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Quantification of glycosidase activities in selected yeasts and lactic acid bacteria

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Using a model system, the activities of α-L-arabinofuranosidase, β-glucosidase, and α-L-rhamonopyranosidase were determined in 32 strains of yeasts belonging to the genera *Aureobasidium, Candida, Cryptococcus, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Saccharomyces, Torulaspora* and *Brettanomyces* (10 strains); and seven strains of the bacterium *Leuconostoc oenos*. Only one *Saccharomyces* strain exhibited β-glucosidase activity, but several non-*Saccharomyces* yeast species showed activity of this enzyme. *Aureobasidium pullulans* hydrolyzed α-L-arabinofuranoside, β-glucoside, and α-L-rhamnopyranoside. Eight *Brettanomyces* strains had β-glucosidase activity. Location of enzyme activity was determined for those species with enzymatic activity. The majority of β-glucosidase activity was located in the whole cell fraction, with smaller amounts found in permeabilized cells and released into the growth medium. *Aureobasidium pullulans* hydrolyzed glycosides found in grapes.

Keywords: *Saccharomyces cerevisiae*; *Brettanomyces*; lactic acid bacteria; glycosidases; glycosides; wine phenols; grape phenolics

Introduction

Grape-derived aroma and flavor compounds are present as free volatiles and, in part, as sugar-bound precursors including glycosides [2,47]. Compounds bound to a sugar molecule are known as aglycones, and in grapes may be aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, or shikimic acid metabolites such as phenols [2,40–42,48]. Glycosides which contain aroma and flavor aglycones may affect wine quality after hydrolysis. Research to increase grape glycoside production and hydrolysis and subsequent release of aglycones may lead to enhanced product quality [2,16,47].

Glycosides may exist as disaccharide complexes such as α-L-rhamnopyranosyl-β-D-glycopyranosides or α-L-arabinofuranosyl-β-D-glycopyranosides [8]. Glycoside hydrolysis may occur either enzymatically or by acid, potentially releasing volatile compounds [16,17,23,46]. Complete enzymatic catalysis of monoterpenyl glycosides occurs through two successive steps: (1) glucose is separated from the terminal sugar by a hydrolase (α-L-arabinofuranosidase, α-L-rhamnosidase, or β-apiosidase); (2) the bond between the aglycone and glucose is broken by β-glucosidase [24]. The hydrolase needed to break the disaccharide bond may have specific or broad activity [24].

Limited hydrolysis of glycosides occurs during berry maturation by endogenous fruit β-glucosidases [7,8] and during vinification by microbial enzymes and acid-catalyzed hydrolysis [23,49]. Sefton [43] showed that products of acid hydrolysis of Merlot and Cabernet Sauvignon wines contributed 'intense berry and plum-like aromas' while products from enzyme hydrolysis were undetectable. In contrast, Abbott *et al* [1] found that acid hydrolysis products enhanced varietal aroma, particularly 'non-berry' attributes. Some acid-catalysis products may cause undesirable alteration of aroma, making enzymatic hydrolysis more favorable [22,48]. These differences may be the result of contrasting mechanisms between enzymatic and acid hydrolysis [46]. Enzymatic hydrolysis cleaves the glycosidic linkage without altering the aglycone, while acid hydrolysis may split alcohol aglycones and produce a reactive carbonation [43].

Glycosidase activities have been documented in enological yeasts (endogenous to wine or the vineyard) [11,13,15]. A few *Saccharomyces* strains exhibit hydrolytic enzyme activity, but greater activity has been found in non-*Saccharomyces* yeast active in native, uninoculated fermentations [25]. Non-*Saccharomyces* yeasts tend to be prevalent in the initial stages of uninoculated fermentation, but decline as alcohol content increases. Strains of *Saccharomyces* generally complete a fermentation [32,35,36]. Species isolated from native fermentations include *Hanseniaspora uvarum*, *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Hansenula anomala*, among others [34]. Each of these species has been shown to produce β-glucosidase *in vitro* [37]. The belief that native fermentations enhance aroma [19] may be supported, in part, by higher hydrolytic enzyme production in these genera. Therefore, quantification of glycosidases may allow winemakers to make informed choices concerning the yeasts used in fermentation initiation.

Co-culture of *Brettanomyces* with *Saccharomyces cerevisiae* can cause aromas and flavors similar to malolactic fermentation, such as enhanced complexity, augmented fruitiness, and diminished vegetative odors [18]. Although commonly viewed as a spoilage organism, *Brettanomyces* may enhance aroma [18]. Shantha Kumara *et al* [44] suc-

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cessfully isolated and purified an α-glucosidase from *Brettanomyces lambicus* found in a secondary lambic beer fermentation.

Although enological yeasts may produce glycosidases, acidic wine conditions may cause denaturation of these enzymes and inhibition of activity [13]. In typical wine conditions, β-glucosidase production and activity is strongly inhibited by low pH, as well as high alcohol concentration, lack of oxygen and presence of glucose [22]. The degree to which these factors inhibit β-glucosidase production and activity depends on the species and strains of the organisms involved [3,13,31,37]. For instance, Delcroix *et al* [13] monitored three strains of *Saccharomyces cerevisiae* and illustrated a 95% loss in hydrolytic enzymatic activity at wine pH. In contrast, Rosi *et al* [38] illustrated the optimum pH for a *Debaryomyces hansenii* β-glucosidase was 3.2.

Malolactic fermentation (MLF) can alter the acidity and sensory characteristics of wine [27–29]. An increase in glucose coinciding with MLF fermentation has been documented [9,10]. This increase could be caused by hydrolysis of glycosides [30]. Linking glycoside hydrolysis and bacterial enzymatic activity has proven difficult because the increase in glucose could also result from residual grape or yeast hydrolytic enzymes [10]. However, De Cort *et al* [12] successfully isolated and purified an α-glucosidase from *Lactobacillus brevis* isolated in Belgian iambic beer during a secondary fermentation.

The objective of this study was to determine the ability of certain enological organisms to hydrolyze glycosides. Selected strains of enological yeasts and lactic acid bacteria were assayed for α-L-arabinofuranosidase, β-D-glucosidase, and α-L-rhamnopyranosidase activities, using different cellular and extracellular fractions. The cultures used included ten vineyard-isolated yeasts, ten strains of *Brettanomyces intermedius*, five strains of commercial *Saccharomyces cerevisiae*, and seven strains of commercial lactic acid bacteria.

Materials and methods

Cultures

The yeast genera and species used in this study are listed in Tables 1 and 2. The vineyard isolates were obtained and identified by Cohen [6]. The strains of *Brettanomyces intermedius* were provided by Lallemand Inc, Montreal, Canada. Commercial strains of *Saccharomyces cerevisiae* were obtained as follows: M1, VL1, and ICV-D47 were provided by Lallemand (Montreal, Quebec, Canada); Fermiblanc was from Gist-brocades (Seclin cedex, France); and Prise de Mousse was from Universal Foods Corp (Milwaukee, WI, USA). All strains were re-isolated to assure that a pure culture was utilized, and maintained on Yeast Mold Agar (Difco, Detroit, MI, USA) slants, pH 5.0. *Leuconostoc oenos* strains were obtained as follows: OSU, 3X, MBR, and MT-01 were from Lallemand Inc. *Leuconostoc oenos* has been officially reclassified as *Oenoccocus oeni* [14], but the common and supplier's listing of *L. oenos* will be used hereafter (Lallemand, Inc). *Vinaflora oenos* was from Chris Hansen's Laboratory (Milwaukee, WI, USA), and MCW and Bitec D were provided by Vinquiry (Healdsburg, CA, USA). Bacterial cultures were isolated and maintained on Apple Rogosa slants as described by Fugelsang [19].

Table 1 Enzyme activities for vineyard isolates and commercial yeasts (expressed as nmol of hydrolyzed β-glucoside or analogue per ml assay medium per gram dry cell mass per 48 h)

Organism	Arabino-furanoside	Gluco-pyranoside	Rhamno-pyranoside
Aureobasidium pullulans	37a	1774 a	653 a
Candida guillermondii	nd	824 c	26 b
Candida parapsilosis	nd	1744 a	nd
Cryptococcus albidus	nd	nd	nd
Hanseniaspora uvarum	nd	nd	nd
Hansenula anomala	nd	719 d	nd
Kloeckera apiculata	nd	1322 b	nd
Metschnikowia pulcherrima	nd	633 e	nd
Pichia guillermondii	<LOD	349 f	nd
Saccharomyces (italicus) cerevisiae	nd	nd	nd
Torulaspora delbrueckii	nd	nd	nd
Saccharomyces cerevisiae (ICV-D47)	nd	nd	nd
Saccharomyces cerevisiae (M1)	nd	< LOD	nd
Saccharomyces cerevisiae (VL-1)	nd	110 g	nd
Saccharomyces cerevisiae (Prise de Mousse)	<LOD	nd	nd
Saccharomyces cerevisiae (Fermiblanc)		nd	nd

nd, None detectable. Values are the averages of duplicate assays. Different letters within columns indicate significance with a *P*-value $\leq$0.05. Limit of detection: 19 nmol ml^{-1}.

Table 2 Enzyme activities for *Brettanomyces intermedius* (expressed as nmol of hydrolyzed β-glucoside or analogue per ml assay medium per gram dry cell mass per 48 h)

Brettanomyces intermedius strain	Arabino-furanoside	Gluco-pyranoside	Rhamno-pyranoside
211	nd	575 e	nd
212	nd	1694 c	nd
213	nd	nd	nd
214	nd	2656 a	nd
215	nd	1765 bc	nd
216	nd	2018 b	nd
brux	nd	nd	nd
'ave'	nd	931 d	nd
souche 'o'	nd	nd	nd
souche 'm'	nd	819 d	nd

nd, None detectable. Values are the averages of duplicate replications. Different letters indicate significance with a *P*-value $\leq$ 0.05. Limit of detection: 19 nmol ml^{-1}.

Hydrolysis of purified glycosides by cells

The procedure of Blondin *et al* [4] with modifications by Charoenchai *et al* [5] was used to determine hydrolytic enzyme activities on three substrates: α-L-arabinofuranoside, β-D-glucoside and α-L-rhamnopyranoside. Cells were grown in 10 ml liquid culture consisting of 6.7 g L^{-1} Yeast Nitrogen Base (YNB, Difco) and 5 g L^{-1} arbutin (Sigma, St Louis, MO, USA). The medium was buffered to pH 5.0 by the addition of 0.6 g L^{-1} K_2HPO_4 and 0.2 g L^{-1} tartaric acid. After 48 h at 30°C, cultures were centrifuged (5000 $\times$ *g*, 10 min, 4°C), washed with cold sterile saline (0.7% NaCl) and re-centrifuged. Each pellet was transferred to

10 ml filter-sterilized growth medium containing 6.7 g L^{-1} YNB and 1 mM substrate: *p*-nitrophenyl-[α-L-arabinofuranoside], [β-glucopyranoside], or [α-L-rhamnopyranoside] (Sigma). The medium was buffered to pH 3.5 with tartaric acid and K_2HPO_4 (approximately 0.9 and 1.0 g L^{-1}, respectively). The reaction tubes were incubated for 48 h at 30°C. The supernatant fluid was assayed for liberated *p*-nitropheny(*p*-NP): 1.0 ml was mixed with 2.0 ml sodium carbonate buffer (0.2 M, pH 10.2) and measured spectrophotometrically (Genesys5™, Spectronic Instruments, Rochester, NY, USA) at 400 nm. A series of standards was prepared that contained 0–200 nM *p*-NP. A substrate blank (buffer and substrate) and sample blanks (cell preparation and buffer) were prepared, and any absorbance was subtracted from experimental absorbance readings. All assays were performed in duplicate.

Enzyme activity location

Cultures: Strains which demonstrated substantial enzymatic activity (>1000 nmol ml^{-1} g dry cell $mass^{-1}$ for *Brettanomyces* cultures, and >300 nmol ml^{-1} g dry cell $mass^{-1}$ for vineyard isolates) were further analyzed to determine the generalized location of enzyme activity (whole cells, permeabilized cells, and culture supernatant fluid) as described by Rosi *et al* [37]. A loopful of culture was transferred from stock slants to 10 ml of liquid medium (YNB 6.7 g L^{-1}; arbutin 5 g L^{-1}; and pH to 5.0). After 48 h, 0.2 ml of the inoculum was added to 125-ml screw-capped bottles filled to 80% of their volume and incubated at 30°C for 3 days.

Whole cells: Cells were harvested from 1 ml of culture (centrifuged at $5000 \times g$, 10 min, 4°C) and washed twice with cold distilled water. The pellet was resuspended in 0.2 ml of citrate-phosphate buffer (100 mM, pH 5.0) and then assayed for activity.

Permeabilized cells: The procedure of Salmon [39], modified by Rosi *et al* [37] was used. The culture (5 ml) was centrifuged ($5000 \times g$, 10 min, 4°C), and the pellet was washed with 5 ml of cold distilled water. The pellet was resuspended in 1 ml of imidazole buffer (75×10^{-3} mmol L^{-1}, 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 min and then centrifuged. The pellet was suspended in 5 ml of cold distilled water, 1 ml of this suspension was centrifuged and the pellet was washed with cold distilled water. The final pellet was resuspended in 0.2 ml citrate-phosphate buffer (100 mM, pH 5.0). Therefore, the permeabilized fraction consisted of washed cells, which had the cell wall compromised.

Supernatant: The supernatant fraction was comprised of 0.2 ml of the unconcentrated growth medium.

Enzyme activity location: Supernatant fluid, whole, or permeabilized cells (0.2 ml each) were mixed with 0.2 ml of 5 mM solution of *p*-NP glycopyranosidase in 100 mM citrate-phosphate buffer (pH 5.0). The reaction mixture was incubated at 30°C for 1 h. The addition of 1.2 ml of carbonate buffer (0.2 M, pH 10.2) stopped enzyme activity, and then the reaction mixture was centrifuged at $10\,000 \times g$ for 2.5 min. Liberated *p*-NP was measured spectrophotometrically as described previously. A series of standards was prepared from 0–200 nM *p*-NP. All assays were performed in duplicate.

Hydrolysis of grape glycosides

Isolation of glycosides: Viognier grapes grown in northwestern Virginia were pressed and the juice was partially fermented (4.5% alcohol, 16°Brix). Viognier glycosides were isolated using Waters (Milford, MA, USA) C-18 reverse phase Sep-Pak columns that were activated by passing through 10 ml of methanol followed by 10 ml of deionized water. After loading 10 ml of undiluted juice, the columns were washed three times with 15 ml of deionized water. Glycosides were eluted with 5 ml methanol. Ten elutions were combined per aliquot, concentrated to dryness, and stored at −20°C. Glycoside concentration was estimated by the analysis of glycosyl-glucose [47].

Glycoside hydrolysis: Culture (50 ml) grown in YNB arbutin was centrifuged, and the pellet was resuspended in 50 ml of 100 mM citrate-phosphate buffer (pH 5.0) and added to an aliquot of Viognier glycosides (111 μM glycosyl-glucose). Pectinolytic enzyme (0.03 g L^{-1}) (AR2000™, Gist-brocades, Seclin cedex, France) in buffer was used to verify the potential for glycoside hydrolysis. After a 48-h incubation at 30°C, liberated glycosyl-glucose was measured spectrophotometrically at 340 nm using an enzymatic glucose assay (Boehringer Mannheim, Indianapolis, IN, USA).

Dry weight

Cell dry weight was determined by filtering 50 ml of culture on pre-weighed filters (0.45-μm; Pall Gelman Sciences, Ann Arbor, MI, USA). Filters were placed in tared aluminum pans, dried overnight at 100°C, and then reweighed.

Statistical analysis

All data were analyzed statistically using SAS (SAS Institute, Cary, NC, USA) and Minitab™ (Minitab, State College, PA, USA). The statistical method employed was Duncan's Multiple Range Test. Determination of enzyme activities required regression analysis of the series of standards.

Results and discussion

Aureobasidium pullulans displayed arabinofuranosidase activity, and two cultures (*A. pullulans* and *C. guillermondii*) showed rhamnopyranosidase activity (Table 1). Both of these organisms also had β-glucosidase activity. These activities may have been caused by the specific activity of more than one enzyme (α-L-arabinofuranosidase, α-L-rhamnosidase, or β-apiosidase) or the broad activity of a single hydrolase. For example, *Hanseniaspora vineae* displayed a lack of specificity, hydrolyzing sugars

with β (1–4) or β (1–2) bonds [45]. Similar characteristics have also been reported for *C. molischiana* [20]. Genera such as *Candida* and *Kloeckera/Hanseniaspora* are reported to grow during early fermentation stages [26,34]. The high β-glucosidase activity found using *A. pullulans*, *C. parapsilosis*, and *Kloeckera apiculata* (Table 1) may support winemakers' claims that uninoculated fermentations result in a more aromatic, flavorful wine [19]. Some of the commercial *Saccharomyces* strains assayed in our study were chosen based upon manufacturer's claims of 'enhanced varietal expression'. However, these strains conventionally used for alcoholic fermentation displayed little or no detectable enzymatic activity (Table 1), similar to other studies using *S. cerevisiae* [5,37]. The one exception was limited β-glucosidase activity exhibited by VL-1 (110 nmol ml^{-1} g cell dry $mass^{-1}$ 48 h^{-1}; Table 1). Zoecklein *et al* [49] noted that fermentation by VL-1 resulted in higher total free monoterpenes and aromatic alcohols than ICV-D47 or Prise de Mousse, and their observations also suggest limited hydrolysis. Seven of ten *B. intermedius* strains displayed β-glucosidase activities, ranging from 575 to 2650 nmol ml^{-1} g dry $cell^{-1}$ 48 h^{-1} (Table 2).

Cultures with high β-glucosidase activity were further analyzed for location of this activity. On average, the whole cell (parietal) fraction contained the majority of the enzyme activity, followed by the (intracellular) permeabilized fraction, and little extracellular (supernatant) activity was detected (Tables 3 and 4). Variances in the location of hydrolytic enzyme activity from the general activities reported in Tables 1 and 2 may have occurred due to cell wall differences. The Rosi *et al* [37] assay is influenced by the ability of the cell culture to form a pellet. In cultures where cell pellets were less cohesive, some cells might have been lost in the assay, causing an artificially low concentration. The extent of this reduction would be dependent on the loss of cell mass. An extreme example was VL-1, for which the lack of parietal (whole cell) enzyme activity seems suspect (Table 3). The activity in the permeabilized cell fraction may have been lower than the whole cell fraction due to denaturation by released digestive enzymes. Additional reduction of detected activity may have occurred through enzyme loss during the wash of permeabilized cells. Limited amounts of enzyme activity in the culture supernatant fluid might have been due to cell lysis. Darriet *et al* [11] found that β-glucosidase is located in the periplasmic space of the yeast cell, and would be released during cell death. A small portion of the enzyme activities in the supernatant fraction may have been due to release of enzyme during autolysis. The extracellular enzyme activities found were slightly higher than those found by Rosi *et al* [37] for the species *Kloeckera apiculata* and *Hansenula anomala*.

Table 3 β-glucosidase activities by location of enzyme activity for vineyard isolates and a commercial yeast strain

Organism	Whole[1]	Permeabilized[1]	Supernatant[2]
Aureobasidium pullulans	2279 a	268 c	nd
Candida guillermondii	230 d	nd	23 c
Candida parapsilosis	1313 b	151 d	20 c
Hansenula anomala	587 c	84 f	6 d
Kloeckera apiculata	621 c	393 b	260 a
Metchnikowia pulcherrimma	1359 b	112 e	73 b
Pichia guillermondii	105 e	57 g	nd
Saccharomyces cerevisiae (VL-1)	nd	726 a	4 d

Values are the averages of duplicate replications. Different letters within columns indicate significance with a *P*-value ≤0.05. Limit of detection: 3 nmol ml^{-1}.
[1] Activity is expressed as nmol *p*NP per mg cells per 30 min (dry weight).
[2] Activity is expressed as nmol *p*NP per ml per 30 min.

Table 4 β-glucosidase activities by location of enzyme activity for *Brettanomyces intermedius* strains

Brettanomyces intermedius strain	Whole[1]	Permeabilized[1]	Supernatant[2]
211	129 e	70 ab	3 b
212	1609 a	1568 bc	6 b
214	747 b	427 a	15 a
215	161 d	156 d	3 b
216	844 c	206 cd	<LOD

Values are the averages of duplicate replications. Different letters within columns indicate significance with a *P*-value ≤ 0.05. Means with the same letter are not significantly different. Limit of detection: 3 nmol ml^{-1}.
[1]Activity is expressed as nmol *p*NP per mg cells per 30 min (dry weight).
[2]Activity is expressed as nmol *p*NP per ml per 30 min.

On average, the 10 *Brettanomyces* strains examined had higher permeabilized and supernatant enzymatic β-glucosidase activity than the vineyard-isolated yeasts (Table 4). Extra and intracellularly produced (α) glucosidase activity has been previously demonstrated from *B. lambicus* isolated in a lambic beer [45].

Hydrolytic enzyme activity was not detected in the *Leuconostoc oenos* strains (data not shown). The fastidious nature of the organisms and the lack of a complex growth medium may have contributed to the lack of enzyme production. During preliminary trials, the lactic acid bacteria dried preparations were added directly to the YNB arbutin without prior isolation. Using this method, *Vinaflora oenos* had limited α-L-rhamnopyranosidase (90 nmol ml^{-1} g^{-1} 48 h^{-1}) production and OSU had minor β-glucosidase activity (111 nmol ml^{-1} g^{-1} 48 h^{-1}). However when isolated colonies were used, no production was observed.

Viognier glycosides (111 μM glycosyl-glucose) were used to determine enzyme hydrolysis of a natural substrate by *A. pullulans*, *K. apiculata*, and *C. parapsilosis*. *A. pullulans* was able to hydrolyze 68% of the glycosides available (final concentration: 35 μM glycosyl-glucose). However, no detectable activity was found in *K. apiculata* or *C. parapsilosis*. Rosi *et al* [38] found that the extent of the glycoside hydrolytic ability of *Debaryomyces hansenii* was grape cultivar-dependent. The grape glycosides may have been present as further substituted disaccharides. In this case, the cellular β-glucosidase could not liberate the aglycones until the terminal sugars were removed. Therefore, the ability of *A. pullulans* to hydrolyze the Viognier glycosides may have been due to its arabinosidase and rhamnosidase activities. Utilization of a natural substrate by *Brettanomyces* cultures was not analyzed due to the lack of detection of arabinosidase or rhamnosidase activities in these yeasts in the first portion of the study (Table 2). The

use of a natural substrate illustrated the potential of *A. pullulans* to hydrolyze grape glycosides.

Further investigation regarding culture conditions in both the yeast and bacteria are warranted. Aerobic conditions, lack of ethanol, elevated pH and temperature, and removal of end product (glucose inhibition) could enhance microbial enzyme activity [37], but these are not common fermentation parameters. Feedback inhibition through glucose concentrations less than 0.5% (w/v) is a common constraint with production of some hydrolytic enzymes [25,37]. Some grape enological yeast enzymes are more resistant to glucose inhibition than others [3,13,15]. The optimum glucose concentration for *D. hansenii* was 2–8% [39]. *Candida wickerhamii* retained 44% of its activity in grape juice (500 mM glucose) [25]. In this study, glucose inhibition was avoided by the use of arbutin as the carbon source in growth media. However, the arbutin with its β-D bond may have failed to induce production of α-L-arabinofuranosidase or α-L-rhamnopyranosidase.

Grape β-glucosidases can exhibit a 60% loss of activity at ethanol concentrations of 3.5% [3,22]. However, many fungal and yeast β-glucosidases are not inhibited by the concentrations of ethanol in table wine [3,13,31]. Among the documented species showing ethanol-stable glycosidases are *Hanseniaspora vineae* [45], *Dekkera intermedia* [4], and *Candida molischiana* [20]. Guegen *et al* [21] demonstrated that β-glucosidase from *Candida entomophila* was stimulated by alcohol up to a concentration of 3.5%, but was inhibited at higher concentrations, likely due to protein denaturation. Permberton *et al* [33] hypothesized this could be due to a glycosyl transferase activity of the enzyme. Ethanol acts as an acceptor for the intermediary glycosyl cation and has better nucleophilic character than water. Further research should focus on the influence of a model wine environment on hydrolytic activities.

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