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Synergistic effect of fumaric acid and chitosan on the inhibition of malolactic fermentation

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Abstract. During wine storage and aging, microorganisms capable of degrading malic acid in an undesirable manner can proliferate. In order to control wine stability and preserve malic acid levels, winemaking strategies using chitosan and fumaric acid at doses of 600 mg/L have been developed. The objective of this study was to evaluate the synergistic effect of chitosan and fumaric acid at reduced doses to inhibit malolactic fermentation (MLF). For this purpose, six fermentation conditions were evaluated in triplicate: a control of uninoculated wine, a treatment with 600 mg/L fumaric acid added, a control of wine inoculated with *Oenococcus oeni*, a treatment of inoculated wine with 600 mg/L fumaric acid added, a treatment with inoculated wine and 200 mg/L chitosan and, finally, a treatment of wine inoculated with 150 mg/L fumaric acid and 100 mg/L chitosan. The trials were carried out in chambers at 20°C, using 500 mL ISO flasks for MLF. General oenological parameters were monitored daily by FTIR and the concentration of malic acid and lactic acid was determined by enzymatic analysis. Once MLF was completed, color and pH parameters were evaluated and optical microscopy and sensory analysis were performed. The results showed a high effectiveness of fumaric acid as an inhibitor of MLF and as an acidifier, presenting a lower pH than the samples treated with chitosan (3.603) and the control (3.623), and a sensory perception of a lower pH (3.403-3.433). In turn, they confirmed a synergistic effect of fumaric acid with chitosan inhibiting MLF at low concentrations.

1. Introduction

Recently, wineries located in warm areas have faced difficulties in protecting malic acidity against microbial degradations during wine stabilization or aging, leading to a decrease in the perception of freshness [1].

Traditionally, the most widely used oenological additive to prevent lactic acid bacteria (LAB) activity has been sulphur dioxide (SO_2) [2].

In order to improve wine stability, the use of fumaric acid has been proposed for its acidifying and antimicrobial role, which helps to control the physicochemical and oxidative stability of wine. Fumaric acid has recently been approved by the OIV (OIV-OENO Resolution 581A-2021) as an inhibitor of malolactic fermentation (MLF) at a dose of 600 mg/L. Its use at the currently authorized dose allows control of bacteria with inhibition of MLF, reduction of pH, preservation of malic acidity and reduction of volatile acidity [3,4]. The mechanism of action of fumaric acid is based on its ability to cross the cell membrane and release protons into the cytosol, which generates an imbalance in cell homeostasis. It has been shown to be highly effective against various bacterial species, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and lactic acid bacteria [4]. However, this acid does not interfere with alcoholic fermentation or affect yeast development, and at the authorized doses it has no sensory impact.

Another complementary strategy to improve wine stability is the use of chitosan, a positively charged polysaccharide derived from the deacetylation of chitin. Its use is permitted in winemaking both as a fining agent and for its antimicrobial action during its contact time with wine. Chitosan has been shown to have a synergistic effect with fumaric acid as an MLF inhibitor, which reinforces protection from malic acidity [5,6].

The aim of this trial is to evaluate the synergistic effect of fumaric acid and chitosan with the purpose of reducing the necessary doses of both compounds and, at the same time, reducing the SO_2 content thanks to their combined antimicrobial capabilities.

2. Materials and methods

2.1. Initial Verdejo white wine

Table 1. Physicochemical parameters of the wine used for MLF.

Density (g/L)	990
Ethanol (%v/v)	11.3
pH	3.44
Reducing sugars (g/L)	3.3
Volatile acidity (mg/L)	0.48
Total acidity expressed as tartaric acid (g/L)	6.6
Malic acid (g/L)	2.17
Lactic acid (g/L)	0.00

MLF were carried out in the Food Technology laboratory of the ETSIAAB (Universidad Politécnica de Madrid, UPM) with wine fermented from Verdejo grape must from the José Pariente winery.

2.2. Lactic acid bacteria strain used for MLF

A freeze-dried Alpha lactic acid bacteria strain of *Oenococcus oeni*, supplied by the Lallemand company, was used for the MLF of Verdejo wine following the manufacturer's instructions.

2.3. Malolactic fermentation

Six fermentation conditions were evaluated in triplicate (Table 2): an uninoculated wine control (condition 1), a treatment to which 600 mg/L fumaric acid was added (condition 2), a wine control inoculated with *O. oeni* (condition 3), a treatment of inoculated wine to which 600 mg/L fumaric acid was added (condition 4), a treatment with inoculated wine and 200 mg/L chitosan (condition 5) and, finally, a treatment of wine inoculated with 150 mg/L fumaric acid and 100 mg/L chitosan (condition 6), in order to evaluate the possible synergistic effect of both compounds.

Table 2. Test conditions.

Strategy	*O. oeni* (CFU/mL)	Fumaric acid	Chitosan
1	-	-	-
2	-	600 mg/L	-
3	$2 \cdot 10^6$	-	-
4	$2 \cdot 10^6$	600 mg/L	-
5	$2 \cdot 10^6$	-	200 mg/L
6	$2 \cdot 10^6$	150 mg/L	100 mg/L

The *O. oeni* strain was inoculated at an average concentration of $2 \cdot 10^6$ CFU/mL. All strains were rehydrated in 20 times their weight in water at 20 °C for 15 min.

The wine was fermented in 500 mL ISO flasks. The assays were carried out in chambers at 20ºC in the Food Technology laboratory of the ETSIAAB (UPM). Periodically, samples were taken from the different treatments to carry out an analysis of general oenological parameters and to determine the concentration of malic acid and lactic acid. At the end of fermentation, parameters of color, pH, fermentative volatile compounds and sensory analysis were also evaluated.

2.4. Analysis of general oenological parameters

The density (g/L), total acidity expressed as tartaric acid (g/L), malic acid concentration (g/L) and nitrogen content (mg/L) of the initial wine were analyzed using an OenoFoss instrument (FOSS Iberia, Barcelona, Spain), a Fourier transform infrared spectrophotometer (FTIR). During fermentation, alcohol content, glucose, fructose and volatile acidity were determined using the same instrument. In addition, periodic determinations of pH evolution were carried out using the pH 80 pH meter (XS Instruments).

2.5. Enzymatic analysis

In order to evaluate the acidifying capacity or physicochemical characteristics of the wine, the concentrations of malic acid and lactic acid were determined using the Y25 enzymatic analyzer (BioSystems, Barcelona, Spain).

2.6. Color analysis

Measurements of absorbance at 420, 520 and 620 nm, color intensity, hue and CIELab coordinates were carried out using the Smart Analysis spectrophotometer (DNA Phone, Parma, Italy).

2.7. Sensory analysis

Sensory analysis was carried out where visual, olfactory and taste parameters were evaluated. The parameters were scored using a scale from 1 (lowest intensity) to 5 (highest intensity). The parameters analyzed were color intensity, hue, cleanliness, aromatic intensity, aromatic quality, herbaceous, floral, fruity, reduced and oxidized aromas, body, astringency, bitterness, acidity and overall perception. The analyses were carried out by a panel of seven tasters of both genders and ages ranging from 20 to 50 years old. They belonged to the staff of the Department of Food Chemistry and Technology (UPM).

2.8. Statistical analysis

Means, standard deviations, analysis of variance and significant difference test ($p < 0.05$) were calculated using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA).

2.8. Optical microscopy

Sediment samples were taken from the vials in which the wine was fermented and observed with an optical microscope in order to identify yeasts and malolactic bacteria.

3. Results and discussion

3.1. Analysis of general oenological parameters

Table 3 shows the final general enological parameters analyzed at the end of fermentation. The ethanol content varied between 11.4% and 11.6% by volume, finding no significant differences (p=0.05) between the wines after the treatments applied in the MLF stage. With the periodic determination of malic acid and lactic acid, it was observed that malic acid began to decrease seven days after inoculation with lactic acid bacteria, coinciding with the production of lactic acid and indicating the beginning of MLF. It was concluded that the only wines that showed MLF were those inoculated with *O. oeni* and those inoculated with *O. oeni* to which 200 mg/L of chitosan was added, which was not sufficient to cause inhibition. The wines in which MLF was carried out, in turn, presented a higher pH.

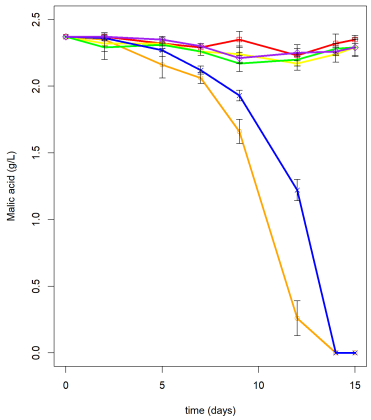

Figure 1. Evolution of malic acid concentration (g/L) during fermentation. The control is shown in red squares; in orange circles, the wines inoculated with *O. oeni*; in yellow triangles, the wines to which fumaric acid was added; in green plus signs, the wines inoculated with *O. oeni* to which fumaric acid was added; in blue crosses, the wines inoculated with O. oeni to which chitosan was added; and in purple rhombuses, the wines inoculated with *O. oeni* to which fumaric acid with chitosan was added.

However, in the wines treated with 150 mg/L fumaric acid and 100 mg/L chitosan, a synergistic effect was observed that inhibited MLF.

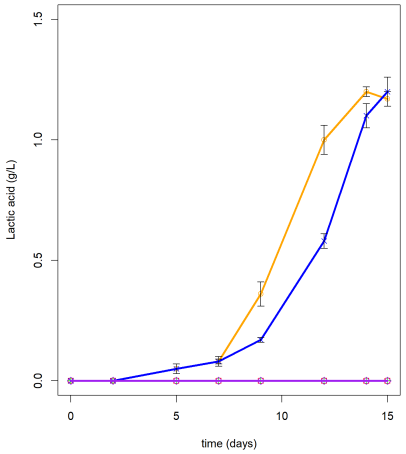

Figure 2. Evolution of lactic acid concentration (g/L) during fermentation. In orange circles, the wines inoculated with *O. oeni*; in blue crosses, the wines inoculated with *O. oeni* to which chitosan was added; and in purple rhombuses, the rest of the conditions, overlapped by the absence of lactic acid production.

3.2. Color analysis

The results analyzed in the spectrophotometer showed significant differences between the different samples (p=0.05) in color intensity, chroma, Hue and the a* and b* coordinates of CIELab space.

Mainly, samples inoculated with *O. oeni* were characterized by significantly higher intensity.

Significantly higher a* and b* values indicated that the samples presented a more yellow and less greenish hue, coinciding with a lower Hue value.

Table 3. General oenological parameters of wines.

Strategy	Ethanol (%vol)	pH	Reducing sugars (g/L)	Volatile acidity (g/L)	Malic acid (g/L)	Lactic acid (g/L)
Control (1)	11.45±0.06^{a}	3.623±0.009^{b}	2.80±0.12^{c}	0.56±0.02^{a}	2.347±0.017^{b}	0.00±0.00^{a}
600 mg/L Fumaric acid (2)	11.57±0.06^{a}	3.403±0.007^{a}	1.40±0.06^{a}	0.707±0.007^{b}	2.29±0.03^{b}	0.00±0.00^{a}
O. oeni (3)	11.43±0.11^{a}	3.62±0.01^{b}	2.13±0.03^{b}	0.580±0.015^{a}	0.00±0.00^{a}	1.170±0.015^{b}
600 mg/L Fumaric acid + *O. oeni* (4)	11.62±0.04^{a}	3.413±0.012^{a}	1.47±0.12^{a}	0.71±0.00^{b}	2.29±0.04^{b}	0.00±0.00^{a}
200 mg/L chitosan + *O. oeni* (5)	11.51±0.02^{a}	3.603±0.009^{b}	2.60±0.10^{bc}	0.583±0.009^{a}	0.00±0.00^{a}	1.20±0.03^{b}
150 mg/L Fumaric acid + 100 mg/L chitosan + *O. oeni* (6)	11.56±0.04^{a}	3.433±0.007^{a}	1.63±0.13^{a}	0.730±0.006^{b}	2.29±0.04^{b}	0.00±0.00^{a}

Table 4. Colorimetric parameters of wines.

Strategy	Color intensity (absorbance units)	Tonality (adimensional)	Chroma	Hue (°)	L	a	b
Control	0,12±0,003^{ab}	3,4±0,7^{a}	4,96±0,18 ^{a}	94,5±0,6^{c}	97,9±0,6^{a}	-0,39±0,06^{a}	4,95±0,17 ^{ab}
Ácido fumárico	0,094±0,009^{a}	3,5±0,5^{a}	4,58±0,11^{b}	93,0±0,7^{bc}	98,4±0,3^{a}	-0,24±0,05^{a}	4,57±0,11^{ab}
O. oeni	0,23±0,04^{b}	1,9±0,4^{a}	5,6±0,7^{ab}	85,2±1,6^{ab}	94,6±1,5^{a}	0,51±0,24^{b}	5,61±0,64^{b}
Ácido fumárico + *O. oeni*	0,104±0,003^{a}	2,9±0,1^{a}	4,71±0,11^{ab}	90,3±1,5^{abc}	98,13±0,03^{a}	-0,05±0,12^{ab}	4,71±0,11^{ab}
Quitosano + *O. oeni*	0,19±0,04^{ab}	1,9±0,3^{a}	4,7±0,4^{ab}	87,9±1,7^{ab}	95,5±1,1^{a}	0,20±0,15^{ab}	4,7±0,4^{ab}
Ácido fumárico + Quitosano + *O. oeni*	0,17±0,02^{ab}	1,7±0,2^{a}	3,92±0,21^{a}	86,7±1,6^{ab}	95,8±0,7^{a}	0,22±0,10^{ab}	3,91±0,21^{a}

On the contrary, those samples where MLF inhibition took place, which coincided with the samples treated with fumaric acid, showed a lower color intensity, with less predominance of yellow and a more greenish hue.

3.3. Optical microscopy

Optical microscopy of the wine vial deposit at the end of MLF was carried out. In this way, *S. cerevisiae* could be observed in all the wine samples and, in the wine inoculated with *O. oeni* and in the wine treated with chitosan, which was also inoculated, bacteria were observed, as shown in Figure 4.

3.4. Sensory analysis

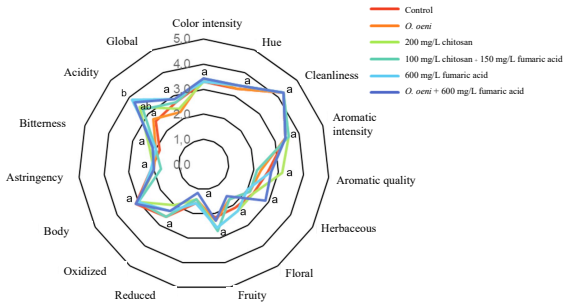

Figure 3. Radar chart with the results of the sensory analysis carried out by 7 tasters.

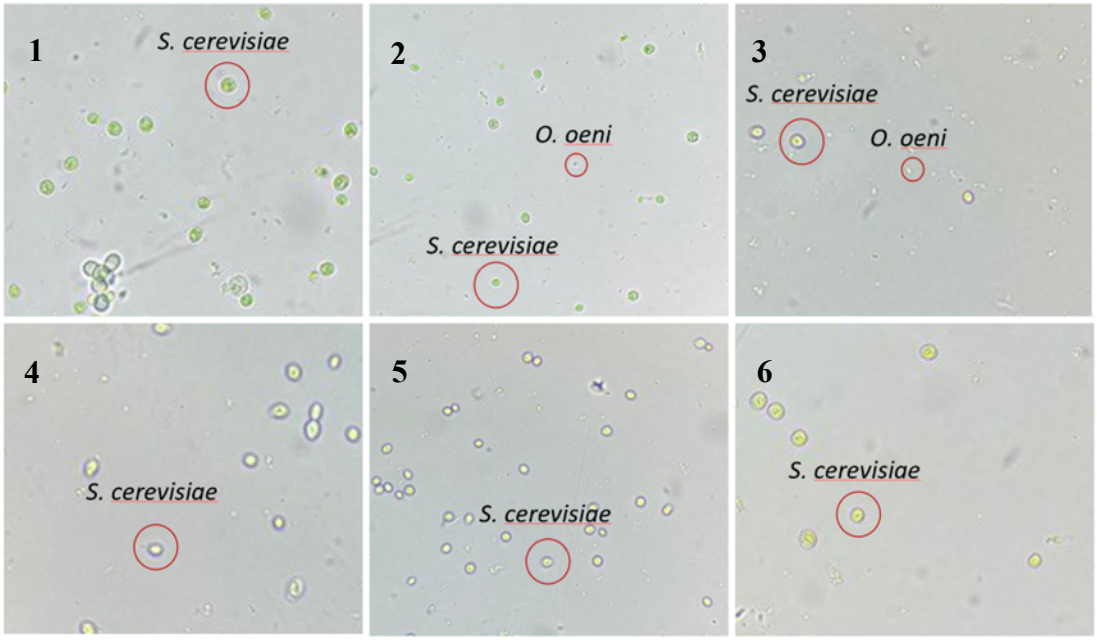

Figure 4. Optical micrographs of the wine bottle deposits. In image 1, the micrograph of the control is shown; in image 2, the control inoculated with *O. oeni*; in image 3, the wine treated with chitosan; in image 4, the wine treated with chitosan and fumaric acid; in image 5, the treatment with fumaric acid; and in image 6, the treatment with fumaric acid inoculated with *O. oeni*.

The data collected in the sensory analysis indicated significant differences in the perception of acidity among the different wines. Wines treated with fumaric acid showed significantly higher acidity than the control and the wine inoculated with *O. oeni*. It should be noted that, at the time of the sensory analysis, a determination of malic acid and lactic acid was carried out, proving that, during the time the wines were refrigerated at 4ºC, the control underwent MLF. In the wines that underwent MLF, sweeter aromas were identified.

4. Conclusions

Wines treated with fumaric acid presented a higher malic acid content and a lower pH, being perceived by tasters as more acidic. The study has shown that fumaric acid at a concentration of 150 mg/L, four times lower than the maximum concentration authorized by the OIV, in combination with chitosan is capable of inhibiting MLF in white wines, allowing the desired malic acidity to be maintained.

It should be noted that the control, where *O. oeni* was not inoculated, ended up carrying out MLF, highlighting the importance of using inhibitors such as fumaric acid or chitosan.

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