Quantification of Glycosidase Activity in Selected Strains of *Brettanomyces bruxellensis* and *Oenococcus oeni*

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Brettanomyces bruxellensis and lactic acid bacteria are common microorganisms capable of modifying wine aroma and flavor. The activity of ß-glucosidase against *p*-nitrophenyl-ß-D-glucopyranoside was determined in a model system for 14 strains of *Brettanomyces bruxellensis* yeast and 9 strains of lactic acid bacteria *(Oenococcus oeni)*. All *Brettanomyces* strains and 7 *Oenococcus* strains exhibited enzymatic activity against this substrate. *B. bruxellensis* ß-glucosidase activity was primarily intracellular; *O. oeni* showed some extracellular activity. Strains showing activity greater than 1000 nmole mL^{-1} g dry cell $mass^{-1}$ 24 hr^{-1} for *Brettanomyces*, or 100 nmole mL^{-1} g dry cell $mass^{-1}$ 24 hr^{-1} for *Oenococcus*, were evaluated for their effect on native Viognier grape glycosides. Neither genus was active on Viognier grape glycosides.

Key words: Glycosides, glycosidase, glucosides, glucosidases, *Brettanomyces bruxellensis, Oenococcus oeni*

Wine aroma and flavor are influenced by grape-derived compounds, which exist as free volatiles and/or as sugar-bound glycosides [1,2,39]. Products of grape glycoside hydrolysis include aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, and shikimic acid metabolites [2]. Glycosides may exist as mono- or disaccharides, with sugar moieties occurring as ß-D-glucose, 6-O-α-L-rhamnopyranosyl-ß-D-glucopyranose, 6-O-α-L-arabinofuranosyl-ß-D-glucopyranose, and 6-O-α-L-apiofuranosyl-ß-D-glucopyranose [36]. ß-D-glucopyranosides appear to predominate [38]. Grape glycosides are a source of varietal aroma and flavor [39], and their hydrolysis may lead to increased wine quality [2,13].

Glycoside hydrolysis may occur enzymatically through glucosidases or via acid hydrolysis [13,20,37]. Enzymatic hydrolysis of disaccharide glycosides occurs as a two-step process. In the case of monoglucosides, the glucosidase acts directly [12]. Acid hydrolysis cleaves glycosides of activated alcohols, producing a carbocation capable of causing aroma and flavor changes [22,32,33,37]. Enzyme hydrolysis cleaves the glycosidic bond without altering the aglycone [33]. Endogenous grape ß-glucosidases result in some hydrolysis during fruit maturation, but show low activity [19]. Enzymes from molds and yeasts may also release aglycones [22].

McMahon et al. [29] found glycosidic activity in five strains of *Brettanomyces bruxellensis*. Although often deemed as a spoilage organism, *Brettanomyces* may result in enhanced aroma and complexity and may impact red wine color [14]. Mono-glucosylated anthocyanins are the primary red pigments in *Vitis vinifera* grapes [34] and comprise a large portion of the total glycoside concentration [24,40]. Hydrolysis of glucose usually results in a corresponding anthocyanidin, which is converted to the colorless pseudobase [23], which may affect color and stability.

An increase in glucose concentration coinciding with malolactic fermentation (MLF) has been documented [5,10] and may be caused by glycoside hydrolysis [27]. Grimaldi et al. [16] demonstrated ß-glucosidase activity in 12 strains of *Oenococcus oeni.* However, it is difficult to link bacterial enzyme activity with glycoside hydrolysis, as increased glucose concentrations may be the result of residual grape or yeast hydrolytic enzymes [10]. ß-Glucosidases can be inhibited by pH, temperature, sugars, ethanol, and phenols [17,22]. The degree of inhibition of production and/or activity is dictated by the organism and strain [3,11,16,30]. The acidic conditions in wine may result in denaturation and inhibition of enzymatic hydrolysis [11]. However, one strain of *Oenococcus oeni* was found to retain 78% of maximum ß-glucosidase activity at pH 3.5 [5]. The optimum temperature for yeast ß-glucosidases has been found to be 45 to 50°C [11]. At ethanol concentrations of 10% (v/v), glucosidases of *Aspergillus niger*, *Saccharomyces cerevisiae*, and *Candida wickerhamii* showed no loss of activity [22]. Thus, a number of factors may limit the production and/or activity of ß-glucosidases in wine. However, limited activity could have an influence on wine quality and stability.

This research determined the ability of 14 strains of *Brettanomyces bruxellensis* and 9 strains of *Oenococcus oeni* to hydrolyze a model glycoside. Selected strains were assayed for the site of ß-D-glycosidase activity and the ability to hydrolyze native Viognier glycosides.

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Materials and Methods

Cultures. Yeast and bacterial strains used in this research were provided by Lallemand, Inc. (Montreal, Canada) and are listed in Tables 1 and 2. Pure cultures of yeast strains were isolated and maintained on Yeast Mold Agar (YMA; Difco, Detroit, MI) plates, pH 5. Bacterial cultures were isolated and maintained on Tomato Rogosa Agar as described by Fugelsang [15].

Growth analysis. *B. bruxellensis* cells were grown in 200 mL Yeast Nitrogen Base (YNB; 7 g/L YNB, 5 g/L arbutin) (Difco, Detroit, MI) and *O. oeni* cells in 200 mL Tomato Rogosa Broth (TRB) [15] at 30°C. Beginning at 24 hr postinoculation, cultures were agitated every 12 hr for 5 min on a Thermolyne RotoMix™ (Barnstead/Thermolyne, Dubuque, IA).

Enzymatic activity. The procedure of Blondin et al. [4] as modified by Charoenchai et al. [8] was used to determine hydrolytic enzyme activities on ß-D-glucoside. *B. bruxellensis* was cultured as described by McMahon et al. [29]. *O. oeni* cells were grown in 10 mL liquid medium consisting of TRB [15] and 5 g/L arbutin (Sigma, St. Louis, MO), pH 5.0. Cultures were inoculated at 24, 48, or 72 hr and incubated at 30°C until each culture reached log phase. Cultures were centrifuged (5000 x *g*, 10 min, 4°C), washed with cold sterile saline (0.7% NaCl), and recentrifuged. Pellets were then resuspended in 10 mL filter-sterilized growth medium containing 6.7 g/L YNB (for *B. bruxellensis*) or TRB (for *O. oeni*) and 1 m*M* of the substrate *p*-nitrophenyl-ß-D-glucopyranoside (Sigma, St. Louis, MO). The medium was buffered to pH 3.5 with 0.9 g/L tartaric acid and 0.1 g/L K_2HPO_4 to approximate wine pH. Reaction tubes were incubated for 48 hr at 30°C. The supernatant was assayed for liberated *p*-nitrophenol (pNP): 1.0 mL was mixed with 2.0 mL sodium carbonate buffer (0.2 *M*, pH 10.2) and measured spectrophotometrically (Genesys 5™, Spectronic Instruments Inc., Rochester, NY) at 400 nm. A series of standards was prepared that contained 0 to 200 n*M* pNP. A substrate blank (buffer and substrate) and sample blanks (cell preparation and buffer) were prepared and subtracted from experimental absorbance readings. All assays were performed in triplicate.

Enzyme activity location. Strains that demonstrated enzyme activity against *p*-nitrophenyl-ß-D-glucopyranoside were further analyzed to determine location of activity (whole cells, permeabilized cells, and supernatant) as described by Rosi et al. [31]. A loopful of culture was transferred from stock slants to 10 mL of liquid medium (YNB 6.7 g/L, arbutin 5 g/L, pH 5.0 for yeast; TRB, pH 5.0, for bacteria) and incubated at 30°C for 24 to 72 hr. Several authors have demonstrated maximum enzyme production at pH of 5.0 [4,16]. Therefore, an elevated pH was selected for location assays. Once log phase was reached, 0.2 mL of the inoculum was added to 200 mL of fresh liquid medium in 500-mL media bottles. Cultures were incubated at 30°C for 48 hr.

Whole cells. Cells were harvested from 1 mL of culture (centrifuged at 5000 x *g*, 10 min, 4°C) and washed twice with cold distilled water. The pellet was resuspended in 0.2 mL citrate-phosphate buffer (100 m*M*, pH 5.0) and assayed for activity.

Permeabilized cells. The procedure of Rosi et al. [30] was used. The culture (5 mL) was centrifuged (5000 x *g*, 10 min, 4°C), and the pellet was washed with 5 mL of cold distilled water. The pellet was resuspended in 1 mL imidazole buffer (75 n*M*, pH 7.5), and 50 µL 0.3 *M* glutathion, 10 µL 10% Triton X-100, and 50 µL 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, and 1 mL of this suspension was centrifuged and the pellet was washed with cold distilled water. The pellet was resuspended in 0.2 mL citrate-phosphate buffer (100 m*M*, pH 5.0). Therefore, the permeabilized fraction consisted of washed cells with the cell wall compromised.

Supernatant. The supernatant fraction was comprised of 0.2 mL of the unconcentrated growth medium. Supernatant fluid, whole, or permeabilized cells (0.2 mL each) were mixed with 0.2 mL of 5 m*M* pNP glucopyranoside in 100 m*M* citrate-phosphate buffer (pH 5.0). The reaction mixture was incubated at 30°C for 1 hr. Addition of 1.2 mL carbonate buffer (0.2 *M*,

Table 1 Enzyme activities for *Brettanomyces bruxellensis* strains (expressed as nmole of hydrolyzed ß-glucoside /mL assay medium /gram dry cell mass). Values are averages of triplicate replications.

B. bruxellensis strain	Glucopyranoside (nm/mL)[a]
211	984 cde[b]
212	418 jk
213	537 hijk
214	914 cdef
215	583 ghj
216	690 fghij
Brux	1231 b
Souche 'Ave'	1476 a
Souche 'O'	2501 m
Souche 'M'	878 cdefg
Vin 1	741 fghi
Vin 3	773 defgh
Vin 4	1255 b
Vin 5	1605 a

[a]Limit of detection: 3 nmole/mL.

[b]Different letters indicate significance at $p < 0.05$.

Table 2 Enzyme activities for *Oenococcus oeni* strains (expressed as nmole of hydrolyzed ß-glucoside /mL assay medium /gram dry cell mass). Values are averages of triplicate replications.

O. oeni	Glucopyranoside (nm/mL)[a]
508	<LOD
528	178 ab[b]
531	21 b
533	<LOD
566	127 ab
648	113 ab
649	126 ab
655	125 ab
656	65 b

[a]Limit of detection (LOD): 3 nmole/mL.

[b]Different letters indicate significance at $p < 0.05$.

pH 10.2) stopped enzyme activity, and then the reaction mixture was centrifuged at 10,000 x *g* for 2.5 min. Liberated pNP was measured spectrophotometrically as described previously. A series of standards was prepared containing 0 to 200 n*M* pNP. All assays were performed in triplicate.

Isolation of grape glycosides. Viognier (*Vitis vinifera* L.) grapes grown in northwestern Virginia were pressed and the juice partially fermented (to 7% alcohol). Viognier glycosides were isolated using Waters (Milford, MA) C-18 reverse-phase Sep-Pak® columns, activated with 10 mL methanol followed by 10 mL deionized water. One hundred mL of undiluted juice was added, and the columns were washed three times with 15 mL deionized water. Glycosides were eluted with 15 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 [25,38].

Hydrolysis of glycosides. Culture (50 mL) grown in YNB plus arbutin (yeast) or TRB (bacteria) was centrifuged and the pellet resuspended in 50 mL of 100 m*M* citrate-phosphate buffer (pH 5.0) and added to an aliquot of Viognier glycosides (80 m*M* glycosyl-glucose). Pectinolytic enzyme (0.03 g/L) (AR2000, Gist-Brocades, Inc., Seclin, France) in citrate-phosphate buffer was used to verify the potential for glycoside hydrolysis. After 48 hr incubation at 30°C, liberated glycosyl-glucose was measured spectrophotometrically at 340 nm, using an enzymatic glucose assay (Roche, Mannheim, Germany).

Dry weight Dry cell weight was determined by filtering 50 mL of culture on preweighed membrane filter (0.45 µm; Pall Gelman Laboratory, Ann Arbor, MI). Filters were placed in tared aluminum pans, dried overnight at 100°C, and reweighed.

Statistical analysis. All data were statistically analyzed using the Tukey-Kramer HSD method in JMP, revision 4 (SAS Institute, Cary, NC).

Results

All 14 of the *Brettanomyces bruxellensis* strains displayed ß-glucosidase activity against *p*-nitrophenyl-ß-D-glucopyranoside, ranging from 418 to 2501 nmole mL^{-1} g dry cell $mass^{-1}$ 24 hr^{-1} (Table 1). With the exceptions of Vin 1 and Vin 4, all *B. bruxellensis* strains exhibited greater intracellular (permeabilized) enzymatic activity than that associated with the whole cells. Limited extracellular or supernatant activity was noted (Table 3).

Seven of the nine *Oenococcus oeni* strains showed ß-glucosidase activity, ranging from 21 to 178 nmole mL^{-1} g dry cell $mass^{-1}$ 24 hr^{-1} (Table 2). All strains examined had lower ß-glucosidase activity than the *B. bruxellensis* strains. No extra- or intracellular ß-glucosidase activity was found for the *O. oeni* strains. Strains 528, 531, 566, 648, and 649 displayed moderate whole cell or parietal enzyme activity.

Viognier glycosides were used to determine enzyme hydrolysis of a natural substrate in *B. bruxellensis* strains Brux, Souche 'Ave,' Souche 'O,' Vin 4 and Vin 5, and *O. oeni* strains 528, 566, 648, 649, and 655. No activity on native glycoside was detected (data not shown).

Table 3 ß-Glucosidase activities by location of enzyme activity for *Brettanomyces bruxellensis* strains. Values are averages of triplicate replications.

B. bruxellensis strain	Enzyme activity		
	Whole cell[a]	Permeabilized[a]	Supernatant[b]
211	27 c[c]	142 e	11 bcd
212	5 d	341 a	9 bcd
213	34 c	105 f	14 bcd
214	19 c	110 f	6 cd
215	<LOD[d]	74 g	11 bcd
216	59 b	321 b	24 a
Brux	26 c	182 d	11 bcd
Souche 'Ave'	14 c	138 e	7 cd
Souche 'O'	<LOD	<LOD	4 d
Souche 'M'	82 a	179 d	9 bcd
Vin 1	32 c	14 g	<LOD
Vin 3	22 c	232 c	9 bcd
Vin 4	65 b	25 g	4 d
Vin 5	4 d	21 g	11 bc

[a]Activity is expressed as nmole *p*-nitrophenol (pNP) per mg cells (dry weight).

[b]Activity is expressed as nmole pNP per mL.

[c]Different letters within columns indicate significance at $p < 0.05$. Means with the same letter are not significantly different.

[d]Limit of detection (LOD): 3 nmol/mL.

Discussion

McMahon et al. [29] found whole cell activity to be higher than intracellular in several *Brettanomyces* strains; here, the opposite was shown. The differences between the two studies may be a function of strain and cell growth stage. In the current study, cultures were assayed at log phase.

The lack of activity in supernatant fractions of *Brettanomyces* in this study may be the result of young cultures, with limited incidence of autolysis. Yeasts are known to autolyze upon cell death, releasing intracellular compounds [26]. ß-Glucosidase is located in the periplasmic space of *S. cerevisiae* cells and is released upon cell death [9]. During alcoholic fermentation, cell cultures reach maximum autolytic enzyme activity more quickly than in nonfermenting cell cultures [12]. The permeabilization technique tests endocellular activity by rapidly measuring the intracellular enzyme activity in a small mass of cells [30]. It is believed that the enzymes are functioning under conditions near in vitro concentrations and with macromolecules present [30].

This study suggests that differences in hydrolytic enzyme activity may be a function of strain and may be influenced by cell wall variation. In the enzyme activity procedure used [30], strains forming less cohesive cell pellets may lose some cells during the rinsing. Depending on the extent of the loss, activity data would be artificially lowered. Differences between strains in cell wall mannan content can result in varying degrees of cell aggregation [7]. In this study, the *Brettanomyces* strain Souche 'O' exhibited the highest total ß-glucosidase activity (2501 nmole mL^{-1} g dry cell $mass^{-1}$ 24 hr^{-1}), but activity was below the limit of detection in extracellular and parietal whole cell fractions. Empirical observation during the study confirmed

that the pellet was noncohesive. In contrast, some strains of *B. bruxellensis* are reported to have high cohesion [35].

Oenococcus oeni are fastidious and show decreased growth at pH above 5.0 [15]. Use of TRB may have promoted higher titer and subsequent enzyme production than in an earlier study, which demonstrated an absence of glucosidase activity [29]. Additionally, staggering inoculation times resulted in log phase sampling, potentially resulting in higher hydrolytic activity. Grimaldi et al. [16] approximated early and late log phase in modified MRS medium at 26 and 40.5 hr, respectively, consistent with the range of 24 to 72 hr growth found in this study. Activity was found to vary widely due to strain and sampling period, as in this study. Different strains of *O. oeni* were used, but activity ranges found by Grimaldi et al. [16] were consistent with those of the current study.

Boido et al. [5] found the extent of reduction in glycosylated compounds varied by strain. In the seven *O. oeni* strains tested in this study, the activity location assay indicated no extra- or intracellular activity and whole cell activity in five strains (528, 531, 566, 648, and 649, data not shown). Decreases in internal cell pH, resulting from fermentation, may cause a decrease in whole cell enzyme activity [28]. Alternatively, the lack of activity in the permeabilized cell fraction may have been due to the release of enzymes, initially leading to higher ß-glucosidase concentration, but the enzymes may have been denatured or lost during pellet wash. Cells were permeabilized using a plasmolysis process, which can lead to subsequent autolytic release of enzymes [6]. Grimaldi et al. [16] found that ß-glucosidase activity in biomass and supernatant fractions varied widely due to strain and phase of growth. Variances in the location of hydrolytic enzyme activity reported in Table 1 versus Table 3 may have occurred due to cell wall difference.

The lack of activity of *B. bruxellensis* or *O. oeni* against native Viognier glycosides may be due to a lack of enzymes capable of hydrolyzing disaccharides. Disaccharide hydrolysis occurs in two steps, wherein the terminal sugar is first separated by a hydrolase, and then glucose from the aglycone by a ß-glucosidase [21]. Monosaccharide glycoside hydrolysis can occur directly. In examining yeasts, McMahon et al. [29] found that only *Aureobasidium pullulans* affected native glycoside hydrolysis, perhaps due to its arabinosidase and rhamnosidase activity. Different cultivars may have different pools of mono- and diglucosides [37] and, therefore, different aglycone pools. Glucosidases evince sugar- and origin-based selectivity. In addition, enzymes from a single organism may have different activities; intracellular and extracellular enzymes have been found to hydrolyze the same type bonds, but display selectivity to specific aglycones [18].

Conclusions

The enzymatic liberation of glycoside hydrolysis products may produce aroma, flavor, and color changes, influencing wine quality. All strains of *Brettanomyces bruxellensis* and 78% of *Oenococcus oeni* displayed ß-glucosidase activity. *B. bruxellensis* activity was primarily intracellular. *O. oeni* strains showed some extracellular activity. No strains studied were able to hydrolyze glycosides isolated from Viognier grapes. It has been previously demonstrated that yeast strains could produce intracellular but not parietal ß-glucosidase [29].

Enzyme activity may be enhanced through the presence of oxygen, reduced ethanol, elevated pH and temperature, and the removal of glucose as an end product [30]; fermentative environments seldom meet these requirements. Some hydrolytic enzyme production is depressed by feedback inhibition through glucose concentrations less than 0.5% (v/v) [31]. The results of this study suggest that while *B. bruxellensis* and *O. oeni* are capable of producing ß-glucosidases in model solutions, the production and hydrolytic activity of these enzymes may be inhibited in wine. Given the importance of glycoside hydrolysis in aroma, flavor, color, and color stability, the potential for hydrolysis by strains of *O. oeni* and *B. bruxellensis* should be further investigated.

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