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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · SEPTEMBER 2003

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Hydrolysis of Wine Aroma Precursors during Malolactic Fermentation with Four Commercial Starter Cultures of *Oenococcus oeni*

MAURIZIO UGLIANO, ALESSANDRO GENOVESE, AND LUIGI MOIO*

Dipartimento di Scienza degli Alimenti, Università degli Studi di Foggia, Via Napoli 25, 71100 Foggia, Italy

The ability of four commercial preparations of *Oenococcus oeni* lactic acid bacteria (EQ 54, Lalvin OSU, Uvaferm Alpha, and Lalvin 31) to hydrolyze wine aroma precursors was evaluated by measuring the concentration of free and bound aroma compounds at the end of malolactic fermentation carried out in model wines containing a mixture of glycosides extracted from Muscat wine. At pH 3.4 there was a decrease in glycosylated compounds matched by a concomitant increase in free forms in all starter cultures tested. When malolactic fermentation was carried out at pH 3.2, a significant decrease in the ability to hydrolyze aroma precursors was observed for two of the cultures tested (Uvaferm Alpha and Lalvin 31). Large differences in the extent of hydrolysis and in the specificity of this activity toward specific aroma precursors were observed and appeared to be related to the chemical structure of the aglycon as well as to individual characteristics of each starter culture. The amounts of glycosylated aroma compounds released during malolactic fermentation suggest that *O. oeni* can alter the sensory characteristics of wine through the hydrolysis of aroma precursors.

KEYWORDS: Malolactic fermentation; β-glycosidase activity; aroma precursors; wine aroma

INTRODUCTION

It is widely accepted that naturally occurring glycosylated aroma precursors play a primary role in the expression of the flavor characteristics of grape and wine. Studies on these secondary metabolites have demonstrated that they constitute a reserve of powerful odor-active compounds, which can potentially be released during processing or storage, with consequent improvement of wine aroma complexity (*1*−*6*). Glycosylated precursors are largely present in grapes as β-D-glucosides, often structured as simple D-glucopyranosides in which the volatile aglycon is bound to a glucose moiety through a β-glycosidic linkage. More complex disaccharides, in which the glucose moiety is further substituted with a second sugar such as α-L-arabinofuranose, α-L-rhamnopyranose, or β-D-apiofuranose may also occur frequently (*7*, *8*). Heat-catalyzed acid hydrolysis and use of β-glycosidase enzymes have been thus considered as possible methods for increasing the rate of glycoside hydrolysis and enhancing wine aroma complexity. However, for different reasons, both of these approaches showed drawbacks that limited their applicability during wine-making. Whereas cleavage of the β-glycosidic linkage by acid hydrolysis can promote unwanted rearrangements in the structure of the aglycons (*9*), β-glycosidase enzymes have often been unpredictable in their behavior, being impure mixtures of glucosidases and other enzymes such as esterase and oxidases (*10*). Moreover, these enzymes have been proven to be inhibited by typical wine-making conditions such as low pH and high concentrations of sugar and ethanol (*11*).

Saccharomyces cerevisiae and other yeasts of enological interest have been investigated in order to detect strains with efficient glycosidase activity. Many studies have shown that a large decrease in the concentration of glycoconjugates occurs during alcoholic fermentation and storage of wine over yeast lees (*12*−*14*). Nevertheless, in vitro experiments revealed that, although some *S. cerevisiae* yeast strains may produce β-glycosidases, these enzymes are unstable at wine pH and may be inhibited by high concentrations of sugar and ethanol (*15*−*19*). Although nothing was known about the fate of bound aroma compounds diminished in concentration by alcoholic fermentation, these findings suggest *S. cerevisiae* yeasts can marginally contribute to the enhancement of varietal flavor of wine through the hydrolysis of glycosides of aroma compounds.

To date, little attention has been given to the possibility that a significant hydrolysis of aroma precursors may occur during malolactic fermentation (MLF) as a result of the metabolic activity of lactic acid bacteria. MLF is known to cause several changes in the chemical composition of wine, among which the most important is the transformation of malic acid into lactic acid through decarboxylation. As a result, wines that have undergone MLF exhibit lower titratable acidity and a softer mouthfeel. In several cases, changes in the aroma profile of wine have also been reported as a consequence of MLF (*20*−*22*), although the mechanisms involved in these modifications

* Author to whom correspondence should be addressed (telephone +39-0881-589235; fax +39-0881-740211; e-mail l.moio@unifg.it).

10.1021/jf0342019 CCC: $25.00
Published on Web 07/22/2003

are still poorly understood. It is well-known that several metabolites, mainly carbonyl compounds, are released by lactic acid bacteria during MLF (*21*). However, because none of these compounds, except diacetyl, is present in sufficiently large amounts to influence the aroma of wine (*22*), unique odors and flavors typically associated with MLF arise probably from other biochemical processes promoted by lactic acid bacteria. The observation that the impact of bacterial metabolism on the aroma characteristics of wine varies largely according to the type of grape employed for wine-making (*21*) suggests that varietal aroma compounds may be involved in the flavor modification related to MLF. Moreover, the concentration of β-damascenone, a compound originating from the degradation of glycosylated aroma precursors (*2*), has been shown to increase in Chardonnay wines after MLF (*23*). The ability of lactic acid bacteria to reveal bound aroma compounds by the release of glycosidic enzymes active under wine-making conditions may contribute to the modification of the flavor character of wine after MLF. Several species of LAB are able to conduct MLF in wine. Nevertheless, the species *O. oeni* is particularly well adapted to the low pH and high ethanol concentration of wine and, therefore, is the one most frequently associated with MLF. The few data on the β-glycosidase activity of *O. oeni* are somewhat contradictory. In an early study, McMahon et al. (*19*) reported the lack of glycosidase activity in various commercial cultures of *O. oeni*. However, more recently, Grimaldi et al. (*24*) observed a significant β-glucosidase activity in several *O. oeni* strains, whereas Boido et al. (*25*) reported a decrease of glycosylated aroma compounds during MLF of Tannat wine. On the other hand, Mansfield et al. (*26*) detected the production of β-glucosidase enzymes in several strains of *O. oeni*, although cultures of the same strains failed to hydrolyze native grape glycosides.

Many industrial starter cultures are now available for the induction of MLF, and their use is becoming popular among wine-makers for the prevention of the drawbacks associated with spontaneous MLF. Moreover, the use of selected strains allows for a greater control over the sensory impact of MLF, as it has been shown that bacterial strain can influence the sensory quality of wine (*22*, *27*).

This paper reports the results of a study on the hydrolysis of wine aroma precursors during MLF carried out with four different *O. oeni* starter cultures in model wine solutions. The objective was to evaluate whether the metabolic activity of these bacteria can alter the wine volatile fraction through the liberation of glycosidically bound aroma compounds.

MATERIALS AND METHODS

Preparation of the Aroma Precursors Extract. Wine aroma precursors used for the preparation of model wine solutions were extracted from 8 L of Muscat wine by means of 10 g of C-18 solid phase extraction (SPE) cartridges (Supelco), as described by Williams et al. (*2*). The extract obtained was dried under vacuum and redissolved in 100 mL of water. Residual volatiles were removed by liquid−liquid extraction with dichloromethane (3 × 15 mL).

Fermentation. The four different commercial preparations used for this study were Lalvin OSU, EQ 54 (MBR), Uvaferm Alpha (MBR), and Lalvin 31 (MBR). Dried preparations were kindly donated by Lallemand Italy (Castel d'Azzano, VR), and according to the manufacturers' literature they were all single-strain cultures with the exception of Lalvin OSU, which was a mixed culture of Er1a and Eysd strains.

Two different studies were carried out.

Study 1. Fermentations were carried out in a synthetic wine containing nutrients and other bacteria requirements at the concentrations reported in **Table 1**. The composition of this growth medium was similar to the one described by Grimaldi et al. (*24*), but the concentration of some constituents such as ethanol and reducing sugars was modified to reproduce the composition of wine. For the same reason, tartaric and acetic acids were added. Four hundred milliliters of synthetic wine was transferred into sterile Erlenmeyer flasks under sterile conditions and inoculated with 15 mg/L starter cultures previously rehydrated in 20 mL of sterile water at 35 °C for 30 min, as described in the manufacturers' instructions. A noninoculated reference sample was prepared by adding 20 mL of sterile water to 200 mL of synthetic wine, and MLF was inhibited by means of 50 mg/L of sulfur dioxide. Samples were incubated at 25 °C until malic acid was no longer detected using an enzymatic kit (Roche, Mannheim, Germany).

Table 1. Composition of the Synthetic Wine[a]

ingredient	amount[b]
ethanol (% v/v)	12.5
tartaric acid	5.0
L-malic acid	3.5
acetic acid	0.6
D-glucose	2.0
D-fructose	2.0
NaCl	0.2
$(NH_4)_2SO_4$	1.0
K_2HPO_4	2.0
$MgSO_4 \cdot 7H_2O$	0.2
$MnSO_4$	0.05
yeast extract	2.0
hlycoside extract (mL/L)	12.3

[a] pH was adjusted to 3.2 or 3.4 with KOH pellets; medium was sterilized by filtration at 0.2 μm. [b] Expressed in g/L unless otherwise specified.

Study 2. The same conditions described for study 1 were adopted except that the pH was adjusted to 3.2 in order to evaluate the influence of this parameter on the glycosidic activity of the four *O. oeni* preparations.

For both studies all fermentations were carried out in duplicate.

Extraction and Analysis of Free and Bound Aroma Compounds. At the end of MLF samples were centrifuged at 5000 rpm for 10 min, and the supernatant was filtered at 0.4 μm. Extraction of free and bound aroma compounds was carried out by means of C-18 SPE cartridges (Phenomenex, Torrance, CA), as proposed by Di Stefano (*28*). Accordingly, 25 mL of synthetic wine was diluted 1:1 with water, loaded on a previously activated C-18 cartridge containing 1 g of sorbent, and eluted at ~3 mL/min. The cartridge was then washed with water, followed by dichloromethane for the recovery of free aroma compounds and methanol for the recovery of the glycoconjugated fraction. The dichloromethane fraction was dried over Na_2SO_4 and concentrated first in a Kuderna−Danish concentrator and finally under a stream of pure N_2, prior to GC and GC-MS analysis. The methanol fraction was dried under vacuum, redissolved in phosphate/citrate buffer (pH 5.0), and incubated at 40 °C after the addition of 150 mg of pectic enzyme (Rohapect C, Rohm Tech, Malden, MA). After 16 h, the solution containing free aglycons was loaded on a C-18 SPE cartridge and the volatiles were extracted with 5 mL of dichloromethane. The extract, dried over Na_2SO_4, was concentrated as previously described and submitted to GC and GC-MS analysis. Each extraction was carried out in triplicate. Gas chromatography was performed using a Hewlett-Packard 5890 chromatograph equipped with a split/splitless injector (Hewlett-Packard, Avondale, PA), a J&W DB-Wax column (30 m length × 0.25 i.d. × 0.25 μm film thickness; J&W Scientific, Folsom, CA), and a flame ionization detector (FID). The temperature program used was 40 °C for 3 min, raised at 4 °C/min to 220 °C, 20 min at maximum temperature. Carrier gas (He) velocity was 37 cm/s. Both detector and injector temperatures were maintained at 250 °C. Identification of compounds was performed by comparison of their linear retention indices with those of pure reference standards. Comparison of mass spectra stored in the NIST database with those obtained for each compound on an HP 5972 quadrupole mass spectrometer coupled with an HP5890 gas chromatograph was also carried out. The same column of HRGC was employed during this analysis. Electron impact mass spectra were recorded with ion-source energy of 70 eV.

Table 2. Concentration of Selected Aglycons and Their Precursors in Synthetic Wines at the End of MLF Carried out under the Conditions of Study 1

	concentration[a] (μg/L)									
	free					bound				
compound	ref	Lalvin 31	EQ 54	Lalvin OSU	Uvaferm Alpha	ref	Lalvin 31	EQ 54	Lalvin OSU	Uvaferm Alpha
linalool	nd	3.3 a	11.7 c	4.6 b	nd	211.7 c	202.1 ba	196.8 a	203.8 a	207.5 cb
α-terpineol	2.3 a	13.8 c	21.5 d	15.2 c	11.4 b	47.8 d	35.6 b	31.6 a	33.8 ab	36.5 c
nerol	12.5 a	118.5 c	214.9 e	180.4 d	80.7 b	584.5 d	466.2 b	369.0 a	382.6 a	508.8 c
geraniol	15.2 a	128.6 c	240.2 e	205.6 d	87.8 b	624.2 e	492.4 c	383.8 a	402.1 b	538.5 d
total	30.0 a	264.2 c	488.3 e	405.8 d	185.9 b	1458.2 e	1196.3 c	981.2 a	1022.3 b	1293.3 d

[a] Different letters (a–e) denote a significant difference ($p < 0.05$) according to the LSD test. nd, not detected.

Statistical Analysis. Analysis of variance and least significant difference (LSD) test were used to interpret the differences in means at the 95% confidence level. Elaborations were carried out using Statgraphics 5.0 Plus-PC (Manugistics, Inc.).

RESULTS

Fermentation. Between 14 and 15 days was required for all strains to complete MLF. Under the experimental conditions of study 1, the values of pH of the synthetic medium at the end of MLF were as follows (values of duplicate fermentation batches): 3.61 and 3.64 for Lalvin 31; 3.58 and 3.61 for EQ 54; 3.56 and 3.56 for Lalvin OSU; and 3.60 and 3.54 for Uvaferm Alpha. When fermentation was conducted at pH 3.2 (study 2), the final values of pH were 3.43 and 3.48 for Lalvin 31; 3.45 and 3.46 for EQ 54; 3.43 and 3.41 for Lalvin OSU; and 3.44 and 3.48 for Uvaferm Alpha. In all cases residual malic acid was below the detection limit of the method, attesting to the completion of MLF.

Hydrolysis of Glycosylated Aroma Compounds. *Study 1.* The results of the GC analysis of samples at the end of MLF under the conditions of study 1 are reported in **Table 2**. The four most important terpenols (linalool, α-terpineol, nerol, and geraniol) were measured to evaluate hydrolysis of the aroma precursors during MLF. In all samples there was a significant increase in the concentration of total free terpenols. This increase was accompanied by a concomitant decline of bound terpenols, attesting to the occurrence of hydrolysis of the bound aroma precursors during the experiment. By comparing the data of the fermented samples with those relative to the unfermented reference sample it may be noted that the hydrolysis of aroma precursors appeared to be strongly enhanced by the occurrence of MLF, because free terpenols occurred at very low concentrations in the reference sample, probably as a result of slow acid-catalyzed hydrolysis. It was thus deduced that, in our experimental conditions, all of the commercial preparations tested revealed a significant ability to hydrolyze monoterpenyl aroma precursors. A large variation in the extent of hydrolysis associated with individual bacterial cultures was also observed. Similarities in the values of pH of synthetic wines obtained with different starter cultures suggested that acid hydrolysis was not involved in these differences. The most intense glycosidic activity was found in samples fermented with EQ 54. Indeed, both the increase of free terpenols and the reduction of conjugated forms were larger when MLF was carried out with this commercial culture. The Lalvin OSU culture, which was a mixture of two strains, also revealed considerable hydrolytic activity. Both the decline in precursors as well as the total amount of free terpene detected in samples fermented with Lalvin 31 were significantly lower than in the case of the two previous cultures. Finally, samples fermented with the Alpha starter culture were characterized by the lowest hydrolysis of aroma precursors.

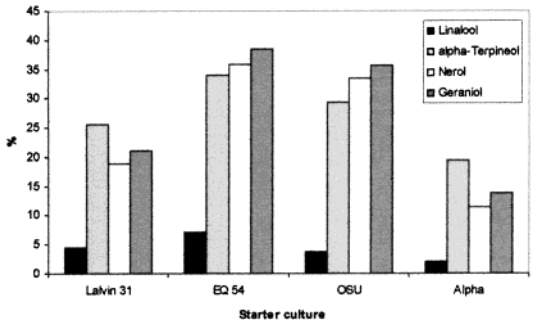

Figure 1. Extent of glycoside hydrolysis, calculated as percentage ratio between the concentrations of glycosides after and before MLF.

The effect of MLF on precursors of the four terpenols measured was also highly variable according to the chemical structure of the aglycon (**Figure 1**). Of the linalool glycosides, 2.0−7.0% of those present in the initial medium were hydrolyzed. α-Terpineol precursors were hydrolyzed 19.46−33.9% of their initial content. There was greater degradation of precursors of nerol and geraniol, which were hydrolyzed from 11.4% to 35.8% and from 13.7 to 38.5%, respectively.

Study 2. Generally, the hierarchy of glycosidase activity of the four cultures did not change when MLF was carried out at pH 3.2 instead of pH 3.4, with EQ 54 showing the highest hydrolytic activity, followed by Lalvin OSU, Lalvin 31, and Uvaferm Alpha. However, the differences in ability to release terpenols from nonvolatile precursors appeared to be more evident under the new conditions of growth (**Figure 2**). Indeed, for starter cultures Lalvin 31 and Uvaferm Alpha, which were those characterized by the lowest glycosidase activity at pH 3.4, a reduction of 0.2 unit in the pH of the growth medium coincided with a significant decline in the release of glycosylated terpenols. This effect was particularly evident in the case of the Uvaferm Alpha, for which the amount of total and selected terpenols detected in the samples fermented at pH 3.2 was less than half that detected after MLF carried out at pH 3.4. Regarding the other two cultures, their ability to hydrolyze aroma precursors was generally not significantly affected by the lower pH, and the only significant difference evidenced between the two experiments was relative to the amount of linalool detected in samples obtained with the starter culture EQ 54, which was significantly lower in samples fermented at pH 3.2.

DISCUSSION

Hydrolysis of native wine aroma precursors by *O. oeni* in a synthetic growth medium is reported here for the first time. In the screening carried out by Mansfield et al. (*26*) none of the seven strains of *O. oeni* tested was able to hydrolyze wine aroma

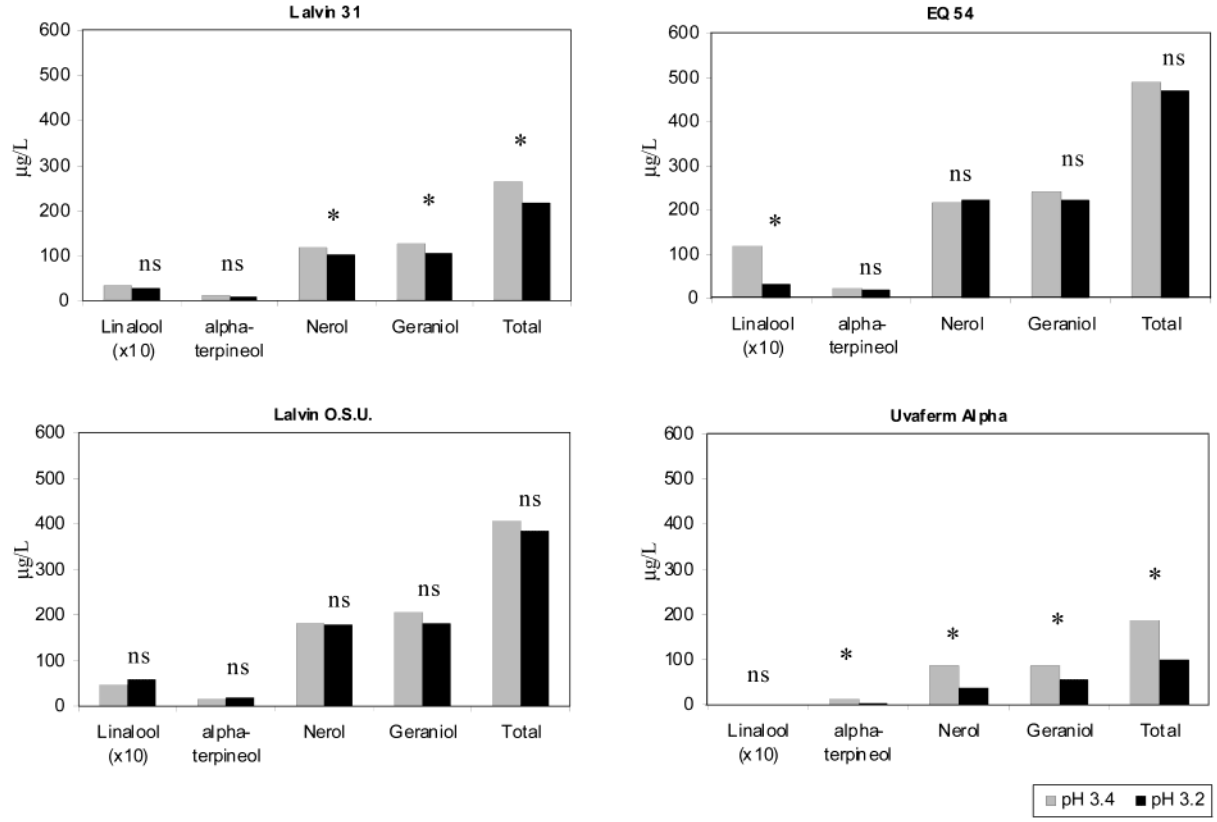

Figure 2. Effect of pH on the concentration of terpenols released during MLF. Asterisks denote a significant difference ($p < 0.05$) according to the LSD test.

precursors, even though these strains were previously selected on the basis of the level of β-glucosidase activity shown against a model glycoside. However, comparisons between that study and the current one are difficult, because the use of different growth media and culture conditions may have influenced the release of hydrolytic enzymes by the lactic acid bacteria. For example, the inclusion of ethanol in the growth medium has been shown to enhance β-glucosidase activity of *O. oeni* (*24*). Generally speaking, however, during MLF under the experimental conditions of this study, glycosides were hydrolyzed from 11.3 to 32.7%, and the decrease of glycoconjugates was matched by a consistent increase in corresponding free aroma compounds. The only exception to this trend was observed for linalool, for which the decrease of glycosylated forms was, in all samples, not followed by a proportional increase of this compound in the free fraction. On the other hand, the concentration of free α-terpineol after MLF was generally larger than the value expected on the basis of the corresponding decrease of glycosylated forms. Rearrangements of linalool into α-terpineol under the pH conditions of wine may have occurred (*12*, *29*).

The large release of glycosylated aroma compounds observed during our experiment suggests that *O. oeni* can actively contribute to the changes of sensory characteristics of wine after MLF through the hydrolysis of aroma precursors. For example, geraniol was released in quantities up to 8 times higher than its odor threshold (*30*). However, according to Boido et al. (*25*), volatile compounds released from precursors during MLF might be adsorbed onto polysaccharides and peptidoglycans produced by *O. oeni*, reducing the effect of glycosidase activity observed during MLF on the composition of the volatile fraction of wine.

Previously published papers concerning the β-glucosidase activity of *O. oeni* describe this characteristic as strain-dependent (*24*−*26*). A large variation in the extent of hydrolysis of glycosylated aroma compounds was also observed for the different commercial starter cultures tested during our experiment. The two cultures exhibiting the strongest hydrolytic activity (EQ 54 and OSU) have been already described as characterized by an intense β-glucosidase activity against a model substrate in a comparative study on several *O. oeni* strains (*24*). However, differences in the amount of terpenols released by the four commercial starters of *O. oeni* under our experimental conditions were never larger than 3-fold. A minor influence of the type of strain on the modifications occurring to the sensory profile of the four experimental wine as a result of the release of bound terpenols may be thus hypothesized.

The primary structure of nerol and geraniol may be a significant factor in explaining the higher percentage degradation of precursors observed for these two compounds, because the rate of enzymatic hydrolysis of primary alcohol precursors is higher than that of tertiary alcohols such as linalool and α-terpineol (*31*). However, if the decline of specific precursors associated with each strain is considered separately (**Figure 1**), it can be deduced that the structure of the aglycon is not the only factor determining the efficiency of hydrolysis of specific glycosides. For example, the ability of EQ 54 and OSU preparations to hydrolyze terpenol precursors was high in the primary alcohols such as nerol and geraniol, but, in the tertiary alcohols, it was high for α-terpineol but relatively poor for linalool, of which the precursors were more abundant than those of the former. Enzymatic hydrolysis of grape monoterpenyl diglycosides occurs through a sequential mechanism involving two steps: first, the linkage between the two sugar units is cleaved by either α-L-rhamnopyranosidase, α-L-arabinofuranosidase, or β-D-apiosidase; second, the aglycon is liberated by the action of a β-D-glucosidase. Only this second step is required in the case of β-D-glucosides (*8*). It can thus be

supposed that different bacterial strains possess pools of glycosidase enzymes with distinct chemical and compositional characteristics, causing differences in the extent of hydrolysis in a mixture of aroma precursors. This aspect appears to be worthy of further investigation.

In study 2 the effect of the pH of the growth medium on the glycosidase activity of the four bacteria strains was evaluated. At pH 3.2 a decrease in the extent of hydrolysis was observed for two of the four dried preparations. This behavior may be attributed to a reduced β-glycosidase activity at lower pH. According to previously published data, β-glucosidase enzymes isolated from several yeasts have shown a rapid fall of activity as pH was reduced (*16*, *32*, *33*). In the case of *O. oeni*, a loss of activity ranging from 57 to 88% of maximal value was observed within the pH range from 3.5 to 4.0 (*24*). The behaviors observed under the conditions of study 2 suggest that the hydrolytic activity of some strains is more strongly affected by pH conditions of the medium, which may limit their contribution to the release of bound aroma compounds during wine-making.

In sum, our results shows that commercial strains of *O. oeni* employed for malolactic fermentation can contribute to the enhancement of wine aroma complexity through the hydrolysis of grape-derived bound secondary metabolites. Significant differences seem to exist between different preparations of *O. oeni* lactic acid bacteria with regard to their ability to hydrolyze glycosylated aroma precursors, which suggests the importance of more extended screening studies for the recognition of strains with extensive glycosidase activity. Further investigations are also necessary to elucidate the biochemical mechanisms involved in the production of glycosidase enzymes by *O. oeni*. Experiments are now in progress to evaluate, during the production of wines obtained from different grape varieties, the influence of some enological variables such as wine composition, cellar practices, and interaction between yeast and bacteria strains on the role of *O. oeni* lactic acid bacteria in the expression of the varietal aroma attributes of wine.

ACKNOWLEDGMENT

We thank Drs. L. Paronetto and P. Vagnoli at Lallemand Italy, and Regione Compania, Se.S.I.R.C.A. We also thank Caroline Turner, MAgrSc Reading, for assistance in the preparation of the manuscript.

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Received for review February 27, 2003. Revised manuscript received May 30, 2003. Accepted June 9, 2003. This study was partially conducted at the Dipartimento di Scienza degli Alimenti, Università di Napoli, and was supported by Lallemand Inc. Italy within a cooperative research project with Università di Napoli.

JF0342019