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ORIGINAL ARTICLE

Selection and characterization of *Oenococcus oeni* strains for use as new malolactic fermentation starter cultures

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Abstract *Oenococcus oeni* (*O. oeni*) strains are critically important for winemaking. They bring microbial stabilization, de-acidification and sensory improvement through malolactic fermentation (MLF) to almost all red wines and some white wines. In this study, 23 strains of *O. oeni* isolated from China were evaluated for use as MLF starter cultures. Strains were characterized and screened in wine-like media with increasing selective pressure, such as low pH and high ethanol concentration. Their abilities to conduct MLF were affected significantly by ethanol concentration and pH. Eight strains were selected and subsequently evaluated by detecting the enzyme-encoding genes involved in biogenic amines synthesis and wine aroma modification. Seven well-performing strains were further assessed in newly alcohol-fermented Cabernet Sauvignon wine. The results show that strains *O. oeni* CS-4b and *O. oeni* ME-5b exhibited better performance than the commercial starter *O. oeni* 31MBR in wine. Therefore, they were selected as potential MLF starters suitable for wine with high ethanol concentration and low pH.

Keywords *Oenococcus oeni* · Malolactic fermentation · Synthetic wine medium · Genetic screening · Cabernet Sauvignon wine

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Introduction

Malolactic fermentation (MLF) is of great importance in winemaking as it improves the microbiological stability and the organoleptic traits of wine (Maicas et al. 1999; Versari et al. 1999; Liu 2002; Bauer and Dicks 2004). Many aroma changes also happen during MLF to improve the aroma complexity of wine (Maicas et al. 1999; Gambaro et al. 2001; Liu 2002; Pozo-Bayón et al. 2005; Bartowsky and Borneman 2011). MLF is a secondary fermentation that usually occurs during the storage of young wines, or several weeks after alcoholic fermentation (AF). Lactic acid bacteria (LAB), mainly *Oenococcus oeni* (*O. oeni*), are the dominant strains responsible for MLF. These strains activate the conversion of dicarboxylic L-malic acid to monocarboxylic L-lactic acid and carbon dioxide, and thus cause the de-acidification of wine. The indigenous wine LAB population includes the genera of *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Van Vuuren and Dicks 1993; Lonvaud-Funel 1999; Versari et al. 1999). However, spontaneous MLF is a slow and unpredictable process, and may cause stuck fermentation, off-flavors, toxic metabolites or even wine spoilage. This is because wine is a hostile environment for bacteria growth owing to the low pH, high ethanol concentration, presence of sulfur dioxide and other inhibiting compounds (such as fatty acids, tannins and various chemical residues) (Versari et al. 1999; Gockowiak and Henschke 2003; Rosi et al. 2003; Bauer and Dicks 2004). Therefore, in the wine industry, MLF often begins with inoculation of extra bacterial cultures, like activated commercial starter cultures or wine undergoing MLF fermentation.

Since the first commercial starter was selected in 1974, related research has been done worldwide for the screening of new commercial starters (Coucheney et al. 2005; Ruiz et al. 2010; Lerm et al. 2011; Bordas et al. 2013; del Mónaco et al. 2014; Li et al. 2014). Selections were made based mainly on the

evaluation of each strain's ability to grow and to degrade L-malic acid. Several criteria have been made for the selection of possible starters (Lerm et al. 2011; Torriani et al. 2011). These criteria include the ability to tolerate low pH, high ethanol concentration and SO_2, good growth characteristics under severe winemaking conditions, and the inability to produce biogenic amines, etc. The aim of the selection process is to obtain new strains that are well adapted to the wine environment, and give desirable MLF benefit.

In the majority of cases, *O. oeni* is used more often than other LAB strains due to its good adaptability to the harsh wine environment as well as its favorable effects on the wine aroma and flavor (Versari et al. 1999; Bauer and Dicks 2004; Bartowsky and Borneman 2011). However, induction of MLF by inoculating with commercially available *O. oeni* starters is not always successful, since *O. oeni* is known to be a fastidious bacterium and the wine environment varies in different areas. There is also an increasing demand for new starters with defined technological and flavor tailoring in the wine industry. Given that the effects of MLF are largely dependent on the strains conducting this process, selection of new starter cultures is an effective approach to improve the quality of MLF and to fulfill increasing industrial needs (Solieri et al. 2010).

In this study, 23 *O. oeni* strains isolated from China were evaluated for the possible use of new MLF starter cultures. The first objective was to characterize the strains' ability to consume L-malic acid and to grow in synthetic wine media with increasing selective pressure, such as low pH (3.8, 3.5, 3.2) and high ethanol concentrations (10 %, 12 % and 14 %). The second objective was to evaluate the MLF capacity of the promising strains in the newly alcoholic fermented wine. This study provided a new framework for the selection of MLF starter cultures with specific technological properties. The results obtained in this study will help researchers understand the MLF properties of the indigenous LAB strains in China.

Materials and methods

Bacterial isolates and culture conditions

The isolates used in this study are listed in Table 1. These strains were isolated from the must of grapes cultivating in Yangling District China, and identified as *O. oeni* using16S rDNA PCR-RFLP analysis (Liu and Yu 2010). *O. oeni* 31MBR, *Lactobacillus brevis* ATCC 367 and *Lactobacillus* ATCC 33222 were used as reference strains.

Representative sampling of the synthetic wine media or wine was carried out for the microbiological enumeration before MLF, and at regular intervals during MLF. The ingredients of the synthetic medium without ethanol are provided in online resource Table S1. Microbiological populations were monitored by plate counting on ATB agar plates. ATB agar contains glucose, 1 g/L; fructose, 5 g/L; L-malic acid, 7 g/L; yeast extract, 5 g/L; peptone, 10 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $MnSO_4 \cdot 4H_2O$, 0.05 g/L; cysteine/ HCl, 0.5 g/L; tomato juice, 250 mL/L; agar, 20 g/L; adjusted to pH 4.8 with NaOH. Synthetic wine media and wine samples were serially diluted ten-fold in 154 mmol/L NaCl, and separately inoculated on Petri dishes with 20 mL ATB agar. Each dilution was plated out in duplicate and incubated at 25 °C for 2–4 days depending on the growth of each strain. After incubation, numbers of bacterial colonies formed on ATB agar were visually counted and expressed as CFU/mL.

An adaptation medium contained ATB 50 g/L, D-fructose 40 g/L, D-glucose 20 g/L, L-malic acid 3 g/L, Tween 80 1 g/L, absolute ethanol 4 % v/v was used in this study. The pH of the adaptation medium was adjusted to 4.6 using sodium hydroxide. The adaptation medium was filter-sterilized using 0.22 μm membrane filter (Whatman International, Maidstone, UK) before use. Strains pre-cultured in the ATB broth were inoculated into the adaptation medium at 2 % (v/v) and cultivated to mid-exponential phase before being inoculated into the synthetic wine media or wine.

Screening and characterization in the synthetic wine medium

The ability of *O. oeni* strains to degrade L-malic acid under the increasing selective pressure was evaluated in synthetic wine media. The synthetic wine media used in this study were made based on the wine environment and the essential nutrient requirements of the wine-related LAB (Terrade and Mira de Orduña 2009). Nine different fermentation conditions were simulated using synthetic wine medium: 10 %, 12 % and 15 % ethanol (v/v, the same below), at pH 3.2, pH 3.5 and pH 3.8. The synthetic wine media were sterilized by membrane filtration using 0.22 μm sterile membrane filter. Twenty-three *O. oeni* strains and the commercial starter culture *O. oeni* 31MBR were inoculated into the synthetic wine media to reach a final concentration of ~ 10^6 CFU/mL. After inoculation, synthetic wine media were incubated at 20 °C under static anaerobic environment to conduct MLF. Bacterial enumeration was carried out during MLF on a 3-day basis. L-Malic acid concentration was detected every day using an enzymatic kit (Megazyme International Ireland, Bray, Co. Wicklow, Ireland).

Genetic screening

Genes encoding histidine decarboxylase (HDC), tyrosine decarboxylase (TDC) and ornithine decarboxylase (ODC) were detected in selected *O. oeni* strains using a multiplex-PCR method. Gene fragments were PCR-amplified with DNA polymerase using 100 ng template DNA and 0.3 μmol/L, 1 μmol/L and 2 μmol/L of primer sets HDC3/HDC2, ODC1/ODC2 and TD2/TD5, respectively. The amplification procedure was 95 °C for 5 min, 35 cycles of 1 min at 95 °C, 52 °C for 1 min, 72 °C for

Table 1 Strains used in this study

Strain	Species	Origin	Strain	Species	Origin
CS-1a	*O. oeni*	Cabernet Sauvignon	ME-3b	*O. oeni*	Merlot
CS-2a	*O. oeni*	Cabernet Sauvignon	ME-4b	*O. oeni*	Merlot
CS-3a	*O. oeni*	Cabernet Sauvignon	ME-5b	*O. oeni*	Merlot
CS-4a	*O. oeni*	Cabernet Sauvignon	ME-6b	*O. oeni*	Merlot
CS-4b	*O. oeni*	Cabernet Sauvignon	ME-2c	*O. oeni*	Merlot
CS-1c	*O. oeni*	Cabernet Sauvignon	ME-4c	*O. oeni*	Merlot
SD-zhy	*O. oeni*	Sauvignon Blanc	ME-6c	*O. oeni*	Merlot
ME-2a	*O. oeni*	Merlot	ME-2d	*O. oeni*	Merlot
ME-3a	*O. oeni*	Merlot	ME-5d	*O. oeni*	Merlot
ME-4a	*O. oeni*	Merlot	ME-6d	*O. oeni*	Merlot
ME-1b	*O. oeni*	Merlot	ME-5e	*O. oeni*	Merlot
ME-2b	*O. oeni*	Merlot			

90 s, with a final extension at 72 °C for 5 min (Marcobal et al. 2005; Coton et al. 2010). Strains with the ability to degrade L-malic acid in synthetic wine media and without any of the genes related to the production of biogenic amines were further screened for the presence of genes encoding β-glucosidase, esterase and phenolic acid decarboxylase (PAD). All primers used in this study were synthesized by Sangon Biotech (Shanghai, China) and were listed in online resource Table S2. *TaKaRa Ex Taq* DNA polymerase (TaKaRa Biomedicals, Shiga, Japan) was used for the PCR experiments according to the manufacturer's instruction.

Aliquots of 5 μL of each PCR sample were analyzed by electrophoresis on 1.0 % agarose gels containing 0.2 μg/mL ethidium bromide. Agarose gels were run for almost 60 min at 80 V in 0.5 × TBE buffer (44.5 mmol/L Tris-boracic acid, 1 mmol/L EDTA). A 200-bp DNA ladder (Sangon Biotech, Shanghai, China) was used as the standard molecular weight marker.

Evaluating the MLF capability in newly alcohol-fermented Cabernet Sauvignon wine

The MLF capacity of individual *O. oeni* strain was evaluated in duplicate in newly alcohol-fermented Cabernet Sauvignon wine. Cells of the selected strains were collected in exponential phase and inoculated into Cabernet Sauvignon wine to reach a final concentration of ~10^6 CFU/mL. Wine samples were filter-sterilized prior to the inoculation of bacterial cultures. *O. oeni* 31MBR served as a control. Newly alcohol-fermented wine without filtration and inoculation was used as a control of the spontaneous MLF. MLF was conducted at 20 °C. L-Malic acid concentration was measured on a 5-day basis to monitor the L-malic acid degradation. Bacterial growth was monitored by plating samples out on Petri dishes with 20 mL ATB agar, supplementing with 0.1 mg/L cycloheximide to inhibit yeasts and 25 mg/L kanamycin sulfate to suppress the growth of acetic acid bacteria.

Results

Screening and characterization of the isolated strains in synthetic wine medium

Synthetic wine media with increasing selective pressure, i.e., low pH and high ethanol concentrations were used to evaluate the strains' ability to grow and to consume L-malic acid in the simulated wine environments. Strains were inoculated into synthetic wine media to reach a final concentration of ~10^6 CFU/mL. After inoculation, L-malic acid concentrations of the synthetic wine media were detected daily. Figure 1 shows the degradation of L-malic acid 7 days after inoculation. As can be seen from Fig. 1, all strains can degrade L-malic acid at the mild condition, pH 3.8 with 10 % ethanol. However, the performance of CS-3a, ME-6b, ME-6d and ME-5e were not ideal. Both CS-1c and ME-6d could degrade L-malic acid in synthetic wine media with 10 % ethanol at pH 3.2, 3.5 and 3.8, while CS-1c was significantly ($P < 0.05$) inhibited, and ME-6d was significantly ($P < 0.05$) activated, by low pH. ME-2b was distinct since it could suffer high ethanol concentration but could not survive when the pH was lower than 3.8. CS-2a, ME-2a, ME-2c, ME-6c, ME-2d, ME-4d and ME-5d could tolerate either low pH (3.2) or high ethanol concentration (14 %). Nine strains, namely CS-1a, CS-4a, CS-4b, ME-3a, ME-4a, ME-1b, ME-3b, ME-5b and SD-zhy were capable of degrading L-malic acid under various conditions in the synthetic wine media and were selected for further screening. In comparison with 31MBR, all

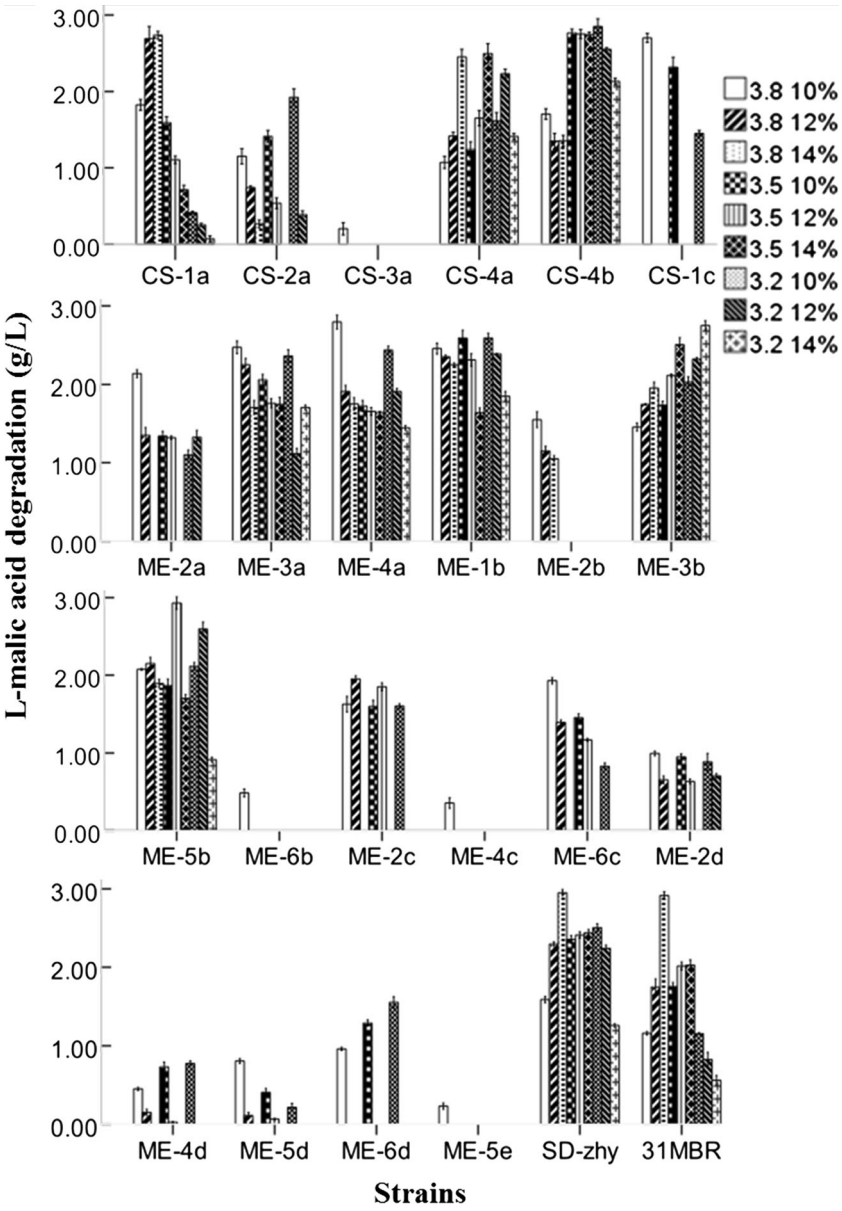

Fig. 1 Degradation of L-malic acid by different *Oenococcus oeni* strains in the synthetic wine media with increasing selective pressure. L-Malic acid degradation was tested 7 days after inoculation. The selective pressure included high ethanol concentration (14 %, 12 %, 10 %) and low pH (3.8, 3.5, 3.2)

these strains except CS-1a degraded more L-malic acid at low pH conditions. Moreover, SD-zhy showed a better performance than 31MBR at high ethanol concentration.

L-Malic acid degradation rate was not the only standard for the selection of MLF starters; the ability to proliferate and to maintain sufficient population in the harsh wine environment should also be considered. Typical results of cell numeration are shown in Fig. 2. Data in Fig. 2 were obtained at different times of MLF in the synthetic wine medium with 14 % ethanol and pH 3.2. Figure 2 shows that all strains display a positive growth. Four strains, CS-4a, CS-4b, ME-3a and ME-5b showed better performance than 31MBR. However, CS-1a showed only a slight increase in population 9 days after inoculation. Therefore, this strain was rejected at this step.

PCR detection of genes involved in biogenic amines production and genes encoding enzymes of enological interest

A total of eight *O. oeni* strains that could successfully proliferate and degrade L-malic acid under the simulated harsh wine conditions (pH 3.2, 14 % ethanol), were selected to detect the genes encoding amino acid decarboxylases. Amino acid decarboxylases are responsible for the biogenic amines production. *Lactobacillus brevis* ATCC 367 and *Lactobacillus* ATCC 33222 served as positive controls for genes encoding HDC, ODC and TDC. The amplification of genes encoding HDC, ODC or TDC was not observed in the eight tested strains (results not given).

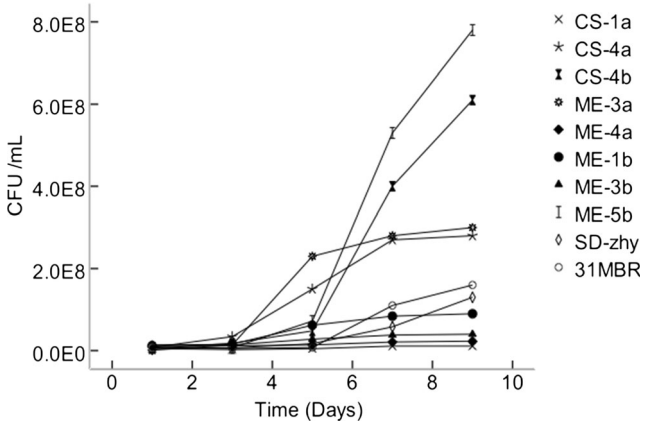

Fig. 2 Cell count results of the nine selected strains in the synthetic wine medium with 14 % ethanol and pH 3.2. Data given show the average variation in cell counts of each strain in duplicate. The relative standard deviation (RSD) is less than 10 % between repetitions

PCR amplification of the genes encoding β-glucosidase, esterase and PAD were conducted for strains without the potential to produce biogenic amines, and the results were presented in Fig. 3. As indicated in Fig. 3, 31MBR was positive for all three detected genes. Six strains were positive for genes encoding β-glucosidase, seven strains were positive for esterase-encoding genes. Only three strains were positive for genes encoding PAD, i.e., CS-4a, ME-1b, ME-3b. In this part, ME-4a was eliminated due to the lack of all these aroma-related genes.

Malolactic fermentation in newly alcohol-fermented Cabernet Sauvignon wine

Strains that were able to survive and to consume L-malic acid in simulated harsh wine condition without any genes involved in biogenic amine production, and with at least one kind of enzyme related with wine aroma, were assessed in the newly alcohol-fermented Cabernet Sauvignon wine at laboratory scale. Parameters of the wine before inoculating of bacterial strains were as follows: L-malic acid 2.97 g/L, pH 3.5, ethanol content 13 % and free SO_2 concentration 20 mg/L.

The results of bacterial numeration at the 1st month of MLF are shown in Fig. 4a. The *O. oeni* population showed a remarkable decrease in the first 5 days, followed by a slight increase in viable cells. ME-5b showed a better ability to proliferate in wine compared with 31MBR. The L-malic acid concentration of the wine inoculated with different strains was detected on the same day, and the results were given in Fig. 4b. Figure 4b shows the L-malic acid concentration stays stable in wine undergoing spontaneous MLF, and decreases obviously in wine inoculated with the selected *O. oeni* strains. Compared with 31MBR, CS-4b and ME-5b exhibited better performance to degrade L-malic acid. Moreover, CS-4b was the only strain that completed MLF in 18 days. Additional experiments proved that ME-5b and 31MBR completed MLF in 33 and 35 days, respectively. Surprisingly, the viable cells of CS-4b stayed at a low level during MLF, although CS-4b was the first strain that finished MLF. Almost no *O. oeni* strain was detected during the spontaneous MLF.

Discussion

This study aimed at characterizing and evaluating the MLF capacity of 23 *O. oeni* strains isolated from China and screening for new MLF starter cultures. MLF capability was first evaluated in synthetic wine media with different pH and ethanol concentrations. Observations on the performance of each strain in the synthetic wine medium presented an indication of the behavior in a similar wine environment. The effects of ethanol and pH on MLF performance were largely dependent on the strains conducting this process. All isolates could survive and degrade L-malic acid under mild conditions (pH 3.8 and 10 % ethanol), but only nine strains could tolerate the harsh condition (pH 3.2 and 14 % ethanol). In this study, *O. oeni* strains showed a better L-malic degradation capability in the synthetic wine medium than previous studies (Capozzi et al. 2010; Ruiz et al. 2010; Lerm et al. 2011). This result was expected since the synthetic wine media used in this study were designed to meet the essential nutrient demands of different *O. oeni* strains. ME-3a was highlighted for its positive reaction to high ethanol concentration and low pH in the synthetic wine media. The results presented above suggested that CS-1a and ME-1b worked well in wine with high pH, CS-4b and ME-3b could be possible starter cultures for wine with low pH, ME-4a and ME-1b presented the potential to conduct MLF in wine with low ethanol content, ME-3b and SD-zhy could be promising strains to conduct MLF in wine with high ethanol concentration.

Genetic screening of the eight strains that could successfully survive and consume L-malic acid under harsh conditions was carried out using PCR-based methods. Biogenic amines can destroy the aroma and wholesomeness of wine and have several health implications (Guerrini et al. 2002; Ancín-Azpilicueta et al. 2008). Therefore, the absence of genes encoding enzymes concerned with biogenic amines formation is an important characteristic for any strain being considered for use as a starter culture. Moreno-Arribas et al. (2000) reported that *O. oeni* was identified as the main histamine producer, and 60 % of the *O. oeni* strains studied by Guerrini et al. (2002) were able to produce histamine. However, in this study the amplification of genes encoding HDC, TDC or ODC was not observed in the selected isolates, indicating that these strains have a low possibility to produce biogenic amines.

The selected strains were further screened for genes encoding enzymes that relate closely to wine aroma modification and enrichment, such as β-glucosidase, esterase and PAD. Bacteria strains with β-glucosidase can hydrolyze the precursors of glucosides, and thus release volatile compounds and

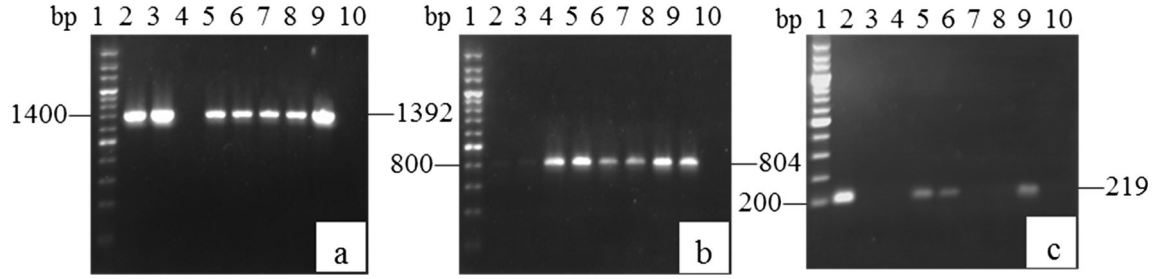

Fig. 3 PCR results showing the presence of genes encoding **a** β-glucosidase, **b** esterase, and **c** phenolic acid decarboxylase. Lanes: *1* 200 bp standard DNA marker, *2* CS-4a, *3* CS-4b, *4* ME-3a, *5* ME-1b, *6* ME-3b, *7* ME-5b, *8* SD-zhy, *9* 31MBR, *10* negative control

enrich the sensory profiles of wine (De Revel et al. 1999; Maicas et al. 1999; Gambaro et al. 2001; Liu 2002; Matthews et al. 2006; Michlmayr et al. 2010; Mtshali et al. 2010; Baffi et al. 2013; Pérez-Martín et al. 2014). Six of the eight selected strains are positive for β-glucosidase-encoding genes, and have the potential to release glycosidically bound flavor compounds. Seven strains possess genes encoding PAD. Due to the presence of PAD, strains are able to metabolize various phenolic acids to generate aroma compounds and add to the complexity of the wine aroma (Cavin et al. 1993; Cabrita et al. 2008). Esterase that originates from wine LAB are responsible for biosynthesis and hydrolysis of esters (Pérez-Martín et al. 2013; Sumby et al. 2013a, b). Different from previous studies, only three strains were positive for esterase-encoding genes (Lerm et al. 2011). The results of the enzymatic screening confirm the presence or absence of the genes, and indicate that these strains have the potential to express the corresponding enzyme; however, they give no indication about the enzymatic activity during MLF in wine. Research on the related enzymatic activity of the potential strains, and the effects of different strains on wine sensory profiles are ongoing.

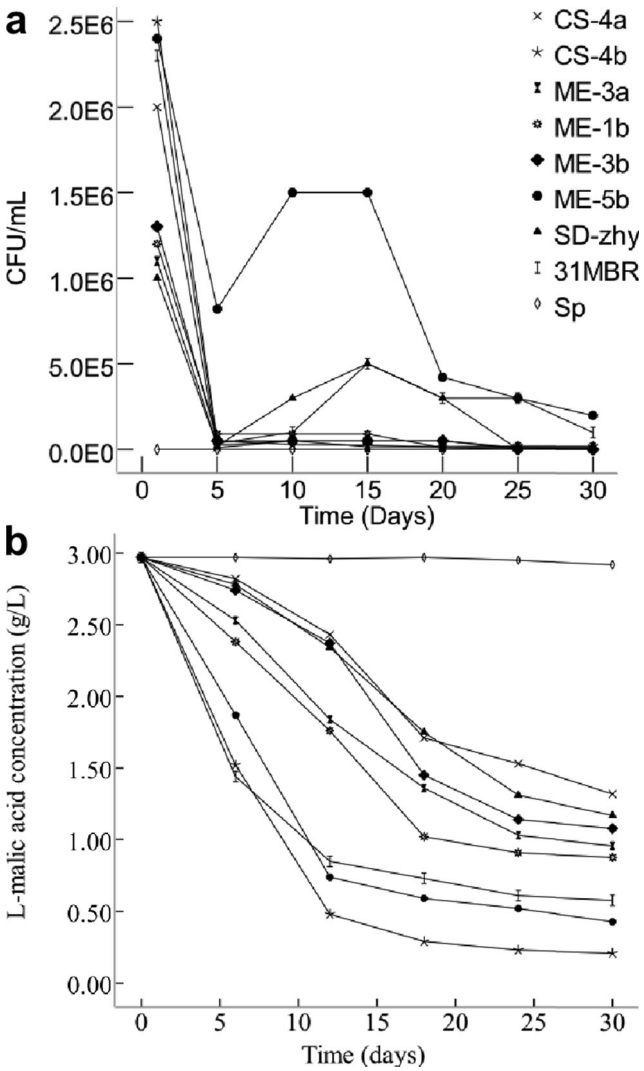

Fig. 4 **a** Cell count results of wine inoculated with seven selected strains, commercial starter culture 31MBR and wine undergone spontaneous malolactic fermentation. *Sp* Spontaneous malolactic fermentation. **b** Changes in L-malic acid concentration in wine inoculated with different *O. oeni* strains. Data given show the average changes in L-malic acid concentration (g/L) of each treatment repeated in duplicate. The RSD is less than 10 % between repetitions

Finally, the MLF performance of the selected strains was evaluated in newly alcoholic fermented wine. Wine is a much more challenging environment for the selected strains compared with the synthetic wine medium due to its low level of nutrients, numerous inhibiting compounds, such as fatty acids, tannins and chemical residues, and, more importantly, the presence of SO_2. SO_2 inhibits the growth of *O. oeni* strains as well as its ability to conduct MLF. A relatively high SO_2 level, 20 mg/L free SO_2, was observed in the Cabernet Sauvignon wine used in this study (Bauer and Dicks 2004). However, both CS-4b and ME-5b showed better MLF performance than the commercial starter 31MBR in the Cabernet Sauvignon wine, and have the potential to be used as starter cultures. Moreover, CS-4b can complete MLF within 20 days, making it attractive for industrial application. The performance of ME-3a in wine was not as good as that in the synthetic wine medium. ME-3a performed well in synthetic wine medium but could not proliferate or induce MLF in Cabernet Sauvignon wine. Given that some *O. oeni* strains have special nutrient requirements, particularly with regard to amino acids and vitamins, and could be generally inhibited by SO_2, tannins or phenolic acid in wine, the results were reasonable (Gockowiak and Henschke 2003; Osborne and Edwards 2006; García-Ruiz et al. 2009; Terrade and Mira de Orduña 2009). ME-3a was isolated from Merlot, which has a

lower tannin level than Cabernet Sauvignon. Hence, we are very interested in evaluating the MLF performance of ME-3a in Merlot, and plan to conduct related research in the future. The viable cells of the tested strains stayed at a lower concentration, and the time needed for MLF was slightly longer than that reported in previous studies since no extra nutrient supplements were added to the wine during MLF (Ruiz et al. 2010; Lerm et al. 2011).

In conclusion, 23 *O. oeni* isolated from China were characterized and screened by assays in wine-like media with increasing selective pressure. Results on MLF performance of different strains under simulated winemaking conditions supports the use of such strains in wine with similar environment. Combined with the results of genetic screening and evaluation in newly alcohol-fermented wine, *O. oeni* CS-4b and *O. oeni* ME-5b were finally selected to be considered as an MLF starter culture suitable for wine with high ethanol content and low pH.

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