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Full Length Research Paper

Abiotic effects on *β*-D-glucosidase activity of two typical (*Oenococcus oeni)* strains in China

Richard Lim, Yang Lee and Feng Yamaha

Center for Viticulture and Enology, College of Food Science & Nutritional Engineering, Tsinghua University, P.O. Box 400165, Beijing, China.

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***β*-D-Glucosidase is one of the most interesting glycosidases for hydrolysis of glycoconjugated precursors to release active aromatic compounds in musts and wines. SD-2a and 31MBR are two typical *Oenococcus oeni* strains widely used in winemaking in China. Up to now, there is still limited information regarding the *β*-D-glucosidase activity of the two strains. *β*-D-Glucosidase activity of the two strains was localized and influence of abiotic stress on the enzyme activity was conducted using synthetic substrate in the present study. Both SD-2a and 31MBR possessed *β*-D-glucosidase activity, activity occurring in whole cells and permeabilized cells but not in the culture supernatant. Whole cells of SD-2a showed higher enzyme activity than that of 31MBR, while 31MBR exhibited higher permeabilized cells activity. *β*-D-Glucosidase from the two strains remained high activity after both bacteria were stressed under winelike conditions for two hours, however, activity decreased sharply when the bacteria were stressed at 45°C, pH 2.5 or ethanol concentration of 16%. SD-2a proved potential for aroma improvement in winemaking, as the whole cells showed high *β*-D-glucosidase activity under winelike conditions.**

Key words: *Oenococcus oeni, β*-D-glucosidase, localization, abiotic stress.

INTRODUCTION

Wine fermentation is a complex process driven by microorganisms such as yeasts and lactic acid bacteria. Malolactic fermentation (MLF) taking place after alcoholic fermentation, is the bacterially driven decarboxylation of L-malic acid to L-lactic acid and carbon dioxide. In addition to decreasing wine sourness via the metabolism of L-malic acid during MLF process, MLF can also lead to flavor modification and improvement of wine quality (Bartowsky et al., 2002; Toit et al., 2010; Gagné et al., 2011). *Oenococcus oeni* is the main lactic acid bacteria to conduct MLF in virtually all red wine and an increasing number of white wine (Bartowsky and Borneman, 2011; Olguin et al., 2011). Over the last decade, it has been increasingly recognized that *O. oeni* exhibits a broad range of secondary metabolic activities during MLF, such as hydrolysis of glycosides, which are of great importance for the taste and flavor improvement of wine (Francis et al., 1999; Liu and Pilone, 2000; Liu, 2002; Matthews et al., 2004). The odorless glycosides remaining in newly made wine could be hydrolyzed enzymatically through glycosidases or via acid hydrolysis (Mansfield et al., 2002; Spano et al., 2005; Michlmayr et al., 2010a). Compared with acid hydrolysis, enzymatic hydrolysis without altering the aglycone is of considerable interest for enhancing natural aroma spectrum of wine during winemaking (Spagna et al., 1998; Mateo and Jimenez, 2000).

β-D-Glucosidase (*β*G) is one important glycosidases to hydrolyze glycosylated aroma precursors, releasing active aroma and flavor compounds during winemaking. Endogenous grape *β*G and fungal *β*G are reported to be strongly inhibited under wine conditions, especially by glucose and high acidity (Chassagne et al., 2005; Saguir et al., 2009). Thus the study of glycosidases from lactic acid bacteria for aroma release in winemaking is of great

*Corresponding author. E-mail: Proff_Rich01@yahoo.fr

interest.

*β*G activity in lactic acid bacteria, mainly *O. oeni*, was evidenced about 20 years ago in synthetic media (Guilloux-Benatier et al., 1993). Over the past decades, numerous studies have been conducted, providing evidence of the potential *β*G activity of *O. oeni* strains for flavor enhancement in wines (Gagné et al., 2011; Michlmayr et al., 2010a; Spano et al., 2005). It has been reported that possession of glycosidic activities is widespread and strain dependent among these strains preferably used for MLF in winemaking (Grimaldi et al., 2005). Usually, *β*G activities are affected by physicochemical factors such as pH, ethanol, and temperature, and the related work has been reported on wine lactic acid bacteria (Barbagallo et al., 2004; Grimaldi et al., 2005; Michlmayr et al., 2010b). The degree of inhibition on enzyme activity depends on organism and strains (Mansfield et al., 2002). However, limited information about the influence of abiotic stress, such as pH, ethanol and temperature stress, on the *β*G activity of *O. oeni* is available. Understanding whether and how *β*G activities are regulated by abiotic stresses is crucial for selecting starters to alter positively wine flavor during MLF.

O. oeni SD-2a and 31MBR are two important strains widely used during winemaking in China. SD-2a is one patent strain screened from spontaneous MLF wines of Yantai, Shandong Province, by College of Enology, NWUAF, China, while 31MBR is one commercial strain prevalent in China with an excellent performance in MLF. Up to now, little information about *β*G activity is available for the two strains.

The present study aimed to assess the *β*G activity of *O. oeni* strains SD-2a and 31MBR and investigated the influence of abiotic stress on the enzyme activity.

MATERIALS AND METHODS

Bacterial strains and cultivation

O. oeni strains SD-2a and 31MBR, stored in our laboratory, were used in the study. Both strains were cultivated in growth medium acidic tomato broth (ATB) containing, glucose 10 g/L, yeast extract 5 g/L, peptone 10 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $MnSO_4 \cdot 4H_2O$ 0.05 g/L, cysteine/HCl 0.5 g/L and tomato juice 250 mL/L. The medium pH was adjusted to 4.8 with KOH. Bacterial cultures were prepared by inoculating 1% (v/v) of precultures into 100 mL of ATB medium and incubated at 25°C until OD_{600nm} reached to about 1.90 (the late exponential growth phase).

Determination of enzyme activity

The procedure of Mansfield et al. (2002) with modifications was used to determine *β*G activity of SD-2a and 31MBR. Bacterial growth was monitored by measuring the OD_{600nm} until the end of exponential growth phase (about 80 h and 40 h for SD-2a and 31MBR respectively). Then 10 mL cultures were centrifuged (5000 g, 10 min, 4°C), washed with cold sterile saline (0.7% NaCl), and recentrifuged. Each pellet was then resuspended in 10 mL filter-sterilized ATB medium containing 1 mM of substrate *p*-nitrophenyl-*β*-D-glucopyranoside (Sigma, USA). Reaction tubes were incubated for 48 h at 25°C. After incubation, the cultures were centrifuged at 10000 g for 10 min at 4ºC to remove cells. The supernatant was assayed for liberated *p*-nitrophenol (*p*-NP): 1.0 mL was mixed with 2.0 mL of 1 M Na_2CO_3 buffer and measured spectrophotometrically (a Beckman DU-800 spectrophotometer) at 400 nm. A series of standards were prepared that contained 0 to 200 µM *p*-NP with intervals of 40 µM (ε_{400} = 6000 L/mol/cm). A blank (medium supernatant with substrate) was prepared and subtracted from experimental absorbance readings. One unit of enzyme activity was defined as µmols of *p*-NP liberated per hour per milligram of dry cell weight.

Enzyme activity location

The strain which demonstrated substantial *β*G activity was further analyzed to determine the generalized location of enzyme activity (whole cells, permeabilized cells, and culture supernatant) as described by Mansfield et al. (2002). Based on the previous studies, maximum enzyme activity was at pH 5.0 (Grimaldi et al., 2000), therefore, this pH was selected for location assays. Bacteria were cultured in ATB medium until the end of exponential growth phase as described earlier.

Whole cells

Cells were harvested from 1 mL of culture (centrifuged at 5000 g, 10 min, 4°C) and washed twice with cold sterile saline (0.7% NaCl). The pellet was resuspended in 0.2 mL of citrate-phosphate buffer (100 mM, pH 5.0) and then assayed for activity.

Permeabilized cells

5 mL culture was centrifuged (5000 g, 10 min, 4°C), and the pellet was washed with 5 mL of cold sterile saline (0.7% NaCl). The pellet was resuspended in 1 mL of imidazole buffer (75×10^{-3} mmol/L, 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, whole cells, or permeabilized cells (0.2 mL each) were mixed with 0.2 mL of 5 mM solution of *p*-NP glycopyranoside in 100 mM citrate-phosphate buffer (pH 5.0). The reaction mixture was incubated at 30°C for 1 h. 1.2 mL of carbonate buffer (1 M, pH 10.2) was added to stop enzyme activity, and then the reaction mixture was centrifuged at 10000 g for 5.0 min at 4°C. Liberated *p*-NP was measured spectrophotometrically as described previously. One unit of enzyme activity was defined as µmols of *p*-NP liberated per minute per milligram of cell dry weight.

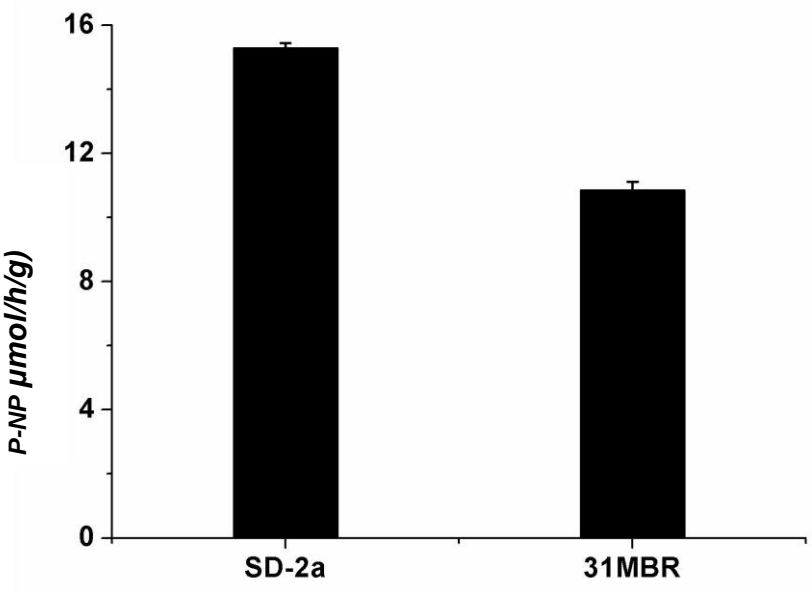

Figure 1. Enzyme activities of *O. oeni* strains SD-2a and 31MBR (expressed as µmols of *p*-NP liberated per hour per milligram of cell dry weight). Values are averages of duplicates.

Influence of abiotic stress on enzyme activity

The abiotic stress treatments were conducted according to the method described previously with some modifications (Spano et al., 2005). Cells at the end of exponential growth phase were harvested from 10 mL culture (centrifuged at 5000 g, 10 min, 4°C) and used for each treatment.

Temperature stress treatment

Prepared cells were resuspended in 10 mL fresh ATB medium, and then incubated at 5, 15, 25, 35 and 45°C respectively for 2 h.

Acid stress treatment

Prepared cells were resuspended in 10 mL fresh ATB medium adjusted to pH 2.5, 3.0, 3.5, 4.0 and 4.5 with HCl/KOH respectively, then incubated at 25°C for 2 h.

Ethanol stress treatment

Prepared cells were resuspended in 10 mL fresh ATB medium containing ethanol concentrations of 0, 4, 8, 12 and 16% respectively, then incubated at 25°C for 2 h.

Based on the result of enzyme localization, permeabilized cells of SD-2a and 31MBR as well as whole cells of SD-2a from each treatment were assayed for enzyme activity with the method described earlier. The condition of the lab is temperature 25°C and humidity 55%RH. All assays were performed in duplicate and were repeated at least three times.

Statistical analysis

Statistical analysis were performed using the software SAS (SAS Institute, Cary, NC, USA). Values were analysed statistically by Turkey's multiple comparison test. Mean values were considered significantly different when $P < 0.01$.

RESULTS

As shown in Figure 1 (Table 1), both SD-2a and 31MBR displayed *β*-D-glucosidase activity against *p*-nitrophenyl-*β*-D-glucopyranoside in growth medium ATB, SD-2a showing higher enzyme activity than 31MBR. Based on this result, both strains were further analyzed for the location of *β*G activity. As shown in Figure 2 (Table 1), fairly low enzyme activity was detected in supernatant for both strains; permeabilized cells of 31MBR exhibited greater enzyme activity than that of the whole cells; for SD-2a whole cells showed enzyme activity almost equivalent to that of permeabilized cells. Similar results were also observed by Barbagallo et al. (2004) that supernatant shows little activity and for whole cells enzyme activity increases greatly after sonication treatment. This would appear to suggest that *β*G of the two strains was not an extracellular type but mainly intracellular form. It was also noted that permeabilized cells of 31MBR showed much higher enzyme activity than that of SD-2a (Table 1), implying 31MBR possessed greater total intracellular enzyme activity than SD-2a. While SD-2a showed higher whole cells activity than 31MBR (Table 1), this could be favorable for winemaking.

The influence of abiotic stress on enzyme activity was conducted on permeabilized cells of both strains as well as whole cells of SD-2a since these three samples possessed high activity in the enzyme location assay.

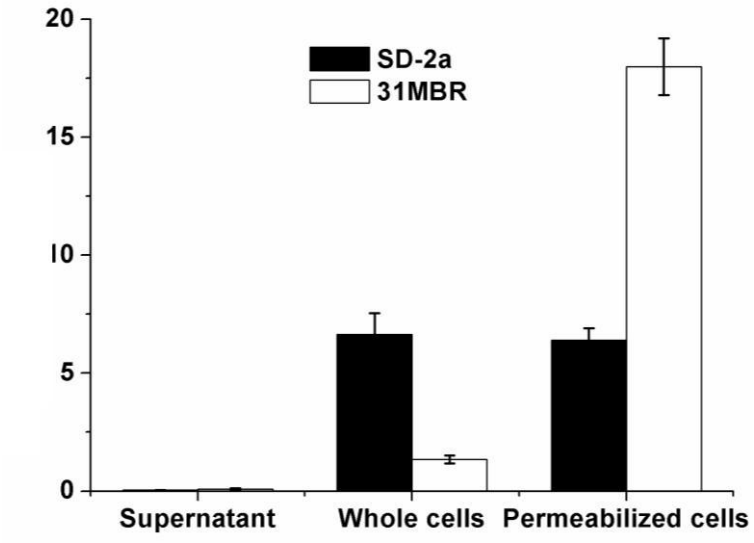

Figure 2. Enzyme localization of *O. oeni* strains SD-2a and 31MBR (enzyme activity expressed as µmols of *p*-NP liberated per minute per milligram of cell dry weight). Values are averages of duplicates.

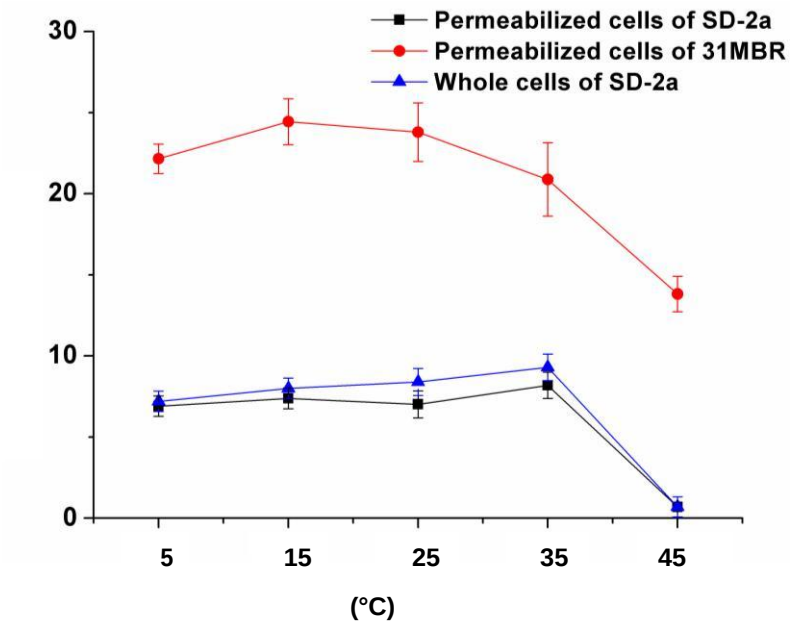

Figure 3. Influence of temperature stress ranging from 5 to 45ºC on *β*-D-glucosidase activity of *O. oeni* strains SD-2a and 31MBR. One unit of enzyme activity is defined as µmols of *p*-NP liberated per min per gram of cell dry weight.

Temperature stress showed moderate impact on *β*G activity of both strains, until strains treated at 45°C enzyme activity reduced by half for 31MBR and almost no enzyme activity remained for SD-2a (Figure 3) (Table 2). The influence of ethanol stress was shown in Figure 4 (Table 2). SD-2a kept high activity when treated at ethanol concentration between 0 to 12%, after treatment at 16% almost no activity can be detected. As for 31MBR, with ethanol concentration increased, enzyme activity exhibited a downward trend, at concentration of 12% about 2/3 of ethanol free activity was left and at 16% still 1/3 remained (Table 2). As for the influence of acid

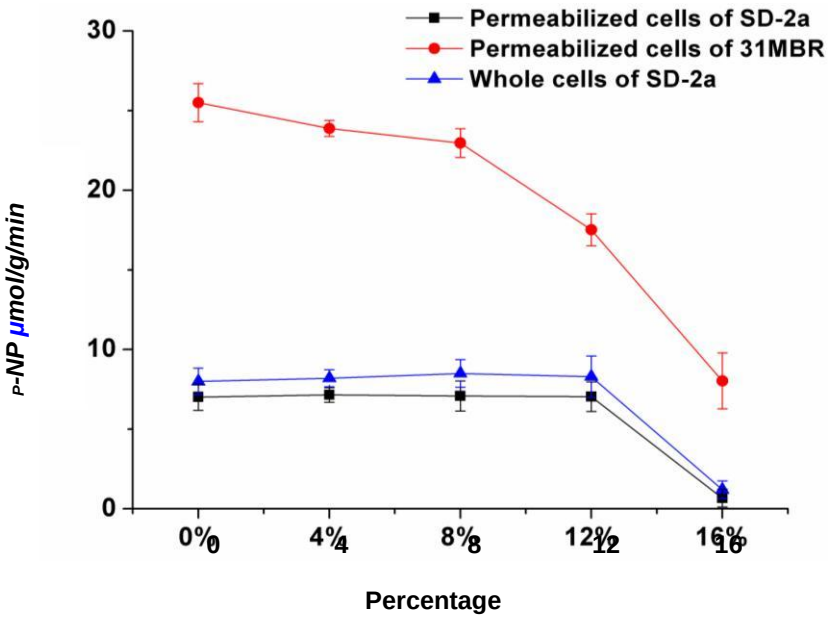

Figure 4. Influence of ethanol stress ranging from 0% to16% on *β*-D-glucosidase activity of *O. oeni* strains SD-2a and 31MBR. One unit of enzyme activity is defined as µmols of *p*-NP liberated per min per gram of cell dry weight.(1A 2B 2B 2 2).

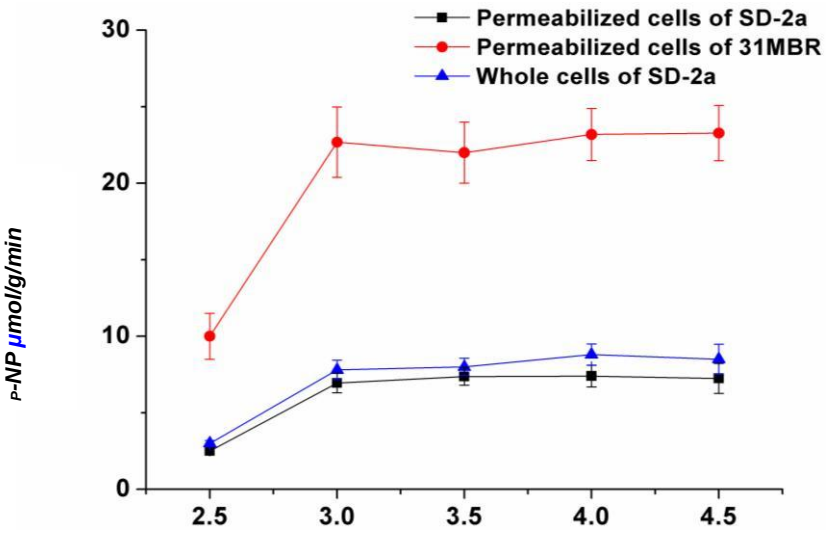

Figure 5. Influence of acid stress pH ranging from 2.5 to 4.5 on *β*-D-glucosidase activity of *O. oeni* strains SD-2a and 31MBR. One unit of enzyme activity is defined as µmols of *p*-NP liberated per min per gram of cell dry weight.

stress, almost no difference between the enzyme activities treated at different pH values was observed for both strains, except stressed at pH 2.5, enzyme from both strains showed decreased activity (Figure 5) (Table 2). Meanwhile, same trends were observed for permeabilized cells and whole cells of SD-2a and whole cells showed higher activity than permeabilized cells (Figures 3, 4 and 5).

DISCUSSION

The present study focused on *β*G activity of *O. oeni* strains SD-2a and 31MBR. For the preliminary research about *β*G activity of the two strains, synthetic substrate *p*-nitrophenyl *β*-D-glucopyranoside was used in present study. Some authors pointed out natural aroma precursors were necessary for an adequate evaluation of

Table 1. Data of Figures 1 and 2.

Strains	Enzyme activity	
	SD-2a	31MBR
Enzyme location	15.3 ± 0.1[a]	10.8 ± 0.3[b]
Supernatant	0.0 ± 0.0[a]	0.0 ± 0.0[a]
Whole cells	6.6 ± 0.9[b]	1.3 ± 0.2[b]
Permeabilized cells	6.4 ± 0.5[b]	18.0 ± 1.2[c]

β-D-Glucosidase activity of *O. oeni* strains SD-2a and 31MBR, and the enzyme location of both strains. Data in the table are means± SE (standard deviation) of triplicate assays. Means with different letters are significantly different ($P < 0.01$).

the glycosidases potential of *O. oeni* strains (Gagné et al., 2011). Related work using natural substrate instead of synthetic substrate to assay *β*G activity of SD-2a and 31MBR is being done in our lab.

The profile of *β*G activity of *O. oeni* strains SD-2a and 31MBR, including enzyme location and influence of physicochemical factors on enzyme activity, has been reported by Li et al. (2012 in press). While in the present study, in order to assess *β*G activity of the two strains more reasonably, assay was conducted with different methods. Firstly, *β*G activity of the two strains was generally evaluated in growth medium ATB. Based on this result, enzyme localization was studied again through penetration treatment instead of sonication treatment. Finally, the influence of abiotic stress on enzyme activity was conducted.

Enzyme assay in growth medium showed that both SD-2a and 31MBR possessed *β*G activity, SD-2a showing higher activity than 31MBR, indicating both strains are potential for aroma enhancement during winemaking. This confirms the previous reports that possession of glycosidic activities is widespread among *O. oeni* strains and strain dependent (Mansfield et al., 2002; Grimaldi et al., 2005). In the enzyme localization assay, whole cells of SD-2a showed great enzyme activity. Capaldo et al. (2011a) reported this could be attributed to the presence of phosphoenolpyruvate dependent phospho transferase system (PEP-PTS). This explanation could well uphold the results reported that whole cells of bacteria showed high *β*G activity while low or no intracellular and extracellular activities were observed (McMahon et al., 1999; Michlmayr et al., 2010b). As for whole cells of 31MBR, low activity was observed. This may be a function of strain or influenced by cell wall variation. Strains forming less cohesive cell pellets may lead to low activity data during enzyme assay (Mansfield et al., 2002). Meanwhile, it should be noted that 31MBR possessed much higher total enzyme activity (permeabilized cells activity) than SD-2a. However, contrary result was observed in Figure 1. This could be explained by the result that whole cells of SD-2a showed greater activity than that of 31MBR. Thus it may be indicated that SD-2a is preferred to 31MBR for aroma enhancement in winemaking since whole cells with great *β*G activity could be used directly in application. These results of enzyme location coincide with and confirm previous report about SD-2a and 31MBR (Li et al., 2012 in press).

Notably, very low enzyme activity was observed in the supernatant of penetration treatment (data not shown), suggesting intracellular *β*G of both strains is insoluble. This is contrary to the previous report that *β*G of both strains is soluble (Li et al., 2012 in press). This difference could be attributed to the different treatment methods during enzyme localization assay; one is sonication treatment while another is penetration treatment. Spano et al. (2005) reported the presence of hydrophobic transmembrane domains in *β*-glucosidase and showed that is widespread among lactic acid bacteria. Thus the transmembrane localization may be true for *β*G of SD-2a and 31MBR. Further study will be conducted to confirm this deduction.

Wine is a medium with very harsh environmental conditions, which can hinder bacterial growth. In the present study, influences of pH, temperature and ethanol stresses on *β*G activity of SD-2a and 31MBR were evaluated. *β*G of both strains kept high activity until the bacteria were treated at 45°C or at ethanol concentration of 16% for two hours. Stress at low temperature tested showed no effect on the enzyme activity. Winemaking conditions classically are below 16% for ethanol concentration and temperatures vary from 10°C to a maximum of 45°C (Capaldo et al., 2011a), enabling *β*G of SD-2a and 31MBR to retain activity in application. The *β*G still remained high activity after both strains treated at pH 3.0, while the activity decreased sharply at pH 2.5. Typically wines range from pH 2.9 to 3.4 for whites and from pH 3.4 to 3.6 for reds (Capaldo et al., 2011b), a pH range across which *β*G of the two strains can remain high activity.

Under stress conditions, stress injury may adversely affect membrane-associated processes, following affect the enzyme inside (Spano and Massa, 2006). For the influence of stress on *β*G activity, no significant difference was observed between whole cells and permeabilized cells of SD-2a, indicating that abiotic stresses may affect the *β*G inside cells not through their action on cell wall or membrane. Both strains, especially SD-2a, whole cells of which showing considerable activity under winelike conditions, may be positive for aroma enhancement in winemaking.

In conclusion, both SD-2a and 31MBR possessed *β*G activity and it was mainly intracellular form. SD-2a possessed great whole cells activity, while 31MBR showed higher total enzyme activity. *β*G from the two strains remained considerable activity after they were stressed under winelike conditions for two hours. SD-2a proved promising for aroma enhancement in winemaking, as whole cells of which showed high *β*G activity after

Table 2. Data of Figures 3, 4 and 5.

Samples	Enzyme activity		
	Whole cells of SD-2a	Permeabilized cells of SD-2a	Permeabilized cells of 31MBR
Temperature			
5°C	6.9 ± 0.6^{a}	7.2 ± 0.6^{a}	22.1 ± 0.9^{a}
15°C	7.4 ± 0.6^{a}	8.0 ± 0.5^{a}	24.4 ± 1.4^{a}
25°C	7.0 ± 0.7^{a}	8.4 ± 0.8^{a}	23.8 ± 1.7^{a}
35°C	8.2 ± 0.8^{a}	9.3 ± 0.7^{a}	20.8 ± 2.1^{a}
45°C	0.7 ± 0.6^{b}	0.8 ± 0.6^{b}	13.8 ± 1.0^{b}
Ethanol			
0%	7.0 ± 0.8^{a}	8.0 ± 0.8^{a}	25.5 ± 1.2^{a}
4%	7.1 ± 0.4^{a}	8.2 ± 0.5^{a}	23.8 ± 0.5^{a}
8%	7.1 ± 0.9^{a}	8.5 ± 0.8^{a}	22.9 ± 0.9^{a}
12%	7.0 ± 0.9^{a}	8.3 ± 1.3^{a}	17.5 ± 1.0^{b}
16%	0.7 ± 0.5^{b}	1.2 ± 0.5^{b}	8.0 ± 1.5^{c}
pH			
2.5	2.5 ± 0.2^{a}	3.1 ± 0.2^{a}	10.0 ± 1.5^{a}
3.0	6.9 ± 0.6^{b}	7.8 ± 0.6^{b}	22.6 ± 2.3^{b}
3.5	7.3 ± 0.5^{b}	8.0 ± 0.5^{b}	21.9 ± 2.0^{b}
4.0	7.3 ± 0.7^{b}	8.8 ± 0.7^{b}	23.1 ± 1.7^{b}
4.5	7.2 ± 0.9^{b}	8.5 ± 0.9^{b}	23.2 ± 1.8^{b}

Influence of temperature, ethanol and pH stress on *β*-D-glucosidase activity of *O. oeni* strains SD-2a and 31MBR. Data in the table are means± SE (standard deviation) of triplicate assays. Means with different letters are significantly different ($P < 0.01$).

stressed under winelike conditions, an important consideration for wine industry. This study contributes to preliminary knowledge about *β*G activity of *O. oeni* strains SD-2a and 31MBR. It will provide some information to aid winemakers in selecting starter cultures for the improvement of wine quality. Further study focusing on the influence of SD-2a and 31MBR on aroma profile during MLF in real wine will be conducted.

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