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Identification of dominant lactic acid bacteria isolated from grape juices. Assessment of its biochemical activities relevant to flavor development in wine

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Fabiana Maria Saguir[1,3]
Iris Eleonora Loto Campos[1]
Carmen Maturano[1]
Maria Cristina Manca de Nadra[1,2,3]

[1]Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina; [2]Centro de Referencia para *Lactobacilos* (Cerela), Tucumán, Argentina; [3]Career Investigators from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina

Abstract: We investigated the dominant lactic acid bacteria (LAB) from grape juice and commencement of malolactic fermentation (MLF) samples of a cellar located in Argentina and assessment of its β-glucosidase activity and butter aroma compounds production. LAB number found in grape juice (approximately $\log_{10}$ 3.3) was lower than that obtained in the MLF samples. *Oenococcus oeni* was predominant, accounting for 68% of the 81 LAB isolated. Majority of whole cells derived from *O. oeni* cultures at the end of the exponential growth showed detectable β-glucosidase activity. Contrarily, the highest proportion of them did not produce diacetyl, acetoin, and 2,3-butylene glycol. A direct relation between both properties among the *O. oeni* strains could not be established. In the selected MS25 strain, L-malic acid was compatible with good enzyme activity and was partially able to annul the negative influence of the low pH (3.8). In different conditions, the aroma compounds were lower than 4 mg/ml, especially at pH 3.8 and in presence of L-malic acid (2.5 g/l). This strain could have adequate characteristics for potential use in winemaking. Finally, the assessment of both biochemical properties in *O. oeni* should be considered as a quality criterion for selecting starter cultures for the improvement of the wines aroma.

Keywords: isolation, lactic acid bacteria, biochemical properties, aroma, wine

Introduction

Lactic acid bacteria (LAB) are widely used in food biotechnology and efficient control of these microbiological processes requires an increase in our knowledge about their ecology and biochemical activities. LAB occur naturally on grapes and their ability to grow in wine had been documented.[1,2] LAB associated with the wine making process belongs to the genera *Lactobacillus, Leuconostoc, Oenococcus*, and *Pediococcus*.[3–5] Malolactic fermentation (MLF) induced by LAB, after alcoholic fermentation (AF), is a suitable process for correct wine acidity. It consists of the L-malic acid decarboxylation to lactic acid, resulting in a wine with a softer mouth feel. At the same time, the MLF provides stability for wines to be aged and the addition of flavors by modifying fruit-derived aromas and producing aroma-active compounds.[1,6]

One of the most evident flavor changes is the development of a 'buttery' or 'butterscotch' character. Diacetyl has been attributed to being a major contributor to this buttery character in wine.[7,8] This metabolite can be formed from citric acid in wine. Saguir and Manca de Nadra[9] demonstrated that L-malic and citric acids favored wine *Oenococcus oeni* strain growth in nutritional stress conditions and that,

Correspondence: Fabiana Maria Saguir
Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491, 4000 Tucumán, Argentina
Tel +54 381 425 1425
Fax +54 381 431 172/400 5600
Email fabianasaguir@fbqf.unt.edu.ar

specifically, citric acid was involved in the biosynthesis of the aspartate-derived essential amino acids. Bartowsky and Henschke,[8] reported that in wine the diacetyl concentration is generally low relative to its flavor threshold and appears to be important to determining wine style. Yeast are also able to synthesize diacetyl during the AF, however, the majority of it is further metabolized to acetoin and 2,3-butanediol.[10]

In the last decades, increased interest has been focused on glycosidic enzymes as a means of flavor modification of wine.[11] In fact, these enzymes hydrolyze glycoconjugated precursors, releasing active aroma compounds such as straight-chain alcohols, norisoprenoids and free phenols. These odorous compounds may occur in a free form in grapes, or bounded to sugar molecules (β-D-glucose or a dissacharide comprising glucose and a second sugar unit being α-L-arabinofuranose, α-L-rhamnopyranose or β-D-apiofuranose) to form an odorless nonvolatile glycosidic complexes.[12] Volatile compounds, present as monoglucosides are liberated via β-D-glucosidase (glucopyranosidase), whereas diglycoside-bound aglycones are liberated through a sequential release by different glucosidase followed by β-D-glucosidase.[13]

Much attention has been paid to *Saccharomyces cerevisiae* (given its importance in wine-making) but this yeast showed very limited glycosidases production, much of which are intracellular.[14] In addition, they are unstable at wine pH and may be inhibited by high sugar and ethanol concentrations.[14] On the other hand, *Aspergillus* sp. is a common source of commercial enzyme preparations that have glycosidic activities; however, these preparations are often impure, requiring resolution before characterization in the laboratory, and they have undesirable effects on the wine.[15] Grapevines produce glycosidases, although these enzymes have little activity against wine glycosides.[16] Thus, strains of LAB well suited to perform the MLF might be a good source of glycosidases. Changes in the glycoside content of Tannat wines during MLF indirectly supported the existence of such activities in the commercial *O. oeni* strains used.[17] More specific data has come from examinations of commercial wines. *O. oeni* isolates,[18,19] which were shown to have the potential for high glycosidase activity against nitrophenyl glycosides. While the use of enzymes and/or selected cultures to liberate aroma compounds from natural grape aroma glycosides is still in the development stage, the findings to date for LAB justify further investigation. On the other hand, no information was reported about some relation between diacetyl production and β-glucosidase activities by wine LAB.

The aim of this work was to investigate the dominant LAB isolated from grape juice and fermented musts at commencement of MLF in a cellar located in north of Argentina and further, to assess its biochemical properties, such as β-glucosidase activity and butter aroma compounds production, that may be relevant to flavor development in wine. At the same time, the influence of some wine parameters on the growth and metabolism of a selected *O. oeni* strain was analyzed.

Materials and methods

Microorganism

Oenococcus oeni strains isolated and identified during this study from grape juice and fermented musts were stored at –20 °C in de Man Rogosa Sharpe (MRS) medium (Oxoid Ltd., London, England) with 15% (v/v) tomato juice (TJ) supplemented with glycerol (30%, v/v).

Samples

Samples were collected aseptically in one cellar at Cafayate, a region located in North of Argentina. Samples consisted of natural juice from red grapes (Malbec variety) and the musts collected on the completion of AF carried out by indigenous or commercial yeast cultures, before MLF had commenced. The pH and ethanol concentration of wine samples were 3.4 and 12%, respectively.

Generally, sample volumes of 500 ml were taken from large tanks, transferred to a sterile glass container, and transported to the laboratory under refrigeration (4 °C).

Processing, enumeration, and isolation of LAB from samples

For enumeration and isolation of LAB contained in the samples, 1.0 ml-aliquots (diluted if necessary) were plated on MRS agar (Oxoid Ltd.) enriched with fructose (5 g/l) and L-malic acid (3 g/l) and adjusted to pH 5.0. The isolating MRS medium was supplemented with two μg/ml of cycloheximide (MRS-C; Sigma, St. Louis, MO, USA) to suppress the growth of yeasts. At the same time, aerobic mesophilic microflora was determined on plate count agar (PCA; Oxoid Ltd.).

Agar MRS-C plates were incubated anaerobically (BBL GasPak Anaerobic System; Becton Dickinson Argentina, Buenos Aires, Argentina) and PCA plates aerobically at 30 °C for 10 days before enumeration. The PCA agar plates were flooded after enumeration with 3% H_2O_2 in order to observe the presence for catalase positive colonies.

In addition, for all samples, isolations on MRS-C agar media were prepared from enrichment cultures conducted in

MRS broth, pH 5.0 (containing cycloheximide as mentioned above) after five days of incubation (when microbial growth became evident).

A total of 72 colonies (24 isolates from each analyzed sample) by random selection were picked up from MRS-C media. A small number of nine isolates were also obtained from PCA media. The isolates were purified by sub-culturing in MRS broth with TJ (15% v/v), pH 4.8 at 30 °C and further characterized. All LAB strains were maintained at –20 °C in MRS with TJ (15% v/v) and glycerol (20% v/v), and grown anaerobically at 30 °C in MRS with TJ (15%).

Phenotypical characterization of isolates

The isolates were characterized for Gram and catalase reaction, cell shape, cytochrome-oxidase activity, spore formation, production of ammonia from arginine,[20] and fermentative catabolism of glucose. Gas and D- or L-lactic acid isomers production from glucose metabolism were determined in Gibson medium,[21] and by using an enzymatic method with Boehringer Kit (Boehringer, Mannheim, Germany), respectively.

Ability of growth, under microaerophilic conditions in BBL GasPak jars (Becton Dickinson Argentina), in which the content of oxygen was reduced by use of a lighted candle, was determined on MRS agar plates incubated at 15 °C, 30 °C, 37 °C, and 45 °C. Ability of growth at different NaCl concentrations (2%, 4%, 6% and 8% w/v) and pH values (4, 5, 5.5, and 6.5) were also investigated. Studies of fermentation of carbohydrates and related compounds were carried out using API 50 CH galleries (BioMérieux, Marcy-l'Etoile, France).

Genotypical characterization by PCR with species-specific primers

Preparation of bacteria for the polymerase chain reactions (PCR) involved growing on MRS medium (Oxoid Ltd.) with TJ (15%) and adjusted at pH 4.8. The DNA extraction was prepared as described by Reguant and Bordons.[4] Concentration of the DNA obtained was estimated by electrophoresis in one% (w/v) agarose gel with Tris–acetate–EDTA buffer (TAE) (Sigma).

Oenococcus oeni strains were identified by the species-specific PCR method,[22] which amplifies a fragment of the malolactic enzyme gene of *O. oeni*. A thermocycler machine (Biometra, Goettingen, Germany) was used for PCR reactions. The 20 µl PCR reaction mix consisted of 0.4 µM of each, On1 and On2 primer,[22] 0.2 mM of each dNTP (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA), 2 mM of $MgCl_2$, 2 µl of 10x reaction buffer (Amersham Pharmacia Biotech, Inc.), 0.5 U Taq DNA polymerase (Amersham Pharmacia Biotech, Inc.), and 10 ng of genomic DNA. The amplification profile was: one cycle at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 64 °C for 2 min, and 72 °C for 2 min. A final extension of 72°C for 10 min was also included. The amplification products were visualized by ethidium bromide (5 µg/ml) staining after gel electrophoresis.

Analytical methods

D-Glucose was analyzed by the glucose oxidase method (Kit from Wiener Laboratory, Rosario, Argentina) and L-malic acid by enzymatic methods (Boheringer Kits; Boheringer). Diacetyl, acetoin and 2,3-butanediol was analyzed as a combined value according to the colorimetric method of Hill and colleagues,[23] modified by Branen and Keenan.[24]

β-glicosidase activity

Enzyme assays were conducted on whole cells and supernatants obtained by centrifugation (4000 g for 20 min) of cultures grown in MRS with TJ (15%), under different conditions, as mentioned in the text, in anaerobic conditions at 30 °C at the final of the exponential growth phase. Enzymatic activity was measured according to D'Incecco and colleagues,[13] using *p*-nitrophenyl-*β*-D-glucopyranoside (*p*-NPG) (Sigma cat no. N7006) as substrate. The assay was performed for 30 min at 37 °C, after which 400 µl of 1 M sodium carbonate solution was added to stop the reaction and allow the development of the yellow color of the *p*-nitrophenolate ion; the samples were then centrifuged. The assay was read against the blank at 400 nm in a spectrophotometer (Spectronic Genesys 5; Milton Roy Company, Rochester, NY, USA) in a 1 cm cell.

The enzymatic activity was expressed as U/g equivalent to µmoles *p*-nitrophenyl released by min and gram of dry weight of cells.

Statistical analysis

The experimental data were analyzed by one-way analysis of variance test. Variable means showing statistical significance were compared using Tukey's test (Minitab student R12). All statements of significance are based on the 0.05 level of probability.[25] Three replicate determinations were carried out.

Results

Isolation and identification of LAB

Determined on selective agar MRS-C media by direct plate culture, the samples of grape juice and fermented musts contained levels of LAB ranging from log 2.9 to log 4.4 cfu/ml.

There was less LAB present in grape juice (3.30 cfu/ml) than in the samples corresponding to commencement of MLF (4.45 and 4.40 cfu/ml when AF was conducted by indigenous or commercial yeasts, respectively).

In grape juice, a count of approximately 1.5 log cycles higher was observed by use of PCA. The majority of colonies on this medium corresponded to yeasts (in the order of 10^4 cfu/ml) and in a lesser extent to LAB. Non-LAB with reaction of Gram and catalase positives was also detected. By contranst, in the samples from beginning of MLF no significant differences in the counts on PCA as regards to MRS-C plates were observed. Moreover, on PCA the majority of colonies were small, grey or white, catalase-negative, Gram-positive able to grow under aerobic conditions suggesting they were LAB.

In any assayed conditions Gram-negative, catalase-positive bacteria belonging to *Enterobacteriaceae* were observed.

On the other hand, when enrichment cultures were used before isolating the presence of LAB, increased significantly (in order 10^6 or 10^8 cfu/ml, depending on the samples used). Similar result was described by Bae and colleagues[26] for LAB isolation from wine grapes.

A total of 72 isolates belonging to LAB group were selected from MRS-C agar media. In addition, nine isolates were obtained from PCA plates (of a total of 81 isolates, 27 corresponded to each analyzed sample). All LAB isolates were Gram-positive, catalase-negative, nonspore-forming, nonmotile cells, and exhibited a fermentative catabolism from carbohydrates. They were obligatory heterofermentative accounting for 74% of the total analyzed strains and exhibited the following characteristics: production of gas and D-lactic acid isomer from glucose, no ammonia formation from arginine, growth at 15 °C but no growth at 45 °C and cocci arranged in chains. Ninety-two percent of isolates belonging to this group were classified as *O. oeni* on the basis that they grew in presence of 10% ethanol, acid pH and at 30 °C or 37 °C and by using of the API 50 CH kit. The homofermentative isolates (26% of the total LAB studied) were mainly characterized as *Lactobacillus* sp. and in a lesser extent as *Pediococcus* sp.

As shown in Figure 1 we observed, that in grape juice *O. oeni* was found to a lesser extent (about 15% lower) than other LAB species such as *Leuconostoc, Lactobacillus*, and *Pediococcus* sp. However, at commencement of MLF independently, of the wild or commercial yeast type employed for conducing AF, almost all isolates were identified as *O. oeni* accounting for 78% and 85%, respectively.

A single product of about 1025 bp, the expected size of the malolactic enzyme gene fragment to be amplified, was obtained only when purified DNA from *O. oeni* isolates phenotypically identified and the reference strain were used as target for specific PCR reaction. Only one of them did not show this positive result and it was excluded from

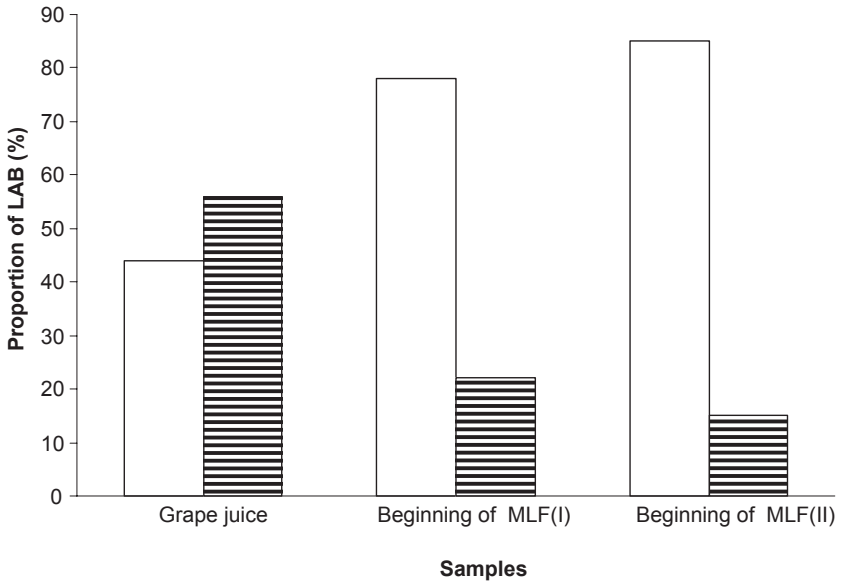

Figure 1 Proportion of *Oenococcus oeni* and other LAB species isolated from grape juice and samples from commencement of MLF, after AF conducted by indigenous yeasts (I) or yeast starter culture (II).
Key: (☐) *Oenococcus oeni*; (≡) other LAB than *Oenococcus oeni.*
Abbreviations: AF, alcoholic fermentation; LAB, lactic acid bacteria; MLF, malolactic fermentation.

this group. Strains of other species than *O. oeni* gave negative reaction (Figure 2).

Growth and pH variation of *O. oeni* strains identified during this study

A total of 54 strains of *O. oeni,* identified during this study were transferred to complex laboratory medium and their maximum population levels and pH values obtained at the end of exponential growth, were determined (Table 1). The extent of bacterial growth varied according to the analyzed strain and it was directly related with the pH diminution. The majority of the strains grew to optical densities (OD) at 560 nm of among 0.66 and 1.00. Thirty-seven percent did not show significant differences ($P < 0.05$) but reached an average final population of $OD_{560\,nm}$: 0.85. Eight strains of the total of *O. oeni* studied showed the highest population levels with an average value of $OD_{560\,nm}$:1.2. One strain that did not show significant difference with those classified as ^{bc} reached the lowest bacterial population level. We focused our attention on those strains of *O. oeni* that were able to achieve growth at absorbance values higher than 0.7 to further investigations. So the strains (11%) classified as ^{c}, or ^{bc} were excluded.

β-glucosidase activity in *O. oeni* strains

Table 2 shows the β-glucosidase activities of whole cells of the *O. oeni* strains obtained from cultures in MRS medium with TJ, pH 4.8, at the end of the exponential growth phase. Majority of tested strains (73%) possessed a detectable and variable level of β-glucosidase activity. Only whole cells derived from 13 out of the 48 strains studied failed to show this enzymatic activity. According to the extent of β-glucosidase activity, they were arbitrary classified into three groups: From one to 20 U/g the enzymatic activity was considered as low, from 20 to 60 U/g it was considered as intermediary and for >60 U/g it was considered as high. Majority of isolates with a detectable activity presented an intermediary level accounting for 40% with an average minimum and maximum value of activity that corresponded to 43.23 ± 2.78, 28.32 ± 2.11, and 53.86 ± 5.13 U/g, respectively (Table 2). Twelve isolates, which correspond to a percentage of 34.3% of the total of positive strains for enzymatic activity showed low activity levels and only nine strains, produced high levels of β-glucosidase enzyme. It is interesting to note that no strains from grape juice (MS1–MS11) corresponded to this last group. Thus, all strains classified as high activity were isolated in a similar proportion from both samples of commencement of MLF. Moreover, of the total of the *O. oeni* isolates with detectable levels of activity 6, 13, and 15 strains corresponded to collected samples from grape juice, begining of MLF after AF by wild or commercial yeast cultures, respectively.

Because no activities were found in the supernatants of all the analyzed cultures, β-glucosidase enzyme was not thought to be extracellular in this bacterium.

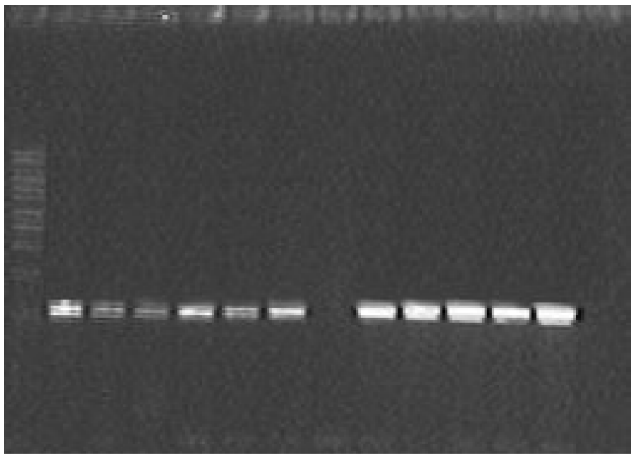

Figure 2 Profiles obtained with PCR primers On1 and On2 of *Oenococcus oeni* isolated from of fermented must at commencement of MLF in agarose gel 1.5%. Line one: marker of DNA molecular weight 1 kb, Lines 2–12: strains phenotypically identified as *Oenococcus oeni*, Line 13: *Oenococcus oeni* X_2L, control positive, Lines 14–15: strains of *Lactobacillus* and *Leuconostoc* sp., controls negative.
Abbreviations: MLF, malolactic fermentation; PCR, polymerase chain reaction.

C_4 aroma compounds formation by *O. oeni*

Diacetyl, acetoin, and 2,3-butilenglycol formation by *O. oeni* strains in MRS supplemented with TJ, pH 4.8 at the end of exponential growth phase was investigated. No butter aroma compounds production was detected in the majority of the analyzed strains (60.4%) and in the cells resting their formation ranged from 0.23 to 25.61 mg/l (Figure 3). This ability was also distributed at a higher frequency in those strains isolated from samples of commencement of MLF (after AF conduced by wild or commercial yeasts) than grape juice and accounting for 42.9%, 38.1%, and 9%, respectively.

Thus, whilst the enzymatic activity was detectable in the majority of the isolates studied (76%), the C_4 aroma compounds formation only occurred in 39% of them. Table 3 shows that the largest size of population that did not produce any studied C_4 aroma compounds produced mainly intermediary levels of β-glucosidase activities whilst most of the strains that formed C_4 aroma compounds in concentrations equal or lower than 4.4 ± 0.22 mg/l did not present β-glucosidase activity or showed it at high levels. Of isolates, 5.5% formed diacetyl, acetoin, and 2,3-butilenglycol in concentrations equal or higher than 9 ± 0.49 mg/l and were mainly unable to produce detectable enzyme levels.

Table 1 Growth of *Oenococcus oeni* isolates in MRS medium supplemented with TJ (15%) and pH variation

Strain	Maximum population*	Final pH	Strain	Maximum population*	Final pH
MS1	0.87 ± 0.01^{afid}	4.46 ± 0.01	MS29	0.92 ± 0.01^{fid}	4.39 ± 0.01
MS2	0.68 ± 0.01^{bc}	4.55 ± 0.07	MS30	0.87 ± 0.01^{afid}	4.45 ± 0.07
MS3	0.78 ± 0.01^{abf}	4.50 ± 0.02	MS31	0.92 ± 0.02^{fid}	4.40 ± 0.02
MS4	0.79 ± 0.02^{abf}	4.51 ± 0.02	MS32	0.89 ± 0.02^{fid}	4.46 ± 0.02
MS5	0.72 ± 0.01^{bec}	4.54 ± 0.02	MS33	0.94 ± 0.05^{id}	4.33 ± 0.02
MS6	0.73 ± 0.02^{abc}	4.55 ± 0.02	MS34	1.10 ± 0.04^{hm}	4.15 ± 0.02
MS8	0.83 ± 0.02^{aef}	4.48 ± 0.02	MS35	0.87 ± 0.04^{afid}	4.46 ± 0.02
MS9	0.87 ± 0.02^{afid}	4.45 ± 0.01	MS36	1.01 ± 0.02^{md}	4.20 ± 0.01
MS10	0.74 ± 0.02^{abc}	4.52 ± 0.01	MS37	1.00 ± 0.03^{md}	4.14 ± 0.01
MS11	0.92 ± 0.01^{fid}	4.41 ± 0.02	MS38	1.10 ± 0.06^{hm}	4.19 ± 0.02
MS12	0.78 ± 0.01^{abf}	4.51 ± 0.01	MS39	0.90 ± 0.02^{fid}	4.36 ± 0.01
MS13	0.61 ± 0.02^{c}	4.59 ± 0.01	MS40	0.84 ± 0.01^{aefi}	4.43 ± 0.01
MS14	0.68 ± 0.03^{bc}	4.56 ± 0.09	MS41	1.43 ± 0.06^{r}	3.92 ± 0.09
MS15	0.66 ± 0.01^{bc}	4.57 ± 0.01	MS42	1.40 ± 0.05^{r}	3.97 ± 0.01
MS16	0.71 ± 0.01^{bec}	4.51 ± 0.01	MS43	1.43 ± 0.04^{r}	3.86 ± 0.01
MS17	0.71 ± 0.02^{bec}	4.52 ± 0.09	MS44	1.37 ± 0.04^{r}	3.90 ± 0.09
MS18	0.68 ± 0.02^{bc}	4.55 ± 0.02	MS45	1.00 ± 0.01^{md}	4.23 ± 0.02
MS19	0.68 ± 0.01^{bc}	4.56 ± 0.01	MS46	1.35 ± 0.05^{r}	3.99 ± 0.01
MS20	0.80 ± 0.01^{abfi}	4.50 ± 0.01	MS47	1.12 ± 0.04^{hm}	4.00 ± 0.01
MS21	1.12 ± 0.08^{hm}	4.00 ± 0.07	MS48	1.38 ± 0.09^{r}	3.97 ± 0.07
MS22	0.88 ± 0.02^{fid}	4.44 ± 0.01	MS49	1.21 ± 0.09^{r}	3.99 ± 0.01
MS23	0.87 ± 0.01^{afid}	4.45 ± 0.01	MS50	1.29 ± 0.10^{h}	4.01 ± 0.01
MS24	0.71 ± 0.01^{bec}	4.53 ± 0.09	MS51	1.41 ± 0.12^{r}	3.96 ± 0.09
MS25	0.92 ± 0.02^{fid}	4.33 ± 0.02	MS52	0.80 ± 0.03^{bfi}	4.49 ± 0.07
MS26	0.92 ± 0.02^{fid}	4.39 ± 0.01	MS53	0.78 ± 0.01^{abf}	4.49 ± 0.10
MS27	0.84 ± 0.01^{aefi}	4.47 ± 0.01	MS54	0.96 ± 0.01^{id}	4.32 ± 0.04
MS28	0.94 ± 0.02^{imd}	4.32 ± 0.01	MS55	1.10 ± 0.03^{hm}	4.23 ± 0.04

Notes: *Absorbance values reached at the end of exponential growth phase were used for maximum bacterial population determinations. Bacterial growth was followed by OD at 560 nm as described in the Materials and methods section. Values are the means of three independent experiments ± standard deviation. Different letters within columns indicate significance with a p value < 0.05 so values with the same letter in the same column are not significantly different ($P < 0.05$). Different times required to reach the end of exponential growth. Smaller time required was three days.

Abbreviations: MRS, deMan Rogosa Sharpe; TJ, tomato juice.

On the basis of its growth response, high enzymatic activity, and an adequate diacetyl, acetoin and 2,3-butilenglycol production, the MS25 strain was selected for further investigations concerning to the influence of some wine parameters on its growth and biochemical activities studied.

Effect of pH and L-malic acid on growth and carbon source utilization

As shown in Table 4 the growth and carbon sources utilization by *O. oeni* MS25 was investigated in MRS medium with TJ under the following condition: absence or presence of L-malate (2.5 g/l) at two initial pH values: 4.8 and 3.8. At pH 4.8 (control), the microorganism grew with a growth rate of 0.067 and with a final $OD_{560\ nm}$ above one. L-malic acid increased both growth parameters of about 13% and 36%, respectively. Similar results were observed by Saguir and Manca de Nadra[11,27] and Aredes Fernandez and colleagues.[28] When the initial pH was adjusted at 3.8, growth rate and final biomass of the MS25 strain significantly decreased. L-malic acid again stimulated growth parameters. At pH 4.8, between 80%–70% of initial glucose was exhausted depending of the L-malic acid presence. At pH 3.2, the sugar consumption, was in a lesser extent than at 4.8. In this condition the organic acid enhanced glucose utilization as well as bacterial growth. L-malic was completely utilized at pH 4.8 and more than 80% at pH 3.8.

Table 2 β-glucosidase activities (U/g) in whole cells of the *Oenococcus oeni* isolates obtained from MRS medium with TJ (15%) at the end of exponential growth phase[a]

Strain ≤ than 20		β-glucosidase activity (U/g)			
		Strain between 20 to 60		Strain ≥ than 60	
Control[b]	0	MS4	53.00 ± 4.04	MS15	70.01 ± 6.59
MS3	18.4 ± 0.95	MS6	46.25 ± 4.02	MS20	80.82 ± 3.50
MS5	19.73 ± 0.67	MS7	43.55 ± 0.61	MS24	64.54 ± 4.36
MS22	3.20 ± 0.38	MS9	39.10 ± 0.21	MS25	128.35 ± 7.84
MS23	6.29 ± 0.54	MS10	51.20 ± 1.41	MS48	60.49 ± 1.92
MS27	9.39 ± 0.45	MS17	41.90 ± 2.22	MS49	89.80 ± 0.69
MS28	4.94 ± 0.12	MS30	46.06 ± 3.21	MS50	69.54 ± 1.62
MS29	6.70 ± 0.58	MS31	28.32 ± 2.11	MS51	115.60 ± 8.77
MS33	18.74 ± 1.2	MS32	47.13 ± 3.24	MS52	114.93 ± 9.72
MS33	18.74 ± 1.2	MS35	33.30 ± 3.04		
MS36	1.98 ± 0.04	MS46	53.86 ± 5.13		
MS39	1.02 ± 0.89	MS47	42.48 ± 3.11		
MS40	8.06 ± 0.51	MS53	43.32 ± 3.93		
MS41	8.60 ± 1.39	MS54	36.09 ± 1.28		

Notes: [a]Different times required to reach the end of exponential growth. Minimum time required was three days. [b]Without enzyme. Values are the means of three independent experiments ± standard deviation.
Abbreviations: MRS, deMan Rogosa Sharpe; TJ, tomato juice.

Effect of pH and/or L-malic acid on enzyme activity and aroma compounds production

Oenococcus oeni MS25 strain appeared able to hydrolyze *p*-NPG under all conditions assayed. However, β-glucosidase activity was positively and negatively influenced by L-malic acid and pH, respectively ranged between 174.2 to 31.2 U/g. At pH 4.8 (control medium), L-malic acid addition markedly increased the activity and it was maximum. By contrast, at pH 3.8 the lowest activity was detected (65% lower than at pH 4.8). In this condition, L-malic acid stimulated about 10% the enzymatic activity, but it was not enough to annul completely the loss of activity compared with that obtained at pH 4.8 (Figure 4).

The initial pH and/or L-malic acid affected negatively the diacetyl, acetoin and 2,3-butilenglycol production by the MS25 strain as regards to the control medium. The more pronounced effect was observed at pH 3.8 and in presence of L-malic acid (Figure 4).

Discussion

Despite the overall significance of LAB in wine production, there are only occasional reports of their isolation from juice grape. In our study numbers of LAB found in grape juice (approximately $\log_{10}$ 3.3) was in accordance with the results reported by du Plessis and colleagues.[29] Lower LAB population was found in grape juice than in the samples from commencement of MLF. This fact may be related with the inability of LAB to compete efficiently with the yeasts and non-LAB population present in the natural medium, or to the metabolic products such as ethanol, SO_2,[30] medium-chain fatty acids excreted by yeasts[31] and/or intracellular and extracellular cationic proteins.[32] In addition, Bae and colleagues[26] demonstrated that in wine grapes initial LAB populations were very low and less than 10^2 cfu/gram. On the other hand at the stage corresponding at commencement of MLF the LAB growth could be helped by the increase in yeast lysis products.

No significant difference in the LAB count between the two samples from beginning of MLF occurred. However, Reguant and colleagues,[33] reported that the LAB population of the wines that had been inoculated with yeast strain C2 decreased at the end of the AF as regards other vinification without it, indicating that the yeast starter culture might have controlled the LAB development.

In grape juice non-LAB presumibly identified as acetic acid bacteria were isolated. The species *Asaia siamensis* considered within the genera of acetic acid bacteria was isolated from wines grapes.[26] No analyzed samples gave detectable *Enterobacteriaceae*. Either they were absent in the grape juice and wines samples or they were overgrown by other species during isolation.

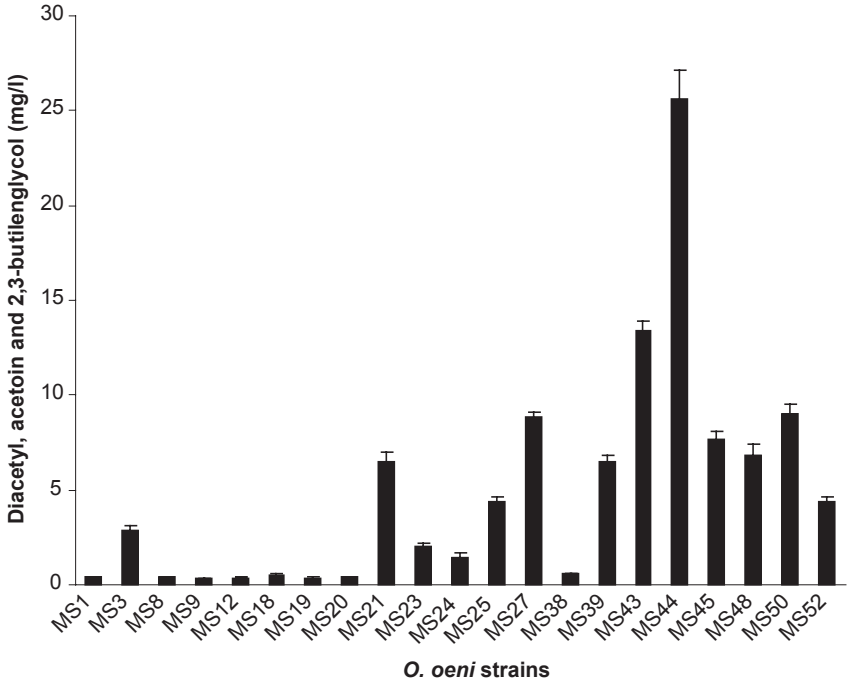

Figure 3 Diacetyl, acetoin or 2,3-butilenglycol production by the *Oenococcus oeni* isolates in MRS medium with TJ, 15%, measured at the end of exponential growth. *Oenococcus oeni* strains that did not exhibit the aroma compounds formation were not included in the graph.
Abbreviations: MRS, deMan Rogosa Sharpe; TJ, tomato juice.

Oenococcus oeni was the most dominant species among the LAB isolates, accounting for 68% of the total isolated strains. However, in grape juice other LAB species than *O. oeni* was the dominant species. This fact could be related with its numerous nutritional requirements for development. It was demonstrated that the majority of amino acids were stimulatory or essential for its growth.[28,32,34] Thus, the decreased growth of *O. oeni* in the natural juice could be due to a nutritional competition. On the other hand the predominance of *O. oeni* at the beginning of MLF (representing 79.6% of the total of LAB isolates), has been also reported in other wines.[1,4,33] du Plessis and colleagues[5] described that *Lactobacillus plantarum* were the dominating species in the grape juice, but their viability decreased significantly during AF, *O. oeni* being the dominating species in 15 of the 23 base wine samples that had undergone spontaneous MLF.

By comparing results obtained from growth responses of *O. oeni* isolates, phenotypical and genotypically were identified, in MRS medium with TJ, we observed that coincidently the lowest final biomass corresponded to isolates from grape juice, where *O. oeni* faced the highest development difficulties.

Studies investigated about the glycosidase activities of *O. oeni*, but an extensive screening including more than 45 wild strains of this species is reported here for the first time. β-glucosidase activities from whole cells of *O. oeni* isolates measured at the end of the exponential growth phase

Table 3 Proportion of *Oenococcus oeni* strains from grape juice and fermented musts in the different groups according to the levels of enzyme and aroma compounds produced

	Proportion of strains (%)			
Diacetyl (mg/l)	**β-glucosidase activity (U/g)**			
Levels	**Not detected**	**Low (1–20)**	**Intermediary (>20–60)**	**High (>60)**
Not detected	13	14.8	28	5.5
≥0.23 ± 0.04 to ≤4.4 ± 0.22	7.4	5.5	3.7	7.4
>4.4 ± 0.22 to ≤8.9 ± 0.26	3.7	3.7	0	1.8
≥9 ± 0.49	3.7	0	0	1.8

Notes: Values are the means of three independent experiments ± standard deviation.

Table 4 Influence of L-malic acid and initial pH on the maximum population and growth rate of *Oenococcus oeni* MS25 and on the residual glucose and L-malic acid of cultures

Culture medium[a]	Initial pH	Maximum population[b]	Growth rate[c] (μ)	Glucose (g/l)	L-malic acid (g/l)
MRS + TJ (CM)	4.8	0.95 ± 0.06	0.067 ± 0.004	5.10 ± 0.42	ND
CM + L-malate		1.30 ± 0.43	0.076 ± 0.004	4.34 ± 0.23	0
MRS + TJ (MCM)	3.8	0.55 ± 0.09	0.049 ± 0.002	8.80 ± 0.51	ND
MCM + L-malate		0.72 ± 0.02	0.056 ± 0.002	7.13 ± 0.38	0.43 ± 0.02

Notes: [a]Initial glucose and L-malic acid concentrations in MRS + TJ, 15% media (CM or MCM) were 20 and 2.5 g/l, respectively. [b]Absorbance values reached at the end of exponential growth phase were used for maximum bacterial population determinations. Bacterial growth was followed by OD at 560 nm. [c]Growth rate was calculated during the exponential phase of growth on at least three linear points determined by plotting the data as Ln (OD_{560} nm) as a function of time of culture. Values are the means of three independent experiments ± SD.

Abbreviations: CM, culture medium; MCM, maximum culture medium; MRS, deMan Rogosa Sharpe; SD, standard deviation; TJ, tomato juice.

demonstrated a range of capabilities. It is interesting to note that the majority of cells produced a detectable activity. By contrast, Barbagallo and colleagues[19], demonstrated that only six out of 11 strains of *O. oeni* studied possessed this activity. However, Grimaldi and colleagues[35] detected activity in all cultures derived from 11 commercial preparations of *O. oeni*. In this study, nevertheless β-glucosidase activity varied according to the strain, we consider that its distribution was quite uniform under assayed conditions, since activity was observed in more than 60% of whole cells, and they produced intermediary or low levels accounting for 74.3% of the total of positive cells for this activity. Strains showing high enzymatic activity were found in the lowest extent and they were isolated from the begining in MLF samples, so this first screening for β-glucosidase activity is very important for selection in further investigations. On the other hand, Bloem and colleagues[36] reported that glycosylated precursors in oak wood are different from those obtained in grape glycosylated precursors, which are bound with a β-D-glucose. So, the further study of other glucosidic enzymes such as α-L-rhamnopyranosidase and α-L-arabinofuranosidase is necessary to complete this first characterization.

An interesting finding was that the highest proportion of *O. oeni* population (61%) did not possess the capacity to produce diacetyl, acetoin, and 2,3-butilenglycol, contrary to that observed for β-glucosidase activty. Saguir and Manca de Nadra[9,27] reported that in one *O. oeni* strain from wine, that no diacetyl, acetoin, 2,3-butilenglycol were found during growing in a complete or N-deficient synthetic media. In our study no direct relation among the *O. oeni* isolates for both biochemical activities could be clearly established. Thus, strains that failed to form the aroma compounds were able to produce different levels of β-glucosidase activity. On the other hand, strains producing the highest concentrations of aroma compounds mainly failed in their enzymatic activities and only a lesser proportion possessed high activity (Table 3). However, considering the relation between the vinification steps, in which the *O. oeni* strains were isolated with the biochemical properties studied of each one, we can establish that both β-glucosidase activity as C_4 aroma compounds formation occurred at a higher frequency in isolated strains from beginning of MLF than grape juice.

It was particularly interesting that the selected MS25 strain was able to grow at pH 3.8. In addition L-malic acid stimulated its growth. This result was in agreement with previous studies for which a stimulating effect of L-malate was observed at pH 4.8 or below.[9,37] Higher values of final pH in the media with L-malic acid than without it (data not shown) would be related with the dicarboxylic acid utilization by the MS25 strain. The fact that L-malic acid was totally consumed at pH 4.8 and more than 80% at pH 3.8 is of particular importance for considering the use of the MS25 strain on wine. Augagneur and colleagues[37] demonstrated that L-malate utilization contributed to the generation of a high ΔpH. They also reported that under low pH conditions the decrease of Δψ, that controls the rate of transport/fermentation coupled with an increased of the ΔpH that drives the transport, contributes to an increased fermentation rate. In *O. oeni* MS25 growing at pH 4.8 in presence of L-malic acid, significant difference in glucose consumption was determined in accordance with the higher biomass production.

L-malic acid stimulated β-glucosidase activity in all conditions, but the initial pH 3.8 had a negative effect on it, despite maintaining a significative enzyme production.

The assays at pH 3.8 and/or with L-malic acid showed a decreased production of C_4 aroma compounds as regards to those obtained at pH 4.8. Nielsen and Richelieu[38] reported that the range of pH values previously recommended for diacetyl production by LAB in various fermented foods was 4.3–4.7.

In conclusion, *O. oeni* was the dominant species among the total of the LAB isolates. The results of screening indicated that the majority of whole cells showed detectable β-glucosidase activity at the end of exponential growth.

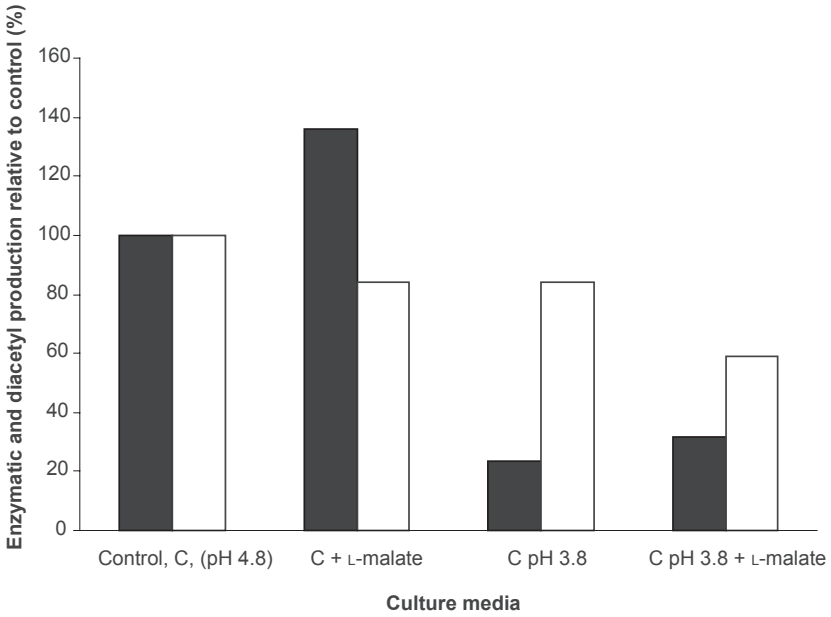

Figure 4 Influence of pH and/or L-malic acid on the β-glucosidase activity and diacetyl production measured in whole cells and supernatant of cultures, respectively at the end of exponential growth phase in *Oenococcus oeni* MS25.

Contrarily the highest proportion of them did not produce diacetyl, acetoin and 2,3-butilenglycol. In the selected MS25 strain the findings indicated that L-malic acid is compatible with a good level of enzyme activity and in part able to annul the negative influence of low pH. In different conditions the aroma compounds were lower than 4 mg/ml, especially at pH 3.8 and in presence of L-malic acid. So this strain would have adequate characteristics for potential use in winemaking, although more experiments are needed to confirm it. Finally, from these results we considerer that the assessment of both biochemical properties studied in the *O. oeni* strains should be taken in account as a quality criterion of selecting starter cultures for the improvement of the organoleptic properties of wines, although further experiments are needed to confirm it.

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