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The effect of organic acids and sulfur dioxide on C4 compound production and β-glucosidase activity of *Oenococcus oeni* from wines under acidic conditions

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Abstract: The purpose of this work was to investigate the effect of l-malic and citric acids and SO_2 on two biochemical properties (diacetyl/acetoin/2,3-butanediol formation and β-glucosidase activity) relevant to flavor development in six *Oenococcus oeni* strains from wines at pH 4.8 and 3.8. Cells were cultured in MRS without citrate (control medium) and combined with l-malic acid (2 g/L), citric acid (0.7 g/L), and SO_2 (80 mg/L) at pH 4.8 and 3.8. All the test strains grew at all conditions tested including in the presence of SO_2 and at initial pH 3.8, even though growth parameters were maximum in the presence of both the acids at pH 4.8. Organic acids were depleted totally regardless of the condition examined, in which degradation of l-malic acid was faster than that of citric acid. Diacetyl, acetoin, and 2,3-butanediol levels significantly varied depending on the strain for a given condition, for example, at pH 4.8 in control medium the highest value (6.55±0.31 mg/L, strain MS25) represented almost threefold the lowest one (2.43±0.22 mg/L, strain MS9). There was also variability for each strain depending on the initial pH (strains MS25, MS27, and MS48) and the presence of organic acids (all strains except MS25) but not SO_2. In addition, among strains there was a trend toward mainly diacetyl formation (55%–75%). *O. oeni* MS9, MS20, and MS46 yielding adequate diacetyl levels were selected for investigating specific β-glucosidase activity and its possible cell localization. Cell suspensions of all the selected strains exhibited positive activities at both pH values which were >4.8. As observed for C4 compounds, organic acids stimulated this activity (28%–49% at pH 4.8; ~20% at pH 3.8), thus partially reverting the inhibition caused by acid stress, while SO_2 did not affect it. The use of different cell fractions (permeabilized cells, cell protoplasts, and cell extracts) associated this activity to the cell surface. Results indicated that diacetyl formation and β-glucosidase activity levels in *O. oeni* strains as influenced by acidity and organic acids are of relevance for vinification decisions.

Keywords: *O. oeni*, metabolism, enzymatic activity, aroma, wine

Introduction

Wine is one of the oldest products where microbiological processes contribute significantly to the final quality of the product. Malolactic fermentation (MLF) that normally occurs after the completion of alcoholic fermentation (AF) consists of the bioconversion of the malic acid in wine to lactic acid and carbon dioxide and is carried out by lactic acid bacteria (LAB), mainly *Oenococcus oeni*. Besides de-acidifying the wine, MLF provides microbiological stability and can improve the final aroma balance by modifying fruit-derived aromas and producing aroma-active compounds by themselves.[1,2] In this

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last regard, diacetyl is considered as one of the most important flavors produced during MLF, its concentration being generally low with respect to its flavor threshold.[2] Presence of this compound at concentrations higher than the sensory threshold (4 mg/L) is regarded by many to be undesirable in the wine or a spoilage character, whereas in the range of 1–4 mg/L and depending on the style and type of wine, it is considered to contribute a desirable "buttery" or "butterscotch" flavor character.[2,3] In wine, diacetyl is derived essentially from citric acid metabolism by LAB, which is formed as an intermediate metabolite in the reductive decarboxylation of pyruvic acid through α-acetolactate to 2,3-butanediol.[4,5] Diacetyl may be reduced further to acetoin which combined with 2,3-butanediol in normal concentrations has no influence on the wine flavor because of their high aroma threshold (~150 and 600 mg/L, respectively).[6] According to Martineau and Henick-Kling,[7] significant differences in final diacetyl concentrations of diacetyl obtained during MLF conducted by different LAB strains were found. However, from few data regarding *O. oeni*, it was found that the influence of physicochemical factors was related to winemaking conditions such as pH, organic acids, and SO_2 in C4 compound formation.[7,8] In this regard, it was reported that sulfur dioxide (SO_2), which is an antiseptic that is generally added to the grape juice before AF and after MLF for its preservation, has the tendency to react with carbonyl compounds.[2,9,10]

On the other hand, glycosidase activities of LAB have a major impact on wine sensory profile.[5,11] Grapes contain compounds called "aroma precursors," of which glycosides are more active.[12] Thus, volatile compounds present as monoglucosides are liberated via β-D-glucosidase (glucopyranosidase), whereas diglycoside-bound aglycones are liberated through a sequential release by different glycosidases followed by β-D-glucosidase.[13] Grimaldi et al[14] demonstrated that *O. oeni* strains possess various glycosidase activities; however, these activities were dependent on wine conditions such as pH and ethanol and residual sugar content. McMahon et al[15] reported that the β-glucosidase activity of *O. oeni* was strongly inhibited by low pH, high alcohol concentration, anaerobic conditions, and presence of glucose. However, information regarding how the organic acids and/or SO_2 affect enzyme β-glucosidase activity in *O. oeni* under acidic conditions is still limited. In a previous work, Saguir et al[16] demonstrated that up to 60% of the total of 54 *O. oeni* strains analyzed that were isolated from Argentinean wines did not produce C4 compounds at the end of the bacterial growth in MRS medium adjusted to pH 4.8, whereas, in this condition, the majority of whole cells showed detectable levels of β-glucosidase activity. On the other hand, it should be noted that the enzymatic activities of greatest interest are those conferred by a single enzyme, ideally with an extracellular localization.

Hence, the objective of this study was to investigate comparatively the influence of L-malic and citric acids and/or SO_2 on the growth parameters, C4 compound production, and β-glucosidase activity in *O. oeni* strains isolated from Argentinean wines at acid pH values, and also at the same time to analyze the cell localization of β-glucosidase enzyme in selected strains, using different cellular fractions. The results obtained will provide a better interpretation of the relationships between physicochemical parameters of enological interest and two relevant biochemical properties that improve aromatic complexity of wines, as a selection characteristic for strains with good malolactic and aromatic potential to be used as starter cultures in the wine industry.

Materials and methods

Bacterial strains

O. oeni MS9, MS20, MS25, MS27, MS46, and MS48 were isolated from grape juice and the fermented musts were collected in one cellar located in North Argentina.[16] The strains were stored at −20°C in MRS medium (Oxoid Ltd., London, England) supplemented with 10% (v/v) of tomato juice and glycerol (30% v/v).

Media, growth conditions, and culture procedures

Modified MRS medium (de Man, Rogosa and Sharpe) without ammonium citrate and supplemented with tomato juice (10%) at pH 4.8 was used as CM (control medium), which was added with L-malic acid (2 g/L) and citric acid (0.7 g/L) (MCCM). MCCM was also modified by the addition of 80 mg/L SO_2 (added as $Na_2S_2O_5$; Sigma Chemical Co., St. Louis, MO, USA) (SMCCM). CM and MCCM were adjusted to pH 4.8 while CM, MCCM, and SMCCM were also adjusted to pH 3.8 with 1N HCl before sterilization at 120°C for 20 minutes. The $Na_2S_2O_5$ solution was sterilized by filtration through a nylon membrane (0.22 μm pore size; EMD Millipore, Billerica, MA, USA) and then added to sterilized medium.

For inoculum preparation, cells cultured in CM at pH 4.8 were harvested at the end of exponential phase (72–80 hours) and resuspended in sterile distilled water to OD_{560nm} =0.90. Cell suspensions (CS) were used to inoculate individually the experimental media at a rate of 2%. For the final cultures in experimental media adjusted at pH 3.8, cells were precultured in CM at pH 3.8 at 30°C before inoculating in

test media at a rate of 2% (v/v) as indicated earlier. It should be noted that in the adaptation medium, an important lag phase was observed (of about 10 hours) before the bacterial growth began.

All cultures were incubated statically at 30°C for 7 days, and supernatants (SNs) were stored frozen (–18°C) for subsequent chemical analysis.

Growth measurement

Bacterial growth was monitored by periodic spectrophotometric measurements at 560 nm using a spectrophotometer (WPA Bioware DNA, Biochrom, England). Cell cultures were diluted, if necessary, with sterile medium prior to measuring OD_{560nm} to maintain linearity between OD and biomass. At the same time, the colony-forming units (cfu/mL) were determined. From these data, it was possible to calculate the average growth rates (μ_{max}) by using the formula: $m = (1/t)(\log_{10}N_t/N_0 \times 2.303)$ where t is the time required for cells to increase from N_0 to N_t.

Analytical methods

D-Glucose, L-malic, L-lactic, and citric acids were measured by enzymatic methods (Boheringer Kits, Mannheim, Germany). Diacetyl and acetoin (C4 compounds) were analyzed as a combined value according to the colorimetric method described by Hill et al[17] modified by Branen and Keenan,[18] while diacetyl was determined by a modification in the spectrophotometric method of Voges–Proskauer–Coblenz[19] under the same conditions that were used for the C4 compound determination.

β-glucosidase activity

Enzyme assay was conducted in *O. oeni* suspension cells (SC) obtained by centrifugation at 4,000 × *g* for 20 minutes at 4°C from different culture conditions at 30°C. Enzymatic activity was measured according to D'Incecco et al[13] using *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NP-*β*-D-glucoside; Sigma Chemical Co.) as substrate. The assay was performed for 30 minutes at 37°C, after which 400 µL of 1 M sodium carbonate solution was added to stop the reaction and to 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 (WPA Bioware DNA) in a 1cm cell. The enzymatic activity was expressed as U/g which was equivalent to micromoles of *p*-nitrophenyl released per minute and gram of dry weight of cells.

In near conditions to vinification (SMCCM medium, pH 3.8) the β-glucosidase enzyme localization was investigated Thus, the aforementioned enzymatic assay was conducted in CS (control), permeabilized cell (PC), cell extract (CE), and cell protoplast (CP). In a previous study, it was demonstrated that there was no activity in culture SNs of test *O. oeni* strains.[16]

PC

Cells were permeabilized according to the procedure of Salmon[20] modified by Rosi et al[21] and optimized in our laboratory. The culture (1 mL) was centrifuged at 5,000 × *g* for 10 minutes at 4°C, and the pellet was washed with 1 mL of distilled water. The pellet was resuspended in 1 mL of imidazole buffer (75×10^{-3} mmol/L, pH 7.5), and then 50 µL of 0.3 M glutathion, 10 µL of 10% Triton X-100, and 50 µL of toluene/ethanol (1:4 v/v) were added. The suspension was placed on a mechanical shaker for 5 minutes and then centrifuged. The pellet was washed with distilled water and resuspended in sodium acetate buffer (0.1 M, pH 5.1). Therefore, the permeabilized fraction consisted of washed cells, which had the cell walls compromised.

CE

Cells grown in SMCCM were harvested by centrifugation at 4,000 × *g* for 20 minutes at 4°C, washed once in sodium acetate buffer (0.1 M, pH 5.1), and then suspended in the same buffer (30%, w/v). Cells were disrupted on ice by sonic treatment (2 minutes; 10 cycles, 80% pressure 1000 psi). Subsequently, CS were centrifuged at 10,000 × *g* for 15 minutes at 4°C, and SNs were assayed for enzyme activity. Protein contents of enzyme extracts were determined using the method of Bradford with bovine serum albumin as a standard.

CP

Protoplasts were obtained according to procedure described by Holt and Ricciardi.[22] At the end of growth, cells (1 mL) were centrifuged at 8,000 × *g* for 10 minutes at 4°C and resuspended with an equal volume of THMS buffer (Tris/HCl, 30 mM; $MgSO_4$, 3 mM; and sucrose 25%) containing 4 mg lysozyme/mL, then incubated for 2 hours at 37°C. The cells were washed twice in THMS buffer and resuspended in 1 mL lysis buffer (sodium phosphate buffer, 50 mM, pH 7.2; dithiothreitol, 1 mM). Acetone/toluene (9:1 v/v) was added (20 µL) and mixed by vortex action for 1 minute. Formation of protoplasts (P) was confirmed by light microscopy. P-solution was stored on ice and assayed for β-glucosidase activity. Enzyme activity was also determined in SN obtained from the two successive centrifugations (after treatment with each buffer), F1 and F2 samples.

Statistical analysis

The experimental data were analyzed by one-way analysis of variance. Variable means showing statistically significant differences were compared using Tukey's test (Minitab student R12). All statements of significance were based on the 0.05 level of probability.

Results

Growth of *O. oeni*, utilization of organic acids, and C4 compounds production under various conditions

Table 1 shows the growth parameters, pH variation, substrate utilization, and production of C4 compounds by *O. oeni* MS9, MS20, MS25, MS27, MS46, and MS48 at pH 4.8. In CM, the strains tested grew with growth rates (μ_{max}) of 0.09/0.08±0.01 hour^{-1} to reach cell densities ranging between 8.20±0.28 and 8.41±0.26 log cfu/mL at 30°C. Addition of organic acids to CM increased the extent of growth of *O. oeni* strains between 1.2- and 1.4-fold as well as $\mu_{max.}$ In this condition, the growth parameters were maxima for the MS27, MS46, and MS48 strains (3.05 log cfu/mL 0.13 hour^{-1}, 2.89 log cfu/mL 0.13 hour^{-1}, and 3.25 log cfu/mL 0.13 hour^{-1}, respectively). Bacterial growths were accompanied by pH reductions of 0.55–0.65 units in CM, whereas in the presence of l-malic acid, the transitory pH increased by about 0.2 units during the first incubation hours and subsequently decreased to final values of about 4.62±0.03 similar to that reported by Saguir and Manca de Nadra.[23] Glucose was consumed by between 20% and 30% of its initial concentration at the end of bacterial growth depending on medium composition, observing the slightly lower consumptions in the presence of organic acids. L-malic and/or citric acids were completely utilized in co-metabolism with glucose. Metabolism of three substrates commenced almost immediately with bacterial growths and occurred faster for l-malic acid which was recovered account ~100% as L-lactic acid, explaining the pH increase during the first culture hours (Table 1). In CM four of the six strains showed detectable levels of production of C4 compounds, whereas MS20 and MS46 did not produce these compounds under experimental condition. Among the positive strains, the mean C4 compound value was 4.19 mg/L, but this varied considerably; the highest value (6.55±0.31 mg/L, strain MS25) representing almost threefold the lowest one (2.43±0.22 mg/L, strain MS9). Addition of organic acids significantly stimulated the production of C4 compounds in all the test strains except in MS25. These increments varied between 1.8- and 2.7-fold depending on strain, thus *O. oeni* MS27 and MS 48 reached C4 compounds values by up to 9 mg/L Interestingly, under various growth conditions, diacetyl represented

Table 1 Parameters analyzed in *Oenococcus oeni* strains in CM and MCCM at pH 4.8

		O. oeni					
Parameter	**Medium**	**MS9**	**MS20**	**MS25**	**MS27**	**MS46**	**MS48**
A[a]	CM	2.26^{aX}	2.21^{aX}	2.22^{aX}	2.36^{aX}	2.40^{aX}	2.30^{aX}
	MCCM	2.60^{aY}	2.85^{abY}	2.55^{aY}	3.05^{abcY}	2.89^{abY}	3.25^{abcY}
μ_{max} (h^{-1})	CM	0.09^{aX}	0.09^{aX}	0.08^{aX}	0.09^{aX}	0.09^{aX}	0.09^{aX}
	MCCM	0.13^{aY}	0.13^{aY}	0.13^{aY}	0.13^{aY}	0.13^{aY}	0.13^{aY}
Final pH	CM	4.25^{aX}	4.23^{aX}	4.22^{aX}	4.17^{aX}	4.17^{aX}	4.20^{aX}
	MCCM	4.65^{aY}	4.60^{aY}	4.66^{aY}	4.61^{aY}	4.62^{aY}	4.58^{aY}
Glucose (g/L)[b]	CM	14.20^{aX}	14.51^{aX}	14.41^{aX}	14.32^{aX}	13.96^{aX}	14.41^{aX}
	MCCM	15.61^{aX}	15.40^{aX}	15.55^{aX}	15.32^{aX}	15.61^{aX}	15.82^{aX}
L-Malic acid (g/day)[b,c]	MCCM	0.81^{a}	0.86^{a}	0.77^{a}	0.79^{a}	0.86^{a}	0.89^{a}
Citric acid (g/day)[b,c]	MCCM	0.23^{a}	0.21^{a}	0.19^{a}	0.19^{a}	0.19^{a}	0.23^{a}
L-Lactic acid (g/L/day)	MCM	1.34^{aX}	1.33^{aX}	1.35^{aX}	1.34^{aX}	1.33^{aX}	1.34^{aX}
	MCCM	1.31^{aX}	1.28^{aX}	1.34^{aX}	1.34^{aX}	1.33^{aX}	1.33^{aX}
C4 compounds (mg/L)	CM	2.43^{aX}	ND	6.55^{bX}	3.62^{acX}	ND	4.17^{cX}
		(80.0)	NA	(77.7)	(69.9)	NA	(52.6)
	MCCM	4.48^{aY}	2.70^{bY}	7.81^{cXY}	9.82^{dY}	2.64^{bX}	11.0^{dZ}
		(72.8)	(78.9)	(73.6)	(67.9)	(72.7)	(66.5)
YBglu	CM	0.08^{aX}	NA	0.18^{bX}	0.11^{abX}	NA	0.08^{aX}
YBglu+cit	MCCM	0.13^{aX}	0.08^{a}	0.20^{abX}	0.28^{bY}	0.07^{a}	0.33^{bY}

Notes: Values are the means of duplicate determinations in three independent experiments. Different letters within row (a,b,c) and columns (X,Y,Z) indicate significance with a *P*-value <0.05 for a given parameter. Means with the same letter are not significantly different. [a]Difference in cell concentration (log cfu/mL) between stationary phase and inocula. [b]Initial concentration of glucose, 20±0.17 g/L; L-malic acid, 2.0±0.08 g/L; citric acid, 0.70±0.03 g/L. [c]Degradation rate (g/day). Both the acids were completely consumed. ( ) represents percentage of diacetyl related to the total C4 compounds produced. YB (%): substrate bioconversion yield (YBglu or YBglu+cit, diacetyl (mM)/glucose or glucose + citrate consumed (mM) in CM or MCCM, respectively) ×100.

Abbreviations: CM, control medium; MCCM, L-malic and citric acids; NA, not applicable; ND: not detected; YB, bioconversion yield.

~70% of the total concentrations of C4 compounds formed. As C4 compounds are produced from pyruvate which can derive from citrate and glucose, calculated net bioconversion yields of glucose (YBglu) or glucose + citrate (YBglu+cit) into diacetyl in CM and MCCM varied from 0.08% to 0.18% and 0.08% to 0.33%, respectively (Table 1).

In order to approximate the enological conditions, studies were performed in the same media but at pH 3.8 and in the presence of SO_2 (SMCCM) (Table 2). At this pH, organic acids stimulated final biomass and μ_{max} of the MS9, MS20, and MS46 strains by ~25% and between 60% and 90%, respectively, but not of the remaining strains in opposition as observed at pH 4.8. Presence of SO_2 decreased μ_{max} by 47%–58% of all the strains tested but not affected their final biomass compared to MCCM. Similar glucose consumptions were obtained for all strains and conditions studied at pH 3.8 which, in general resulted lower than those obtained for the same condition at pH 4.8 coinciding with the lower final biomass formed. However, organic acids were almost completely consumed and >94% of L-lactic acid was recovered from L-malate decarboxylation, in a similar way as observed at pH 4.8 (Table 2). At pH 3.8, the C4 compounds were also not produced by *O. oeni* MS20 and MS46 in CM, whereas the mean value of C4 compounds formed by the remaining strains was 2.25 mg/L, ~90% lower than that obtained at 4.8. In this condition, the levels produced also considerably varied between strains (lowest, 1.05 mg/L strain MS27; highest 3.34 mg/L strain MS25) as observed at pH 4.8. Also the presence of organic acids increased their formation with stimulation percentages varying between 1.6- and 4.5-fold except for strain MS25. In this condition, SO_2 did not produce any significant modification on C4 compound levels formed, compared to MCCM without it. Again diacetyl was mainly formed representing between 55% and 76% of the total concentration of C4 compounds, whereas YBglu+cit into diacetyl varied between 0.12% and 0.24% (Table 2).

Effect of organic acids and SO_2 on β-glucosidase activity in selected *O. oeni* strains

Based on the characterization of C4 compound production, we selected the strains of *O. oeni* MS9, MS20, and MS46 for investigating their specific β-glucosidase activity in CS obtained at the end of exponential growth phase at pH 4.8 and 3.8, under different conditions (Figure 1). In CM, all selected strains showed detectable levels of β-glucosidase activity which varied between 30–60 U/g and 20–35 U/g, at pH 4.8 and 3.8 respectively. *O. oeni* MS20 exhibited the highest activity but at the same time the most marked inhibition percentage (42%) when initial pH was decreased from 4.8 to 3.8 in contrast to the MS46 strain which was slightly inhibited (28%). Addition of organic acids to CM increased β-glucosidase activity, between 28% and 49%, depending on strain at pH 4.8 and to ~20% at pH 3.8. Also, the addition of SO_2 to MCCM, pH 3.8, did not affect the enzymatic activity of CS of the three strains that are studied in this research significantly.

In the condition more near to vinication (SMCCM, pH 3.8), the β-glucosidase sub-cellular location was investigated using different cell fractions (PC, CE, and CP). Assays with CS were

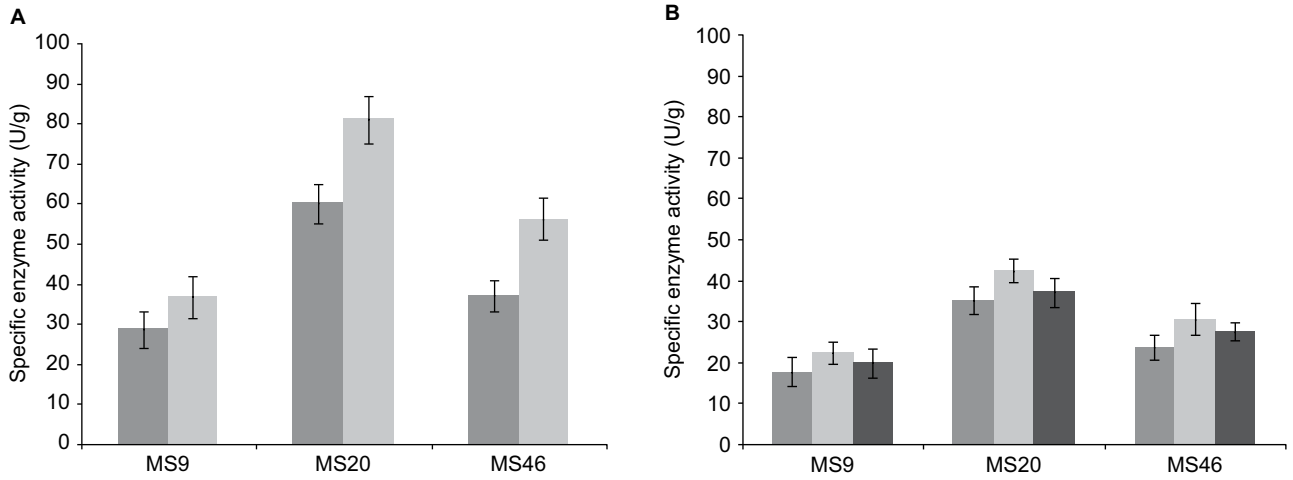

Figure 1 Specific β-glucosidase activity of *O. oeni* MS9, MS20, and MS46 in CM (▒), MCCM (▒) adjusted at pH 4.8 (**A**) and CM, MCCM, and SMCCM (▒) at pH 3.8 (**B**).
Note: Values are the means of duplicate determinations in three independent experiments.
Abbreviations: CM, control medium; MCCM, L-malic and citric acids; SMCCM, addition of SO_2 with L-malic and citric acids.

Table 2 Parameters analyzed in *Oenococcus oeni* strains in CM by MCCM and SMCCM at pH 3.8

		O. oeni					
Parameter	**Medium**	**MS9**	**MS20**	**MS25**	**MS27**	**MS46**	**MS48**
A[a]	CM	1.14^{aX}	1.74^{bX}	1.13^{aX}	1.60^{bcX}	1.62^{bcX}	1.45^{bX}
	MCCM	1.45^{aY}	2.10^{bY}	1.40^{aX}	2.01^{bY}	2.03^{bY}	1.65^{aX}
	SMCCM	1.48^{aY}	2.07^{bY}	1.32^{aX}	1.93^{bY}	1.95^{bY}	1.59^{aX}
μ_{max} (h^{-1})	CM	0.021^{abX}	0.026^{aX}	0.017^{bX}	0.017^{bX}	0.023^{abX}	0.020^{abXY}
	MCCM	0.023^{aX}	0.035^{bY}	0.024^{aX}	0.031^{abY}	0.037^{bY}	0.028^{abX}
	SMCCM	0.011^{abY}	0.017^{aY}	0.008^{bY}	0.014^{abX}	0.015^{abX}	0.012^{abY}
Final pH	CM	3.45^{aX}	3.40^{aX}	3.50^{aX}	3.46^{aX}	3.41^{aX}	3.43^{aX}
	MCCM	3.76^{aY}	3.74^{aY}	3.75^{aY}	3.74^{aY}	3.76^{aY}	3.76^{aY}
	SMCCM	3.77^{aY}	3.76^{aY}	3.70^{aY}	3.76^{aY}	3.78^{aY}	3.75^{aY}
D-Glucose (g/L)[b]	CM	16.90^{aX}	16.92^{aX}	16.72^{aX}	16.43^{aX}	16.81^{aX}	16.81^{aX}
	MCCM	17.41^{aX}	17.33^{aX}	17.42^{aX}	17.12^{aX}	17.91^{aX}	17.89^{aX}
	SMCCM	17.58^{aX}	17.71^{aX}	17.59^{aX}	17.86^{aX}	17.82^{aX}	17.96^{aX}
L-Malic acid (g/L/day)[b,c]	MCCM	0.27^{aX}	0.32^{aX}	0.28^{aX}	0.29^{aX}	0.32^{aX}	0.34^{aX}
	SMCCM	0.17^{aY}	0.20^{aY}	0.16^{aY}	0.18^{aY}	0.20^{aY}	0.20^{aY}
Citric acid (g/L/day)[b,c]	MCCM	0.10^{aX}	0.11^{aX}	0.09^{aX}	0.10^{aX}	0.12^{aX}	0.11^{aX}
	SMCCM	0.05^{aY}	0.04^{aY}	0.03^{aY}	0.04^{aY}	0.04^{aY}	0.05^{aY}
L-Lactic acid (g/L)	MCCM	1.28^{aX}	1.27^{aX}	1.29^{aX}	1.27^{aX}	1.29^{aX}	1.30^{aX}
	SMCCM	1.26^{aX}	1.27^{aX}	1.29^{aX}	1.28^{aX}	1.28^{aX}	1.27^{aX}
C4 compounds (mg/L)	CM	2.57^{aX}	ND	3.34^{aX}	1.05^{bX}	ND	2.05^{abX}
		(75.9)	NA	(79.9)	(75.1)	NA	(63.4)
	MCCM	4.05^{aY}	2.00^{bX}	4.25^{aX}	4.68^{aY}	2.35^{bX}	6.90^{cY}
		(74.8)	(75.0)	(74.6)	(73.5)	(74.9)	(65.1)
	SMCCM	3.47^{aY}	1.87^{bX}	4.05^{aX}	4.22^{aY}	2.13^{bX}	6.38^{cY}
		(70.1)	(65.5)	(65.2)	(59.3)	(59.3)	(56.1)
YBglu	CM	0.15^{abX}	NA	0.25^{bX}	0.05^{aX}	NA	0.09^{aX}
YBglu+cit	MCCM	0.24^{aY}	0.12^{b}	0.24^{aX}	0.24^{aY}	0.12^{b}	0.30^{aY}

Notes: Values are the means of duplicate determinations in three independent experiments. Different letters within rows (a,b,c) and columns (X,Y,Z) indicate significance with a *P*-value <0.05 for a given parameter. Means with the same letter are not significantly different. [a]Difference in cell concentration (log cfu/mL) between stationary phase and inocula. [b]Initial concentration of glucose, 20±0.17 g/L; L-malic acid, 2.0±0.08 g/L; citric acid, 0.70±0.03 g/L. [c]Degradation rate (g/day). Both the acids were completely consumed. ( ) represents percentage of diacetyl related to the total C4 compounds produced. YB (%): substrate bioconversion yield (YBglu or YBglu+cit, diacetyl (mM)/glucose or glucose + citrate consumed (mM) in CM or MCCM, respectively) ×100.

Abbreviations: CM, control medium; MCCM, L-malic and citric acids; SMCCM, addition of SO_2 with L-malic and citric acids; NA, not applicable; ND: not detected; YB, bioconversion yield.

included as control (Figure 2). Specific activities obtained in CE and P resulted in <1–2 U/g regardless of strain tested while PC of the MS9, MS20, and MS46 strains showed the greatest values corresponding to 25, 51, and 43 U/g, respectively, which resulted between 33% and 42% higher than those obtained in CS. On the other hand, the enzymatic activity of the SN fractions (F1 and F2) obtained during protoplast processing represented >90% of the total sum of F1+F2+P (Figure 3).

Discussion

The activity of LAB is of particular interest for the industrial wine production undergoing MLF since it may profoundly affect its aromatic characteristics. In this study, we comparatively investigated how malic and citric acids, SO_2, and/or acid pH may affect two relevant metabolic activities of *O. oeni* for the aroma production in wine.

Interestingly, all test *O. oeni* strains grew under conditions examined and tested including in the presence of SO_2 and at initial pH of 3.8. Growth parameters were maximal, in general, in the presence of L-malic plus citric acids especially at pH 4.8 in concordance with results reported by Saguir and Manca de Nadra[23,24] who demonstrated the beneficial effect of organic acids for the growth rate, the biomass formed, and to fulfill the amino acid requirements of *O. oeni* strains. However, in our study even though organic acids also stimulated the growth of the majority of the test strains at pH 3.8, this effect did not occur in two of them that showed similar growths in both CM and MCCM that showed similar growth responses in contrast as those obtained at pH 4.8. Possibly, a negative impact of citrate on growth related to its end-products, such as acetic acid, could counterbalance the positive effect of L-malate for the biomass formed at low pH, as reported by Augagneur et al,[25] in *O. oeni*. In our study, all tested strains especially MS20, MS27, and MS46 showed good potential to overcome winemaking-related stress factors such as SO_2 addition and low pH. This fact is particularly

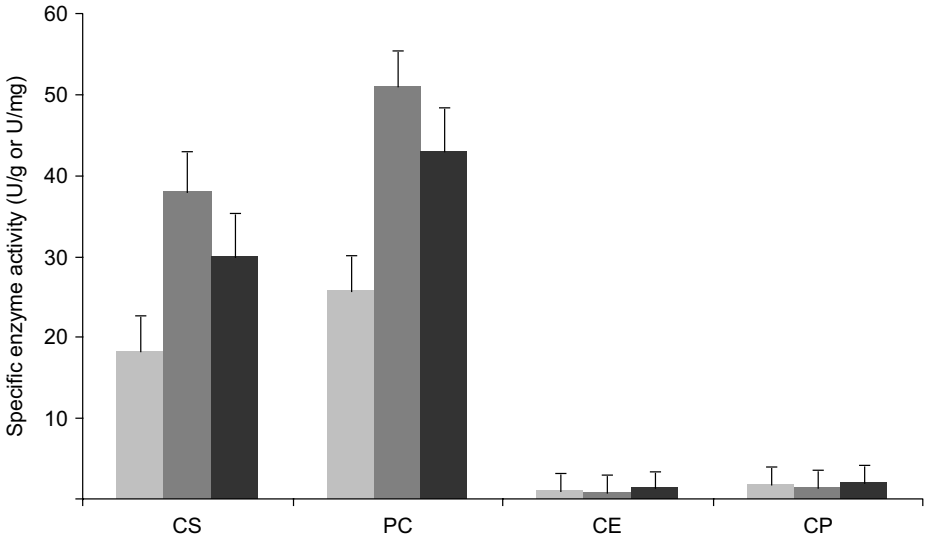

Figure 2 Specific β-glucosidase activity of *Oenococcus oeni* MS9 (□), MS20 (▒), and MS46 (■) in CS, PC, CE, and CP obtained at the end of the exponential phase in SMCCM adjusted at pH 3.8.
Notes: Specific enzyme activity was expressed as U/mg of total proteins in CE and U/g of dry weight of cells in CS, PC, and CP. Results represent the mean of duplicate determinations in three independent experiments.
Abbreviations: CE, cell extract; CP, cell protoplast; CS, cell suspension; PC, permeabilized cell; SMCCM, addition of SO_2 with L-malic and citric acids.

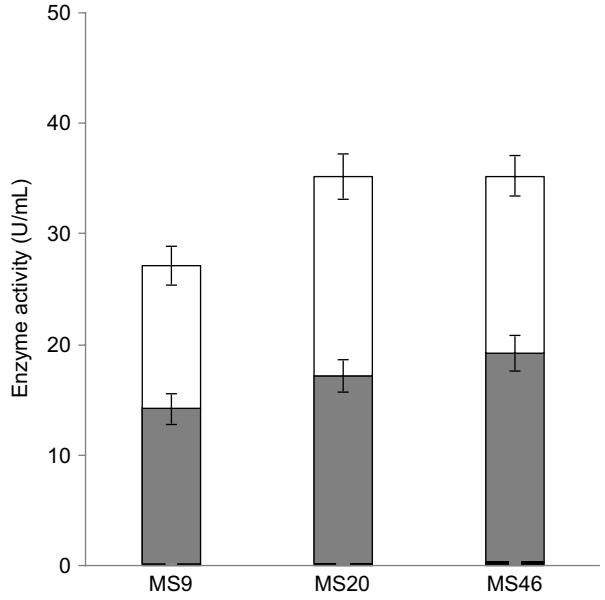

Figure 3 β-glucosidase activity in supernatant fractions, F1 (□) and F2 (▒), obtained during processing of cell protoplasts (■) of *Oenococcus oeni* MS9, MS20, and MS46 at the end of the exponential phase in CM added with L-malic and citric acids and SO_2 (SMCCM) adjusted to pH 3.8.
Note: Results represent the mean of duplicate determinations in three independent experiments.

interesting since du Toit et al[26] and Rojo-Bezares et al[27] described that LAB are more sensitive to SO_2 action than other non-LAB, *O. oeni* being the most sensitive one. In the present work, analysis of sugars and organic acid utilization profiles under different experimental conditions revealed interesting characteristics of Argentinean wines' *O. oeni*: 1) glucose and organic acids are co-metabolized regardless of medium composition and initial pH value; 2) high residual glucose levels are obtained, whereas organic acid degradation proceeds to completion regardless of condition tested; and 3) high L-lactic acid levels recovered from L-malic acid decarboxylation confirm their good malolactic potential.

Regarding the production of C4 compounds by the *O. oeni* strains in CM being influenced by the organic acid catabolism and SO_2 presence at low pH, interesting results were found. The results demonstrated that the majority of the *O. oeni* strains (~67%) produced C4 compounds from glucose catabolism at both pH 4.8 or 3.8, probably as a means of preventing toxic pyruvate levels from accumulating from glycolysis.[28] However, there was variability in the levels formed between strains for a given pH condition which had no direct relationship with the cell growth. Thus, strains could be divided into three groups according to their production levels (no producer, low producer, and intermediate producer). Besides, there was variability in the C4 compound production levels for each strain between the different pH conditions, producing lesser quantities at pH 3.8, which could be associated to lower growths in the more acid condition. However, this occurred in 50% of the analyzed strains, whereas the non-producer and MS9 strains behaved in a similar way as observed at pH 4.8. Thus, there would be another factor in addition to growth extent affecting their production. On the other hand, Nielsen and Richelieu[2] reported that the optimum range of pH values for diacetyl production by LAB in various fermented foods was 4.3–4.7. In our study, L-malic and citric acids, which

were almost completely utilized, significantly increased C4 compound formations by *O. oeni* strains in concordance with the higher biomass formed and the results reported by Nielsen and Prahl.[29] However, the increments obtained on C4 compounds production by the presence of organic acids resulted in general greater than those observed on cell growth, suggesting that the additional energy gain associated with MLF might result in more available pyruvate derived from glycolysis for C4 compound formation pathway other than energy generation. According to Leblanc,[30] the C4 compound formation depends on the energy requirement and redox balance of the cells. It is interesting to note that at pH 3.8 the stimulation percentages on C4 compound production by the organic acids were in general higher than those obtained in the same condition at pH 4.8. According to Phalip et al,[31] the synthesis of the intermediary α-acetolactate is favored under conditions of excess pyruvate and acidic pH, which has been shown to increase the production of C4 aroma compounds. On the other hand, it is known that citric acid leads to the production of C4 compounds, especially diacetyl, acetoin, or 2,3-butanodiol.[32] In our study, the *O. oeni* strains showed a trend toward diacetyl formation indicating that it is a property that is distributed uniformly in these bacteria isolated from Argentinean wines. However, substrate(s) bioconversion yields were in general low even in the presence of citric acid. Therefore, the variability in the C4 compound production levels, mainly represented by diacetyl, between strains for a given condition and for each strain depending on pH (in some cases) and/or presence of organic acids (in the majority of them) clearly indicates the importance of this property for different strains and thus should be taken into consideration when collections of strains are screened. Strains MS9, MS20, and MS46 would be the most suitable for adequate diacetyl yield in vinification conditions; therefore, they were selected for further investigations in relation to β-glucosidase activity.

Interestingly, detectable levels of β-glucosidase activity were found in CS of all the strains selected at the end of the exponential phase, including MS20 and MS46 that did not exhibit detectable levels of C4 compounds in CM. Thus, there was no direct relationship between these two characteristics for a given strain and condition. Nevertheless, similar to that observed for C4 compound formation, there was variability in the activity levels found between the test strains, especially at pH 4.8, in agreement with previous works.[11,16] In addition, these activities were only partially inhibited at pH 3.8 compared to 4.8, although this effect differed according to strain. Thus, strain MS46 whose inhibition was <10% showed an interesting behavior in this regard. In general, organic acids stimulated their specific activity at both pH 4.8 and 3.8, thus partially reversing the inhibitory effect caused by acid stress. Considering the metabolism of these bacteria, in our study, the high residual levels of glucose at the end of exponential growth of the test *O. oeni* strains, especially in the presence of organic acids, could be related to the increased β-glucosidase activity, which is relevant as it provides an additional glucose source.[33] Interestingly, SO_2 treatment did not produce any significant change on enzymatic activity similar to that observed for C4 compound formation profiles. In this regard, it is the first report on the SO_2 effect in relation to this activity.

Studies carried out using different cell fractions demonstrated increased β-glucosidase activity in PC compared to CS, whereas it was not active in SN,[16] CE, or P. On the contrary, McMahon et al[15] did not determine β-glucosidase activity in CP of *O. oeni*, possibly by denaturalization of the enzyme during obtaining process while Barbagallo et al[34] and Michlmayr et al[35] reported the intracellular nature of glycosidase enzyme of wine *O. oeni* strains. However, Mesas et al[36] determined that in *O. oeni* ST81 the β-glycosidase enzyme was located in the periplasmic region of the cell, and Pérez-Martín et al[37] concluded a strain-dependent localization of the β-glucosidase activity in wine LAB. Thus, based on the results obtained in this present study, it can be concluded that the enzyme β-glycosidase of the *O. oeni* strains isolated from Argentinean wines was localized at the level of the cell surface, which gives additional value for potential use in the wine industry. Enzymatic activity found in SN fractions obtained during cell processing for protoplast formation supported this hypothesis. Today, this result is being confirmed by similar experiments using natural glycosides obtained from grapes and by examining the impact of selected strains on wine aroma following MLF in microvinification experiments. In conclusion, statistically significant differences were observed among the final concentrations of C4 compounds formed and the levels of β-glucosidase activity mainly depending on strain and L-malic or citric acid content and to a variable degree on the initial pH but not on SO_2 presence. Thus, both the biochemical features are of relevance for vinification decisions. On the other hand, the fact that the β-glucosidase enzyme was associated with the cell surface of the selected strains esult of great interest for their potential technology application as sources of an enzyme that could improve wine quality and aromatic complexity under vinification conditions.

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Disclosure

The authors report no conflicts of interest in this work.

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