Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(5):136-144

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Screening of lactic acid bacteria with high activities malolactic enzyme and analysis of indigenous flora in red wine

Kai Liu[1], Jianhua Luo[2], Dawei Gao[1], Zhiwei Liu[1], Yanping Liu[1], Nan Li[1], Yanhong Bian[1], Ruiyu Zheng[1] and Xuwu Zhang[1]

[1]Applied Chemical Key Lab of Hebei Province, College of Environmental and Chemical Engineering, Yanshan University, No.438 Hebei Street, Qinhuangdao, China
[2]Huailai County Aristocratic Manor Wine Limited Company, Zhangjiakou, China

---

ABSTRACT

Lactic acid bacteria (LAB) carrying malolactic fermentation (MLF) have an important effect on the quality of red wines. The object was to study LAB biodiversity and screen the strains with the high malo-lactic enzyme activites as oenological starter cultures in MLF. A total of 60 LAB strains were isolated from Yueqiannian dry red wine samples in the fermenter (Changli region). On the basis of morphological observation and physiological assays, the coccis (51 strains) and rods (9 strains) were respectively confirmed as Oenococcus and Lactobacillus. 16S rRNA sequence analyses were used to identify C10, J2, J5 and MJ12 strains to species level. The strains were blasted in GenBank, C10, J2, J5 strains were identified as Oenococcus oeni, and MJ12 was identified as Lactobacillus vini. The 30 isolates were grouped into seven different genotypes at 70% similarity by means of randomly amplified polymorphic DNA (RAPD), indicating a high genetic diversity of Oenococcus spp. In addition, alcohol and acidic resistance experiments for the strains showed that O. oeni sp. C10 and O. oeni sp. J2 has the capability of growing in wine-like conditions [pH 3.0; ethanol concentration of 14% (v/v)]. Moreover, the two strains could conduct MLF, and malic acid degradation rate was 430.625 mg/(L·day) and 76.994 mg/(L·day), respectively. Therefore, O. oeni sp. C10 and O. oeni sp. J2 can be envisaged as starter cultures for MLF in red wines.

Key words: Malolactic enzyme; Dry red wine; Indigenous flora; Lactic acid bacteria; Starter cultures

---

INTRODUCTION

Malolactic fermentation (MLF) in wine is a secondary fermentation that usually occurs at the end of alcoholic fermentation by yeasts, although it sometimes occurs earlier. Malate is decarboxylated to lactate by malolactic enzyme of lactic acid bacteria (LAB). LAB exists naturally on grapes [1] and in wineries [2]. Some species of LAB associated with MLF play an important role in winemaking, because with the conversion of malate to lactate and carbon dioxide during MLF [3], total acidity is lowered, the biological stability is improved and the organoleptic properties of the wines are modified [4]. In high acidity wines produced in cool-climate regions such as Changli County (119°09′E, 39°43′N), lowering acidity by MLF has been proven to be a useful and indispensable way. MLF can occur spontaneously in wines, but it starts randomly and is difficult to control. Therefore, winemakers recently used LAB starter cultures to induce MLF in wine. However, induction of MLF by inoculation with commercially available strains is not always successful, for the bacteria fail to adapt in a very harsh environment [5-6] or because of cellular damage during storage of the commercial malolactic bacteria [3]. Selection of indigenous strains for wine inoculation based essentially on the survival in wine can help solve this problem [5].

LAB appearing in the winemaking process belongs to the *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* genera. The species have been found in grapes must and wine are listed: *L.bobalius*, *L.brevis*, *L.buchneri*, *L.casei*,

L.collinoides, *L.delbrueckii*, *L.fermentum*, *L.fructivorans*, *L.hilgardii*, *L.kunkeei*, *L.lindneri*, *L.mali*, *L.nagelii*, *P.parvulus*, *P.pentosaceus*, *Leuc.mesenteroides*, *Leuc.paramesenteroides* and *O.oeni* [1,7-8]. In most cases, *O.oeni* is the only species identified when the fermentation is completed owning to their higher tolerance towards environmental determinants, such as high ethanol levels, low pH and temperatures and sulphur dioxide [4,9]. For this reason, most commercial starter cultures are strains of *O.oeni* [6].

A complete comprehension of microbial flora in red wine can provide valuable information for future quality control, as well as fermentation monitoring. Using indigenous strains as starter cultures can help overcome the problems encountered in MLF as explained above, make full utilization of local microorganism resources, and brew red wines with local characteristics. However, at the moment little is known about the bacterial population of red wines in Changli County, and the used starter cultures are imported from France, Sweden and Australia. Thus, we isolated LAB in Yueqiannian dry red wine, identified them by means of both physiological assays and molecular techniques, and then screened the most suitable strains as starter cultures.

EXPERIMENTAL SECTION

2.1 Wine samples and reagents

As shown in Table 1, all wine samples (produce date, 2010.11.27) were obtained from Changli Yueqiangnian Winery Co., Ltd (Changli Region, Northeast of China), and confirmed to undergo spontaneous MLF by measuring pH of the wines in winery. Cycloheximide was purchased from Sigma (USA). Lysozyme, proteinase K, primer 27F, primer 1495R, and primer R2 were purchased from Sangon (CHN). PCR Clean-Up Kit was obtained from Sunbiotech (CHN). Methanol was of chromatographic grade and the other reagents were of analytical grade.

Table 1 Wine samples used in the study

	Wine samples	Code
1	Cabernet Sauvignon	C
2	Kyoho	J
3	Muscat	S

The code indicates the source of the strains.

2.2 Isolation and cultivation of strains

The isolation was performed respectively by scratching wine samples onto the plates of ATB and MRS medium. ATB medium (pH 4.8) consisted of peptone (1% m/v), glucose (1% m/v), yeast extract (0.5% m/v), $MgSO_4 \cdot 7H_2O$ (0.2% m/v), $MnSO_4 \cdot 4H_2O$ (0.05% m/v), tomato juice (25% v/v) and agar (2% m/v). MRS medium (pH 6.2) was made up of peptone (1% m/v), beef extract (1% m/v), glucose (2% m/v), sodium acetate (0.5% m/v), ammonium citrate dibasic (0.2% m/v), K_2HPO_4 (0.6% m/v), $MgSO_4 \cdot 7H_2O$ (0.058% m/v), $MnSO_4 \cdot 4H_2O$ (0.025% m/v), tween 80 (0.1% v/v) and agar (2% m/v). Growth of yeast was suppressed by adding 50 mg/L cycloheximide to the media. The plates were incubated anaerobically at 28 ℃ for 7 days. Purified cultures were stored on medium slants at 4 ℃ or maintained at -80 ℃ in 20 % (v/v) glycerol until identification.

2.3 Preliminary identification

Gram staining and catalase tests were performed on each isolate. Gram-positive and Catalase-negative strains were selected and inoculated in glucose. Paper chromatography was used to detect lactic acid in glucose fermentation broth, and operated in n-butyl alcohol/acetic acid/water (100/25/25 v/v) added with phenol bromophenol blue (0.1 % m/v). 2 % (v/v) lactic acid was used as the standard. After drying, the occurrence of yellow spots in the same *Rf* value indicated lactic acid production as compared with the standard.

2.4 Biochemical and physiological assay

The isolates were identified up to genera level through gelatin liquefaction, tryptophan hydroiysis and hydrogen sulfide production tests. The strains were cultivated in gelatin medium, peptone water medium and lead acetate medium at 28℃for 4 days, respectively, then corresponding reaction phenomena was observed [10]. Furthermore, acid and ethanol tolerance abilities were monitored spectrophotometrically by measuring the optical density (OD) at 600 nm, after cultivation in ATB broth added with HCl or absolute ethyl alcohol to achieve various pH values (3.0, 3.2, 3.4) or ethanol concentrations (10%, 12%, 14% v/v), respectively.

2.5 DNA extraction

After growing at 28 ℃ in ATB broth for 48 h, the bacterial cells were harvested by centrifugation at 4,000 rpm for 20 min, washed with TE buffer. Genomic DNA extraction was performed by methods described in [11] with small modifications. Bacterial pellets were dissolved in 500 μL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 50 μL lysozyme solution (50 mg lysozyme in 100 μL of TE buffer). After incubation at 37℃ for 4 h, 125 μL 2% (v/v)

sodium dodecyl sulfate (SDS) and 25 μL proteinase K (10 mg/mL) was added. The solution was vortexed and successively incubated for 1 h at 37 ℃. Afterwards, 650 μL of phenol/chloroform/isoamyl-alcohol (25/24/1 v/v) was added for extractions. This mixture was mixed thoroughly and centrifuged for 10 min at 12,000 rpm. The upper phase was transferred to a new tube, after which 500 μL of trichloromethane was added and the mixture was centrifuged for 5 min at 12,000 rpm. Nucleic acids were recovered by addition of 500 μL of isopropanol and centrifugation for 15 min at 12,000 rpm. The DNA pellet was rinsed with 70% ethanol, air dried, redissolved in 100 μL of TE buffer and stored at -20 ℃.

2.6 16S rRNA analysis

Bacterial small subunit rRNA genes were selectively amplified from purified genomic DNA using the bacteria-specific forward primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′ corresponding to *Escherichia coli* positions 8 to 27) and reverse primer 1495R (5′-CTACGGCTACCTTGTTACGA-3′ corresponding to *E. coli* positions 1514 to 1495) according to Bae et al. (2004) [12] and Chen et al. (2008) [13]. The 25 μL PCR conditions were: one cycle at 94 ℃ for 3 min, 30 cycles of 94 ℃ for 1 min, 58 ℃ for 1 min and 72 ℃ for 2 min. Final extension was carried out at 72 ℃ for 10 min.

The PCR products were purified with a PCR Clean-Up Kit as recommended by the manufacturer, then sequenced by Beijing Sunbiotech Co., Ltd. The resulting sequences were compared with DNA sequences from the National Center for Biotechnology Information (NCBI) database located at http://www.ncbi.nlm.nih.gov using BLAST programme [14]. Phylogenetic trees were generated using MEGA5 software (version 5.05) and displayed using TREEVIEW software.

2.7 RAPD analysis

The PCR protocol proposed by Vigentini et al. (2009) [15] was modified. Amplification was carried out in a total volume of 25 μL using primer R2 (5′-CTGAAGCGGA-3′). The initial denaturation was at 94 ℃ for 3 min, 35 cycles of 94 °C denaturation for 30 s, 36 °C annealing for 30 s and 72 °C elongation for 45 s, final extension at 72 °C for 10 min. Amplified products were resolved by electrophoresis on a 1% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.3) at 5 V/cm for 30 min.

RAPD gels were visualized by UV illumination at 254 nm and photographed with a Zoom Digital Camera (KODAK, USA). The patterns were normalized and further processed using Quantity One pattern analysis software package. This programme grouped the isolates by the pearson product moment correlation coefficient (r) and performed cluster analysis by the unweighted average pair-group (UPGMA) method.

2.8 MLF detection

The strains showing high acid and ethanol tolerance were inoculated in wine samples without undergoing MLF at quantity of 10^5 CFU/mL. Changes of malic acid and lactic acid were detected by high performance liquid chromatography (HPLC). The chromatographic conditions were as followings: Angilent ZORBAX XDB-C18 column (250 mm × 4.6 mm, 5 μm) used at room temperature; Methanol and water (5:95) (v/v) consisting of 1 % (m/v) phosphoric acid used as mobile phase at a flow rate of 0.8 mL/min; UV detector wavelength was 214 nm and injection volume was 20 μL. The peak area response to the concentration of malic acid (r= 0.9973) and lactic acid (r=0.9981) was linear over the range 0.15-20 g/L. pH of the wine samples was measured by precision pH meter (Yidian, Shanghai, CHN).

RESULTS AND DISCUSSION

3.1 Isolation and preliminary identification

Guerrini et al. (2003) [4] demonstrated that a considerable heterogeneity exists among isolates from wine, thus its adaptability to wine and influence on wine quality are strain-specific. LAB naturally existing in must or wines may maintain qualitative and organoleptic characteristics of wine. Therefore, this work was carried out on isolation, identification and characterization of indigenous LAB in dry red wine. In the study, a total of 60 Gram-positive, catalase-negative and lactic acid-productive strains were isolated and identified as LAB. Fourteen of them were collected from wine sample obtained from Cabernet Sauvignon grapes, 25 isolates from Kyoho grapes, others from Muscat grapes. In addition, all of them could produce lactic acid from glucose, so they were identified as LAB. 51 strains mostly growing on ATB medium were coccis and 9 strains isolated on MRS medium were rods. The coccis and rods could be presumptively discriminated by colony morphology and color: the former were gray and less than 1 mm in diameter, the later was milky white and larger in size. Both the coccis and the rods were arranged in pairs or a chain.

3.2 Further identification and tolerance to acid and ethanol

Further identification of LAB based on phenotypic characteristics is regarded as time-consuming and limited in terms of both its discriminating ability and accuracy [16]. Physiological tests showed that none of strains were able to liquefy gelatin, degrade tryptophan or produce hydrogen sulfide, but the coccis could grow in 10% of ethanol. According to Dicks et al. (1995) [17] and Buchanan et al. (1984) [18], the coccis and rods were identified as *Oenococcus* and *Lactobacillus*, respectively. With the increased concentrations of ethanol, the OD value radically diminished down; OD value of C10 strain was 0.409, 0.356 and 0.245 at 10%, 12% and 14% of ethanol, respectively (Fig. 1). The similar trend could be found after acidic-stress treatment (Fig. 2). More specifically, at ethanol concentration of 14%, none strains survived (OD<0.2) except for the C10 and J2 strains (Fig. 1), meanwhile they had a specific activity to grow at low pH even 3.0 (Fig. 2).

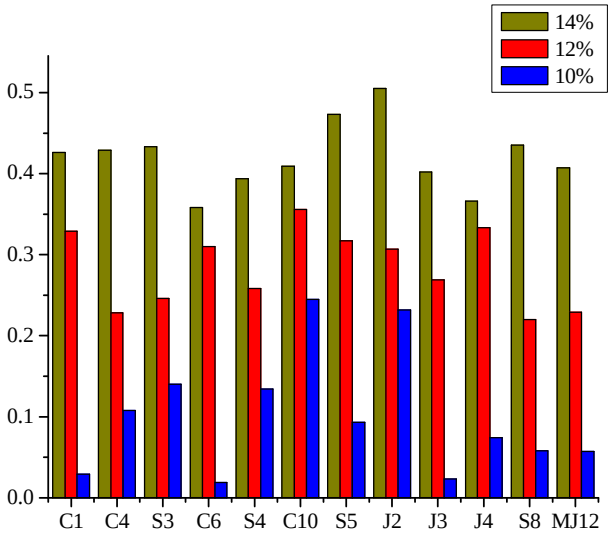

Fig. 1. Ethanol tolerance for partial strains. Strains were cultured at ethanol concentration of 10, 12 and 14% (v/v), respectively. After growth for 96 h, OD_{600} values were measured

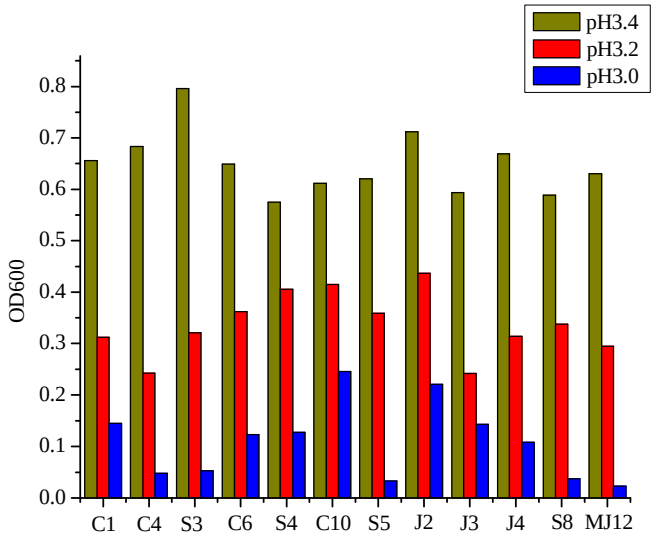

Fig. 2. Acid tolerance of partial strains. Strains were cultured at pH gradient of 3.0, 3.2 and 3.4, respectively. After growth for 96 h, OD_{600} values were measured

Ethanol and acid are the inhibiting factors for growth of *O. oeni* strains, and tolerance to them is prerequisite for starter cultures in MLF. The survey showed that *O. oeni* sp. C10 and *O. oeni* sp. J2 possessed higher survival ability in wine-like medium than the others. Both of them had a specific activity to grow at low pH even 3.0. This behavior would appear to be due to expression of certain stress genes [19]and induction of the small heat shock protein after adaptation at low pH [5,20] . C10 strain performed better in tolerance of acid for higher OD_{600} value at pH 3.0. Contrary to Sico et al. (2008) [21] who reported none of *O. oeni* isolates from Aglianico wines could grow in 14%

of ethanol, *O. oeni* sp. C10 and *O. oeni* sp. J2 could survive in 14% ethanol concentration, indicating that alcohol tolerance appeared to be strain dependent as already described [5,15]. Furthermore, the results showed that the strains could conduct MLF to increase pH of wine. Especially, *O. oeni* sp. C10 had a higher malic acid degradation rate of 430.625mg/(L·d). On account of such characteristics, they had the potential to work as starter cultures for MLF of dry red wine. Meanwhile further assays such as diverse enzymatic activities [22], capacity to produce biogenic amines or resistance to freeze-drying should be performed for industrial applications[6,15].

3.3 16S rRNA sequencing

Sequences of C10, J2, J5 and MJ12 strains were aligned, their phylogenetic positions relative to known sequences were determined (Fig. 3). The closest phylogenetic neighbor of C10 and J2 strains was *O.oeni* (NC008528.1) with an identity of 99%. MJ12 was closest to *Lb.vini* (AHZA01000512.1) with an identity of 99%. The sequence of J5 strain showed a relative low similarity to *O.oeni* (NC008528.1) at 87%. In addition, none was completely identical to any 16S rRNA sequence from GenBank. As reported by Nocker et al. (2004) [23] it was not surprising, because only a relatively small number of bacteria had been sequenced and included in databases.

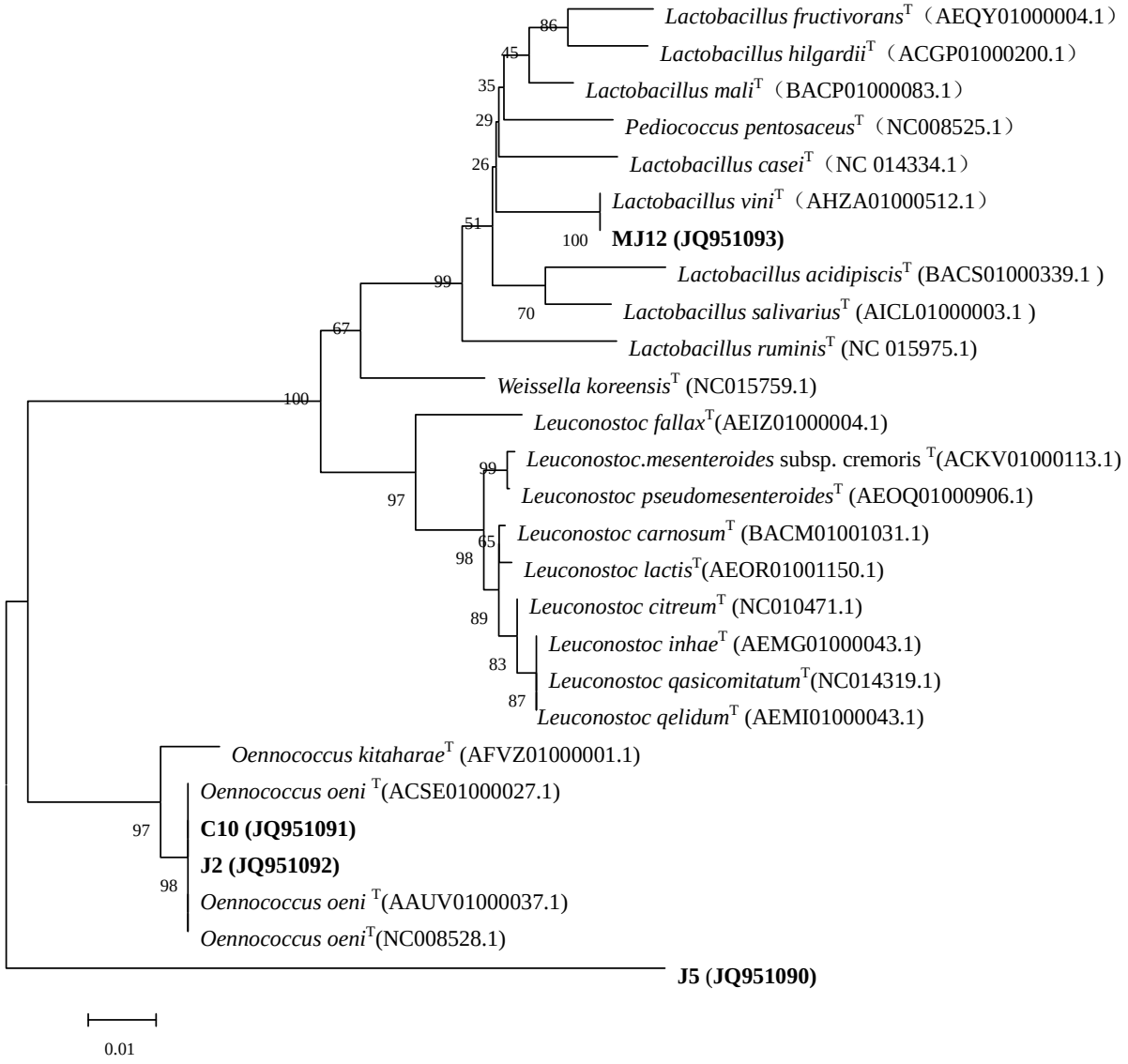

Fig. 3. Phylogenetic relationships of C10, J2, J5 and MJ12 strains based partial 16S rRNA gene sequences

Based on general properties and special inhabitation of LAB, a feasible and reliable protocol was created to distinguish LAB in wine samples: the coccis and rods could be preliminarily identified as *Lactobacillus* and *Oenococcus*, then confirmed by physiological tests (gelatin liquefaction, tryptophan hydroiysis, production of hydrogen sulfide) and the ability to grow at an ethanol concentration of 10%, respectively, and identified up to species level by 16S rRNA analysis. We found most strains of *Oenococcus* were isolated from ATB medium,

whereas all *Lactobacillus* strains were from MRS medium. Thus, we can use ATB and MRS as selective medium for *Oenococcus* and *Lactobacillus*, respectively. This is related to tomato juice composition working as growth factor of *Oenococcus* [16]. The availability of modified MRS medium by addition of tomato juice (20% v/v) for maintaining of *Oenococcus* [24] was consistent with the explanation.

The tree was constructed using the neighbor-joining method. Bootstrap values were based on 1000 replicates. Scale bars represent 0.01 nucleotide substitutions per nucleotide position. GenBank accession number for strains are in parentheses. T represents type strains.

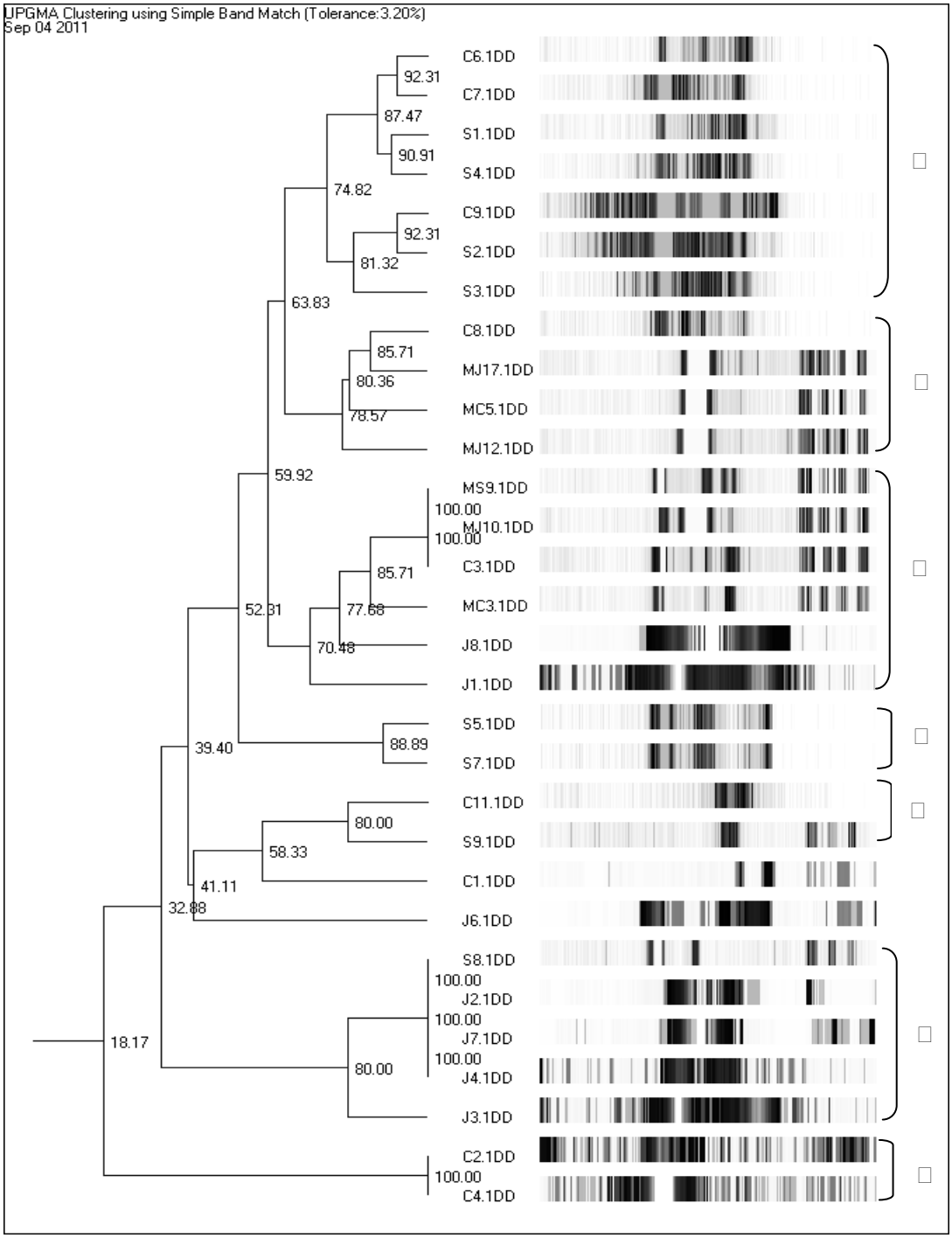

Fig. 4. UPGMA dendrogram based on the RAPD patterns of 30 LAB strains isolated from wine samples in this study

3.4 Molecular clustering of LAB isolates

RAPD has been widely used in molecular typing strains of *Oenococcus*. Zavaleta et al. (1997) [25] used RAPD

analysis to discern group A (50 strains) and group B (20 strains) with a similarity level around 56%, and reported that the calculated correlation coefficient for the RAPD dendrogram was r=0.852. Dendrogram of genetic similarity built on RAPD patterns was shown in Fig. 4. Basically seven clusters were delineated at r =70%. Cluster I comprised 7 strains (C6, C7, S1, S4, C9, S2 and S3) at r =74.82%. 4 strains (C8, MJ17, MC5 and MJ12) formed cluster II at r =78.57%. 6 strains (MS9, MJ10, C3, MC3, J8 and J1) in cluster III grouped at r =70.48%. Cluster IV contained the isolates S5 and S7 at r = 88.89%. Cluster V comprised strains C11 and S9 at r =80.00%. 5 strains (S8, J2, J7, J4 and J3) formed cluster VI at r =80.00%. Cluster VII was constituted by strains C2 and C4 at r =100.00%. Owning to low similarity (58.33% and 41.11%, respectively), the strains C1 and J6 were not grouped into such cluster. The strains of *Lactobacillus* (MC5 and MJ12) clustered together with *Oenococcus* strains in cluster II.

Our results showed that seven clusters of 30 strains were delineated at r=70%, in agreement with Cocolin et al. (2009) [26] who arbitrarily selected a coefficient of correlation of 70% to distinguish the clusters. Bartowsky et al. (2003) [27]reported that *Oenococcus* strains which originated from the same winery were either indistinguishable or closely related to each other. But we found that although isolated from the same winery even the same sample, the strains distributed in different groups. Each wine sample used different wine grape and brewing process, which resulted in formation of special habitat inhibit then dominance of one species even strain or biotype. Marcobal et al. (2008) [28] pointed out that the genus *Oenococcus* lacks the mismatch repair (MMR) gene system giving DNA mutation rates higher. Thus, we supposed that the selection of wine environment and hypermutation of *Oenococcus* strains might be account for above inconsistency. The results also showed that *Oenococcus* strains belong to different group possessed similarity level arrived below 60%, however, strains clustered in a group at higher levels (70%), some even reached to 100%. This would appear to suggest a high genetic diversity of *Oenococcus*, as previously stated [4,15] . Genetic diversity may lead to properties diversity, so we can select strains showing high activity of MLF for enological application.

3.5 Malolactic activity

Malolactic fermentation is a secondary fermentation, and is catalyzed by malolactic enzyme, which takes the harsher malic acid converting to a softer lactic acid in wine. The results of MLF conducted by C10 or J2 strains were shown in Fig. 5. As fermentation went on, the concentration of malic acid declined, and lactic acid rised. The decrease of malic acid in C10-fermented wine was more obvious than that of J2-fermented wine. The malic acid degradation rate of C10 and J2 strains were 430.625 mg/(L·day) and 76.994 mg/(L·day), respectively. After fermentation for 14 days, pH value of the wine samples increased by 0.1.

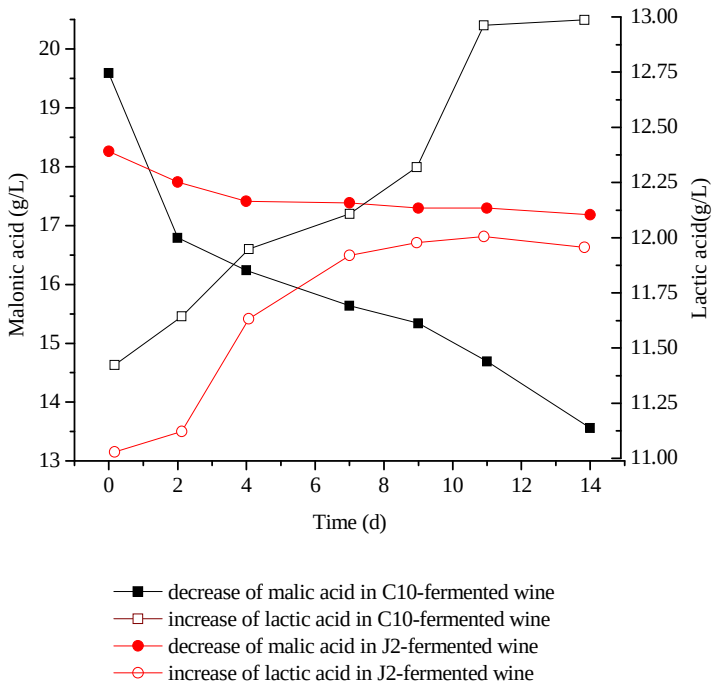

Fig. 5. Change of malic acid and lactic acid in the wine samples during MLF conducted by *O. oeni* sp. C10 or *O. oeni* sp. J2. Concentration of malic acid and lactic acid was determined by HPLC

Alcoholic fermentation has a selection of the LAB in the grape must, and most of these LAB species generally do not multiply and decline towards the end of alcoholic fermentation, with the exception of *O.oeni*. The results

showed that 51 of 60 (85%) *Oenococcus* strains were isolated from the red wines, indicating their dominant status during MLF. The analysis of LAB composition in red wines can help us evaluate the quality of the wine. *O. oeni* strains are the most desirable requirement for malolactic fermentation and so far no sensorial defect has been attributed to them [29]. So we estimate that the simples used to isolate LAB were in good quality in term of MLF. Since it has just been shown that some *O. oeni* strains can produce undesirable products from the hygienic point of view, the relevant indicator must be measured. As for *Lactobacillus* spp., it is generally related to depreciations and diseases, for example *L.fructivorans* and *L.hilgardii* can spoil fortified wines [30]. However, some study has shown some oenological *Lactobacilli* spp., included *L.plantarum* and *L.hilgardii*, have the potential as MLF starter cultures [30]. We trend to believe that the influence of *Lactobacillus* spp. on quality improvement and depreciation of wine is wine-depended and strain-depended. The strain MJ12 has been identified as *L.vini*, which possesses many favorable characteristics suitable for MLF starter cultures: survival on the harsh wine conditions, citric and malic acids metabolism [31], formation no ammonia from arginine [31], and production no biogenic amine [32], which is of importance for aroma and flavor development in wine. Therefore, *L.vini* sp.MJ12 may be used as the next generation of MLF starter culture, although it has a long way to go.

CONCLUSION

In the study, a total of 60 Gram-positive, catalase-negative and lactic acid-productive strains were isolated and identified as LAB. The coccis (51 strains) and rods (9 strains) were respectively confirmed as *Oenococcus* and *Lactobacillus*. The 30 isolates were grouped into seven different genotypes at 70% similarity by means of randomly amplified polymorphic DNA, indicating a high genetic diversity of *Oenococcus* spp. Two strains could conduct MLF, and malic acid degradation rate was 430.625 mg/(L·day) and 76.994 mg/(L·day), respectively. Therefore, they can be envisaged as starter cultures for MLF in red wines.

Acknowledgments
This work was financially supported by the research grant from Hebei Province Natural Science Fund (No.C2011203137, 11965152D).

REFERENCES

[1] HW du Plessis; LMT Dicks; IS Pretorius; MG Lambrechts; M du Toit, *Int. J. Food Microbiol.*, **2004**, 91, 19-29.
[2] P Garijo; R Lopez; P Santamaría; E Ocón; C Olarte; S Sanz; AR Gutiérrez, *Int. J. Food Microbiol.*, **2009**, 136, 142-146.
[3] M Esti; G Volpe; L Micheli; E Delibato; D Compagnone; D Moscone; G Palleschi, *Anal. Chim. Acta.*, **2004**, 513, 357-364.
[4] S Guerrini; A Bastianini; G Blaiotta; L Granchi; G Moschetti; S Coppola; P Romano; M Vincenzini, *Int. J. Food Microbiol.*, **2003**, 83, 1-14.
[5] F Coucheney; N Desroche; M Bou; R Tourdot-Mare´chal; L Dulau; J Guzzo, *Int. J Food Microbiol.*, **2005**, 105, 463-470.
[6] P Ruiz; PM Izquierdo; S Seseña; ML Palop, *Int. J. Food Microbiol.*, **2010**, 137, 230-235.
[7] LMT Dicks; A Endo. *S, Afr. J. Enol. Vitic.*, **2009**, 30, 72-90.
[8] MÁ Pozo-Bayón; I Pardo; S Ferrer; MV Moreno-Arribas, *Afr. J. Biotechnol.*, **2009**, 8, 3995-4001.
[9] H Alexandre; PJ Costello; F Remize; J Guzzo; M Guilloux-Benatier, *Int. J. Food. Microbiol.*, **2004**, 93, 141-154.
[10] P Sheng; XD Cheng, Experiment of microbiology, 4th edition, Higher Education Press, Beijing, **2008**, 112-119.
[11] K Audenaert; K D'Haene; V Messens; T Ruyssen; P Vandamme; G Huys, *Food Microbiol.*, **2010**, 27, 12-18.
[12] S Bae; GH Fleet; GM Heard, *Int. J. Food Microbiol.*, **2004**, 94, 301-312.
[13] HC Chen; SY Wang; MJ Chen, *Food Microbiol.*, **2008**, 25, 492-501.
[14] V Capozzi; V Ladero; L Beneduce; M Fernández; MA Alvarez; B Benoit; B Laurent; F Grieco; G Spano, *Food Microbiol.*, **2011**, 28, 434-439.
[15] L Vigentini; C Picozzi; A Tirelli; A Giugni; R Foschino, *In. J. Food Microbiol.*, **2009**, 136, 123-128.
[16] I Sa´nchez; S Sesen˜a; L Palop, *Int. J. Food Microbiol.*, **2003**, 82, 181-189.
[17] LMT Dicks; F Dellaglio; MD CollIns, *Int. J. Syst. Bacteriol.*, **1995**, 45, 395-397.
[18] RE Buchanan; NE Gibbons. Bergey' Manual of Systematic Bacteriology, 8th edition, The Chinese academy of sciences institute of microorganisms, Science Press, Beijing, **1984**, 797-800.
[19] C Beltramo; N Desroche; R Tourdot-Maréchal; C Grandvalet; J Guzzo, *Res. Microbiol.*, **2006**, 157, 267-274.
[20] S Sugimoto; A Al-Mahin; K Sonomoto, *J. Biosci. Bioeng.*, **2008**, 106, 324-336.
[21] MA Sico; MG Bonomo; G Salzano, *World. J. Microb. Biot.*, **2008**, 24, 1829-1835.
[22] RN Barbagallo; G Spagna; R Palmeri; S Torriani, *Enzyme. Microb. Tech.*, **2004**, 34, 292-296.
[23] A Nocker; JE Lepo; RA Snyder, *Appl. Environ. Microb.*, **2004**, 70, 6834-6845.
[24] I Rosi; F Nannelli; G Giovani, *LWT-Food. Sci. Technol.*, **2009**, 42, 525-530.

[25] AI Zavaleta; AJ Marti´nez-Murcia; F Rodri´guez-Valera, *Appl. Environ. Microb.*, **1997**, 63, 1261-1267.
[26] L Cocolin; P Dolci; K Rantsiou; R Urso; C Cantoni; G Comi, *Meat. Sci.*, **2009**, 82, 125-132.
[27] EJ Bartowsky; JM Mccarthy; PA Henschke, *Aust. J. Grape. Wine. R.*, **2003**, 9, 122-126.
[28] AM Marcobal; DA Sela; YI Wolf; KS Makarova; DA Mills, *J. Bacteriol.*, **2008**, 190, 564-570.
[29] A Lonvaud-Funel, *Antonie van Leeuwenhoek.*, **1999**, 76, 317-331.
[30] M du Toit; L Engelbrecht; E Lerm; Krieger-Weber; S. *Lactobacillus, Food Bioprocess. Tech.*, **2010**, 6, 876-906.
[31] AM Rodas; E Chenoll; MC Macia´; S Ferrer; I Pardo; R Aznar, *Int. J. Syst. Evol. Micr.*, **2006**, 56, 513-517.
[32] JM Landete; S Ferrer; I Pardo, *Food Control.*, **2007**, 18, 1569-1574.