



Article

β -Glucosidase Activity of *Lactiplantibacillus plantarum* UNQLp 11 in Different Malolactic Fermentations Conditions: Effect of pH and Ethanol Content

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Abstract: *Lactiplantibacillus plantarum* strain UNQLp 11 is a lactic acid bacterium with the potential to carry out malolactic fermentation (MLF) in red wines. Recently, the complete genome of UNQLp 11 was sequenced and this strain possesses four loci of the enzyme β -glucosidase. In order to demonstrate that these glucosidase enzymes could be functional under harsh wine conditions, we evaluated the hydrolysis of p-nitrophenyl- β -D-glucopyranoside (p-NPG) in synthetic wine with different ethanol contents (0%, 12%, and 14% *v/v*) and at different pH values (3.2, 3.5, and 3.8). Then, the hydrolysis of precursor n-octyl β -D-glucopyranoside was analyzed in sterile Pinot Noir wine (containing 14.5% *v/v* of ethanol, at different pH values) by headspace sorptive extraction gas chromatography-mass spectrometry (HSSE-GC/MS). The hydrolysis of p-NPG showed that β -glucosidase activity is very susceptible to low pH but induced in the presence of high ethanol content. Furthermore, UNQLp 11 was able to release the glycosylated precursor n-octyl, during MLF to a greater extent than a commercial enzyme. In conclusion, UNQLp 11 could improve the aromatic profile of the wine by the release of volatile precursors during MLF.

Keywords: *Lb. plantarum*; malolactic fermentation; β -glucosidase activity; wine; flavor



Citation: Brizuela, N.S.; Arnez-Arancibia, M.; Semorile, L.; Pozo-Bayón, M.Á.; Bravo-Ferrada, B.M.; Tymczyszyn, E.E. β -Glucosidase Activity of *Lactiplantibacillus plantarum* UNQLp 11 in Different Malolactic Fermentations Conditions: Effect of pH and Ethanol Content. *Fermentation* **2021**, *7*, 22. <https://doi.org/10.3390/fermentation7010022>

Received: 9 January 2021

Accepted: 31 January 2021

Published: 14 February 2021

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1. Introduction

Winemaking is a microbiological process involving two different fermentation processes: alcoholic fermentation (performed by yeasts) and malolactic fermentation (MLF) (performed by lactic acid bacteria (LAB)). During MLF, these LAB decarboxylate L-malic acid into L-lactic acid, increasing the pH of the wine, decreasing its astringency, and reducing the risk of microbial contamination [1,2]. Furthermore, LAB are responsible for the change in the chemical composition of wine, with high impact on its flavor. One of the most relevant reactions is that of the β -glucosidase enzyme (β -D-glucoside glucohydrolase, EC 3.2.1.21). This enzyme is responsible for the hydrolysis of glycoconjugated (non-odorant) terpenes and nor-isoprenoids, which are some of the main grape components that contribute to wine aroma, conferring floral/fruity characteristics [3–6].

Although some ethanol-tolerant enzymes extracted from the fungus *Aspergillus niger* are commercially available [7,8], the application of microorganisms with endogenous β -glucosidase activity would by-pass the cost of production and application of such enzymatic preparations [9]. The enzyme β -glucosidase has been detected in enological strains of the species *Oenococcus oeni* and *Lactiplantibacillus plantarum*, which are the main LAB species responsible for MLF [10–13]. This enzymatic activity is usually evaluated by hydrolysis of p-NPG because it is easy and fast to determine using a colorimetric method [10–12].

However, β -glucosidase by p-NPG hydrolysis is not applicable for red wines because of color interferences. Iorizzo et al. [6] studied same strains of *Lb. plantarum* isolated from Italian wines and all of them were able to hydrolyze the glycoside from octyl- β -D-glucopyranoside and release different amounts of the aglycone 1-octanol. They report that this ability was bacteria-dependent, and therefore different depending on the type of bacteria assayed. However, several authors have reported that the activity of this enzyme can be affected by some environmental factors such as low pH, temperature, presence of sugars, and ethanol [14–16]. Particularly, the ethanol concentration of wines is usually from 12 to 14% *v/v* and the pH between 3.2–3.8. Both, pH and ethanol content are affected by several factors, such as grapes maturity, climate, varieties, winemaking practices, among others [17–21]. Therefore, the physicochemical properties of wines might affect the β -glucosidase activity and the release of volatile terpenes.

Although *O. oeni* is the major LAB used in commercial starter cultures for MLF, in the last decade, several commercial malolactic starters using *Lb. plantarum* have been released in the market [22], because of their ability to tolerate wine harsh conditions, to carry out MLF, and because they present more flavor-related genes than *O. oeni* [23]. The success of these starter cultures is influenced by a variety of factors, including the strain adaptation to the winemaking practices of each wine. For this reason, several authors have recommended the use of autochthonous starter cultures, because they could be well adapted to the conditions of a specific wine region [24,25].

In previous works, we demonstrated that *Lb. plantarum* were present in spontaneous MLF of Pinot Noir from Argentinean North Patagonia region. This region has optimal agro-ecological conditions for high quality viticulture, in which Pinot Noir have found the conditions to express all its oenological potential [12,13,26]. In this sense, the strain of *Lb. plantarum* UNQLp 11 showed higher ability to consume malic acid with a small inoculum size, without pre-acclimation treatment and growing in a low-cost alternative medium, which presents several economic advantages on the production of indigenous starter cultures [13,27]. In addition, UNQLp 11 was able to positively modify the flavor of wine, increasing the concentration of esters and same terpenes in the profile of volatile compounds [28–30]. All these characteristics tend to confirm that UNQLp 11 is an excellent candidate as an autochthonous malolactic starter culture. Moreover, the recent description of its complete genome has allowed us to observe that this strain possesses four loci (genes located in specific fixed position on the chromosome) of the enzyme β -glucosidase [31], but its hydrolysis capacity under harsh conditions of wine has not been studied yet.

For these reasons, the aim of this work was to evaluate the glucosidase activity of *Lb. plantarum* UNQLp 11 during fermentation with different concentrations of ethanol and different pHs, in order to analyze the ability of this strain to facilitate the release of glycosylated precursor under harsh environmental conditions. As a first screening, β -glucosidase activity of UNQLp 11 was measured in synthetic wine (at pH 3.2, 3.5, and 3.8 and with 0, 12, and 14% *v/v* of ethanol content) by hydrolysis of p-NPG, and then the β -glucosidase activity was analyzed in Patagonian Pinot Noir wine (14.5% *v/v* of ethanol) at different pH values (3.2, 3.5, and 3.8) by HSSE-GC/MS, using n-octyl β -D-glucopyranoside as precursor.

2. Materials and Methods

2.1. Growth, Acclimation, and Wine Inoculation

Lactiplantibacillus plantarum UNQLp 11 (GenBank Accession Number—complete genome CP031140) was isolated from a Patagonian Pinot Noir wine vintage 2012 [13] and previously was studied by our group [13,28–30]. UNQLp 11 was grown in MRS (de Man, Rogosa and Sharpe) broth (Biokar Diagnostics, Beauvais, France), at 28 °C and pH 6.5 for 48 h (early stationary phase, $\sim 10^{10}$ colony forming units (CFU)/mL). Then, the culture was washed with physiological solution, centrifuged (at 4 °C, for 10 min at 5000 $\times g$), and inoculated in synthetic wine or Pinot Noir wine.

2.2. β -Glucosidase Activity during Fermentation in Synthetic Wine

UNQLp 11 was inoculated in synthetic wine following Bravo-Ferrada et al. [32], with some modifications: 5 g/L tartaric acid, 4.5 g/L malic acid, 0.6 g/L acetic acid, 2 g/L glucose, 2 g/L fructose, and 12 or 14.0% *v/v* ethanol (and wine without ethanol as control). For each ethanol concentration, the pH values were adjusted to 3.2, 3.5, or 3.8, using concentrated HCl (6 mol/L). Synthetic wines were supplemented with 50 μ mol/mL of p-NPG as substrate, inoculated with *Lb. plantarum* UNQLp 11 ($\sim 5 \times 10^8$ CFU/mL), and incubated at 21 °C for 15 days. As control, the different wines (without inoculation) were supplemented with a commercial winemaking β -glucosidase from *A. niger* (containing 21.6 UI/g), at the concentration recommended by the supplier (10 mg/L).

The survival of *Lb. plantarum* UNQLp11 was studied before and after incubation in synthetic wine on days 2, 10, and 15. The number of viable cells was measured by plate count in MRS agar at 28 °C for 48 h, in aerobic conditions. The malic-acid consumption was also monitored using Enology BioSystems enzymatic kits (Barcelona, Spain).

Glucosidase activity on the substrate p-NPG was determined according to the protocol described by Grimaldi et al. [10], with some modifications. Samples of 1 mL of each wine, at different times of fermentation, were diluted with 1 mL of 0.2 mol/L carbonate buffer (pH 10.2), clarified by centrifugation, and the absorbance of the supernatant measured at 400 nm. The concentration of the released p-nitro phenol (p-NP) was calculated from a calibration curve prepared by using standard p-NP solutions diluted in synthetic wine. Enzyme activity was expressed as μ mol of released p-NP from 1 mL of wine.

2.3. Hydrolysis of octyl β -D-glucopyranoside in Sterile Pinot Noir Wine

(a) Vinification conditions

Lb. plantarum UNQLp 11 were inoculated at $\sim 1 \times 10^7$ (low inoculum) and 1×10^8 CFU/mL (high inoculum) at the end of the alcoholic fermentation, in a Pinot Noir wine containing 14.5% *v/v* ethanol, <2.00 g/L residual sugars, 2 g/L malic acid, and 96 mg/L total SO₂, and whose pH values were adjusted to 3.2, 3.5, and 3.8 with HCl (6 mol/L). Wine was sterilized by filtration through a 0.2- μ m pore size (Sartorius Stedim Biotech GmbH, Gottingen, Germany), and 5 mg/L of octyl β -D-glucopyranoside (Sigma-Aldrich) was added to detect the 1-octanol released in wine by β -glucosidase activity [6,33]. The vinification was performed at 21 °C for 15 days.

Wines supplemented with octyl β -D-glucopyranoside (without *Lb. plantarum* inoculation) and with commercial β -glucosidase were also measured as control samples. The commercial winemaking β -glucosidase from *A. niger* (containing 21.6 UI/g) was used according supplier recommendation: 10 mg/L.

After fermentation, viable cells were analyzed by plate count and the remaining malic acid was measured by an enzymatic kit (see above).

(b) Determination of β -glucosidase activity of UNQLp 11 strain was evaluated using a commercial n-octyl glycoside and evaluating of the corresponding aglycone, 1-octanol, by headspace sorptive extraction gas chromatography-mass spectrometry (HSSE-GC/MS).

The released 1-octanol was quantified by the HSSE-GC/MS procedure previously described [34] and explained in Appendix A.

2.4. Statistical Analysis

All determinations were the average of three independent of replicate assays. Data are shown as mean values. Means were compared by one-way ANOVA and Tukey's post-test for multiple comparison, and if $p < 0.05$, the difference was considered statistically significant. All the statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA, 2007).

3. Results

3.1. β -Glucosidase Activity during Fermentation in Synthetic Wine

The survival and β -glucosidase activity, of UNQLp 11 inoculated in synthetic wine supplemented with p-NPG at different conditions of pH and ethanol for 15 days, was analyzed.

Figure 1 shows the survival of this strain in wine at pH values of 3.8 (Figure 1A), 3.5 (Figure 1B) and 3.2 (Figure 1C) at different ethanol concentrations. As expected, the survival of this LAB strain in the wine was more affected when high concentrations of ethanol (12 and 14% *v/v*) were combined with low pH values. At pH 3.2, a drastic decrease in the number of viable cells was observed after two days of incubation in wine with 12 and 14% *v/v* of ethanol. However, in all wine conditions, UNQLp 11 consumed more than 90% of the malic acid concentration (data not shown).

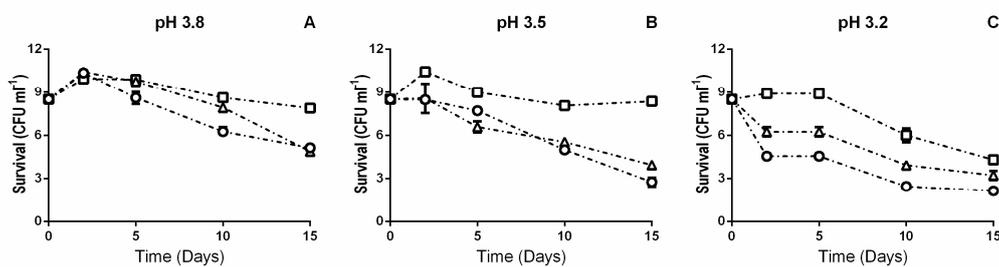


Figure 1. Survival of the *Lactiplantibacillus plantarum* strain UNQLp 11 (log CFU/mL) during fermentation in synthetic wine with different ethanol content (0% (□), 12% (Δ), and 14% *v/v* (○)), at different pH values: 3.8 (A), 3.5 (B), and 3.2 (C).

Figure 2 shows the β -glucosidase activity of UNQLp 11 in synthetic wine at different pH values. At pH 3.8, although the viability decreased in the presence of ethanol, the enzyme activity increased, being higher at 14% than 12% *v/v* of ethanol (Figure 2A). At low pH, β -glucosidase activity of UNQLp 11 was strongly negatively affected. Figure 2B show data at pH 3.5, the activity was only 33% at pH 3.8, being little better in the presence of ethanol 14% *v/v*. Furthermore, at pH 3.2, the activity was strongly inhibited at all ethanol concentrations (Figure 2C).

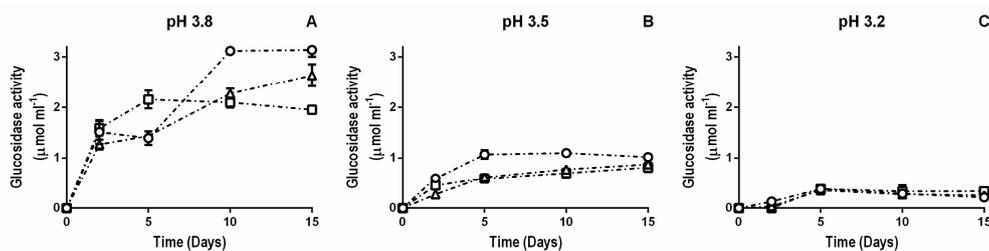


Figure 2. β -glucosidase activity of UNQLp 11 during fermentation in synthetic wine with different ethanol content (0% (□), 12% (Δ), and 14% *v/v* (○)), at different pH values: 3.8 (A), 3.5 (B), and 3.2 (C). The results are expressed as $\mu\text{mol mL}^{-1}$ of p-nitrophenol (p-NP).

Taking into account that β -glucosidase activity of UNQLp 11 is affected by cell viability and that the survival was affected by ethanol and low pH (Figure 1B,C), the ratio of activity/log CFU was analyzed in order to compare different wine conditions. β -glucosidase activity/log CFU shows a linearly relation with the time (Figure 3A), and the slopes of these regressions are compared in Figure 3B. The progressive increase of β -glucosidase activity was observed with the increase of ethanol content, but little changes in the pH, not only negatively affected the cell viability, it also dramatically affected the β -glucosidase activity of UNQLp 11.

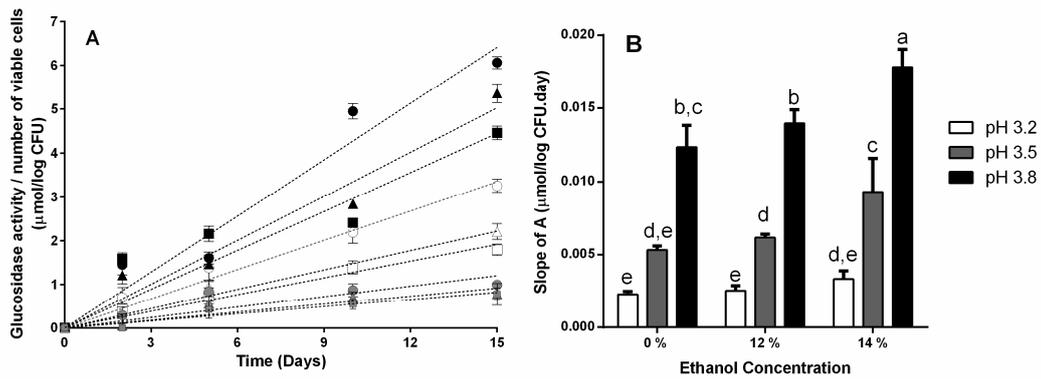


Figure 3. (A) β -glucosidase activity of UNQLp 11/log number of viable cells ($\mu\text{mol p-NP}/\log \text{CFU}$) along time of synthetic wine with different ethanol content (0% (squares), 12% (triangles), and 14% *v/v* (circles) at different pH values: 3.8 (black symbols), 3.5 (white symbols), and 3.2 (gray symbols). (B) Slope of linear regression obtained from Figure 3A. White bars: pH 3.2; gray bars: pH 3.5, and black bars: pH 3.8. Different letters (a, b, c, d, e) represent groups with significant differences ($p < 0.05$). The statistical analysis was done with ANOVA and posterior Tukey test.

Figure 4 shows the control wine using commercial β -glucosidase enzyme. The activity was much higher than UNQLp 11. The activity of commercial enzyme increased in the presence of ethanol and was less sensitive to low pH than β -glucosidase activity of UNQLp 11.

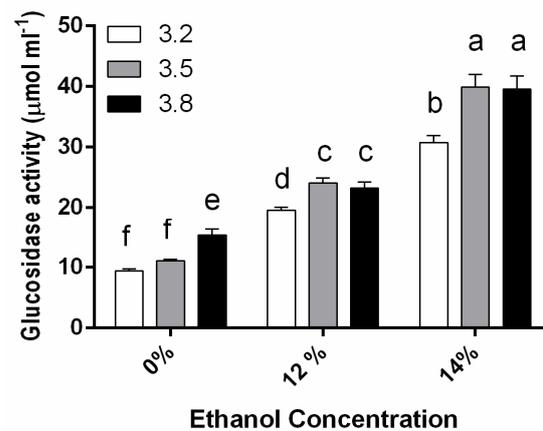


Figure 4. β -glucosidase activity of a commercial enzyme from fungal origin in synthetic wine with different ethanol concentrations (0%, 12%, and 14%) and at different pH values (white bars 3.2, gray bars 3.5, and black bars 3.8) for 15 days at 21 °C. Different letters (a, b, c, d, e, f) represent groups with significant differences ($p < 0.05$). The statistical analysis was done with ANOVA and posterior Tukey test.

3.2. Hydrolysis of Octyl β -D-Glucopyranoside in sterile Pinot Noir Wine

To analyze the β -glucosidase activity in real wine conditions, MLF was carried out at laboratory scale in a Pinot Noir wine containing 14.5 % *v/v* of ethanol and the pH of wine was adjusted to 3.2, 3.5, and 3.8, in the same way that synthetic wine studied above. Pinot Noir wine was supplemented with the glycosylated precursor octyl β -D-glucopyranoside the addition of a known concentration of synthetic n-octyl glycoside is an excellent alternative to explore the activity of enzymatic systems in red wines without color interferences [6,35].

Taking into account that ethanol was not a problem for β -glucosidase activity of UNQLp 11, we focused the study on the effect of different pH values and used lower UNQLp 11 inoculums than in synthetic wine. The sterile wine was inoculated with UNQLp 11 in two different concentrations: high inoculum ($10^8 \text{ CFU}/\text{mL}$) and low inoculum

(10^7 CFU/mL). After 15 days of fermentation at 21 °C, both inoculum (high and low) did not show significant differences in cell viability and the consumption of malic acid was greater than 80% in both cases (data not shown), indicating that MLF was successful.

Figure 5 shows the amount of 1-octanol (mg/mL) (obtained by the hydrolysis of the precursor octyl β -D-glucopyranoside) released in Pinot Noir wine inoculated with UNQLp 11 and compared with the release of commercial enzyme. The controls used were: wine without precursor, wine + precursor, and wine without precursor + commercial enzyme. In all of them, the concentration of 1-octanol was lower than the detection limit for this compound (data not shown).

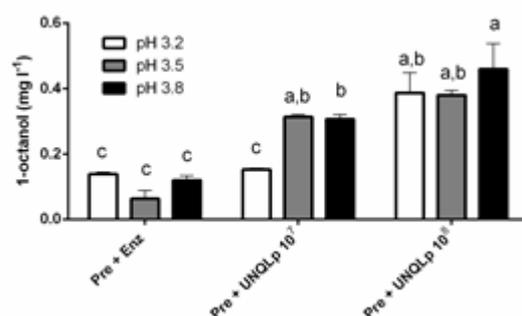


Figure 5. Release of 1-octanol in sterile Pinot Noir wine supplemented with precursor (Pre) and fermented with UNQLp11 (inocula: 1×10^7 or 1×10^8 CFU/mL) or with the commercial enzyme, for 15 days at 21 °C. Wines present different pH values (white bars 3.2, gray bars 3.5, and black bars 3.8). Different letters (a, b, c) represent groups with significant differences ($p < 0.05$). The statistical analysis was done with ANOVA and posterior Tukey test.

Interestingly, the amount of 1-octanol released was higher in wine inoculated with UNQLp 11 than in wine with the commercial enzyme (Figure 5). For high inoculum of UNQLp 11, the hydrolysis of the precursor was $\sim 10\%$ of the precursor added to the wine and not significant differences were observed by the pH of the wine. For low inoculums, the release of 1-octanol decreased significantly at pH 3.2, and the addition of commercial enzyme the values were lower than 50% than obtained with UNQLp 11 (excepted low inoculum at pH 3.2).

4. Discussion

In order to demonstrate that the glucosidase enzymes found in UNQLp 11 have biochemical functionality, the ability of *Lb. plantarum* UNQLp 11 to hydrolyze glycosylated precursors was studied under different wine fermentation conditions.

We observed that the hydrolysis of p-NPG (by UNQLp 11 and commercial enzyme) increase with the presence of ethanol. Other authors have reported similar results with an increase in glucosidase activity in solutions containing alcohol. Narasimha et al. [36] reported a 30% and 80% increase in enzymatic activity of a β -glucosidase from *A. niger* in presence of ethanol and methanol, respectively. Leite et al. [37] reported that a β -glucosidase from *Thermoascus aurantiacus* CBMAI-756 had an increase in enzymatic activity in the presence of ethanol. However, Grimaldi et al. [10] and Barbaragallo et al. [14] reported the decrease of the hydrolysis of p-NPG in the presence of high ethanol content, for the enological strains of *O. oeni*, suggesting a better potential of β -glucosidase activity from *Lb. plantarum* than *O. oeni* for winemaking purposes.

On the other hand, we observed that the commercial enzyme has a greater capacity to hydrolyze p-NPG in synthetic wine and is less sensitive to changes in the pH. However, the uses of specific LAB strains to perform MLF have other benefits, such as other enzymatic activities that improve the flavor, as well as an economic cost of production [9,14]. In this sense, UNQLp 11 was able to remain viable, consume malic acid, and hydrolyze p-NPG in synthetic wine. In addition, in previous works, we demonstrated that UNQLp 11 produces

modifications in the taste of the wine, with increase in the ester concentration and release of some terpenes such as β -citronellol [27,30].

These findings suggest that a more in-depth study of glucosidase activity directly in red wine is needed. Results showed the ability of UNQLp 11 strains to release volatile aglycones from octyl- β -D-glucopyranoside in Pinot Noir wine. Interestingly, UNQLp 11 produced significantly higher amounts of the aglycone than the commercial enzyme (in contrast to the results observed in synthetic wine). Although some authors affirm that commercial fungal glucosidase possess considerable activity for winemaking [38,39], others consider that this activity tends to be inhibited under enological conditions [14,40], under these discrepancies, it is probable that the concentration of the commercial enzyme must be adjusted accordingly to the different types of wine. Particularly β -glucosidase may be inhibited by SO₂ [35], which is present in Pinot Noir but absent in synthetic wine. UNQLp 11 is able to tolerate SO₂ concentrations present in wine and other stress factors such as tannins, yeast metabolites, organic acids, etc. [13].

5. Conclusions

In conclusion, UNQLp 11 strain was able to hydrolyze p-NPG in synthetic wine during MLF. Furthermore, under the stress conditions present in real red wine, was able to hydrolyze n-octyl glycoside in high percentage. Thus, this strain is a good candidate as a malolactic starter culture, easy to grow and with low cost for its application at industrial level, not only able to conduct MLF, but also able to improve the flavor of wine by enzymatic activity and releasing volatile aglycones under harsh wine conditions.

Author Contributions: N.S.B. and M.A.-A. carried out the experiments and analysis. E.E.T. and B.M.B.-F. designed and wrote the manuscript with support from L.S. and M.Á.P.-B. All authors have read and agreed to the published version of the manuscript

Funding: This research was funded by Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain, for the funding (COOPB20346 international cooperation Project), grants from Universidad Nacional de Quilmes (Buenos Aires, Argentina), Agencia Nacional de Promoción Científica y Técnica (ANPCyT; PICT 2016 N°3435), and CONICET (PIP-2017–11220170100898C O).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: L.S. is a member of the Research Career of the Comisión de Investigaciones Científicas de Buenos Aires, Argentina, B.M.B.F. and E.T. are members of the Research Career of the National Scientific and Technical Research Council (CONICET), B.N. is a fellow of CONICET, and P.B.M. is a member of the Research Career of the Instituto de Investigación en Ciencias de la Alimentación (CIAL), Universidad Autónoma de Madrid, Spain.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

The released 1-octanol was quantified by the HSSE-GC/MS procedure previously described [34]. Briefly, 1 mL of sample (wine) was placed in a 20-mL headspace vial (Gerstel) and taken to 5 mL with a hydroalcoholic solution (13% ethanol and pH = 3.5). A 40- μ L aliquot of an internal standard solution (10 mg L⁻¹ 3-octanol) and 1.5 g of NaCl were added to each vial. A preconditioned polydimethylsiloxane stir bar (Twister) (20 mm in length \times 0.5 mm in film thickness), supplied by Gerstel (Mülheima/d Ruhr, Germany), was suspended in the headspace of the vial by using a glass insert. The vials were sealed with septa caps using a hand crimper. The extraction was carried out for 3.5 h at 36.5 °C in a water bath at controlled temperature. After sampling, the twister was removed, dried with a lint-free tissue, and placed in the glass tube for the thermal desorption.

Determination of β -glucosidase activity was quantified using the corresponding calibration curves with 1-octanol standard solution, using the same procedure as described for the wine samples.

All the twistlers were thermally desorbed using a thermal desorption unit (Gerstel) in combination with a cooled injection system (CIS)-4 injector (Gerstel) for cryofocusing the analytes prior to the transfer onto the analytical column. Splitless thermal desorption was programmed from 40 °C to 240 °C (held for 5 min) at 60 °C/min. Cryofocusing was performed with liquid nitrogen at −100 °C. Injection was performed with a ramp of 12 °C/min from −100 °C to 240 °C and held for 5 min.

Analyses were performed in a 6890 N gas chromatographer coupled to an Agilent 5973 N mass spectrometer. Volatile compounds (1-octanol) were separated on a DB-WAX polar capillary column (30 m × 0.25 mm i.d. × 0.50 µm film thickness) from Agilent (J&W Scientific, Folsom, CA, USA). Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 40 °C, later increased to 130 °C at 4 °C/min, then increased to 240 °C at 8 °C/min, and held for 5 min.

For the mass spectrometry system, the temperatures of the transfer line, quadrupole, and ion source were 270, 150, and 230 °C respectively. Electron impact mass spectra were recorded at 70 eV and the ionization current was 10 µA. Spectra were acquired in scan (from m/z 35 to 350) and Selected Ion Monitoring modes. The identification of 1-octanol was based on the comparison of retention times and mass spectra. Mass spectra were compared with those from the NIST 2.0 database.

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