \text{mg})$ , respectively.



**Fig. 7** Deactivation curves of the purified enzyme. (closed triangles) enzyme in 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer supplemented with 0.1 M NaCl, (closed circles) enzyme in 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer

#### Half-life

The stability of the purified enzyme preserved in 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0), and the same buffer supplemented with 0.1 M NaCl was evaluated for 18 and 30 days, respectively. The deactivation curves are shown in Fig. 7. As indicated in Fig. 6, the two curves were noticeably different even though no significant difference was observed in the first 7 days, in the following 10 days the enzyme activity was quite different between the enzyme preserved in phosphate buffer and enzyme preserved in phosphate buffer added with NaCl. The purified enzyme preserved in phosphate buffer exhibited a drastic decrease in enzymatic activity while the enzyme kept in the same buffer added with NaCl showed a moderate decrement in enzyme activity. The purified enzyme possessed a half-life of 30 day when preserved in 0.02 M  $\text{KH}_2\text{PO}_4$ – $\text{Na}_2\text{HPO}_4$  buffer (pH 6.0) supplemented with 0.1 M NaCl, and an obvious reduction (as much as 40 %) was observed for the enzyme kept without NaCl. There has been little information regarding the half-life of MLE, and the explanation for this phenomenon was that NaCl can preserve enzyme activity during storage by its antimicrobial effect or as kosmotropic agent that stabilizes the conformation of the enzyme.

#### Conclusions

From the results presented above, it was hard to escape the conclusion that the ability of *O. oeni* SD-2a to synthesize MLE was highly dependent upon the culture parameters,



particularly L-malic acid supplementation. In this study, MLE was successfully purified from *O. oeni* SD-2a using protamine sulfate precipitation, anion exchange chromatography and gel filtration chromatography. The MLE was purified by 43-fold with a yield of 0.42 % and a nominal molecular mass of 59 kDa, and a theoretical pI of 4.76. The purified enzyme displayed its optimal temperature and pH at 35 °C and 6.0, respectively, which had a better stability with a half-life of 30 days in the presence of NaCl. MLE was inhibited by ethanol, and the relative effectiveness of the divalent cations assayed was  $Mn^{2+} > Ca^{2+} > Zn^{2+} > Mg^{2+} > Fe^{2+} > Cu^{2+}$ . Furthermore, investigation on cloning, sequencing and over expression of genes of MLE from *O. oeni* still remains to be done in the future.

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**Conflict of interest** None.

**Compliance with Ethics Requirements** This article does not contain any studies with human or animal subjects.

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