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Assessment of β-D-Glucosidase Activity of Intact Cells of Two *Oenococcus oeni* Strains with Synthetic and Natural Substrates

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Abstract

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The β-D-glucosidase (βG) activity of intact cells of *Oenococcus oeni* SD-2a and 31MBR was assessed with synthetic and natural substrates, also its partial characterisation was investigated. Results showed that intact cells of both strains showed βG activity, SD-2a performing higher than 31MBR, against either synthetic or natural substrate. The high βG activity of SD-2a intact cells under wine-like temperature, pH, ethanol concentration, and glucose concentration could be of great interest for its utilisation in winemaking. SD-2a proved promising for aroma enrichment in winemaking.

Keywords: *Oenococcus oeni* strains; β-D-glucosidase activity; different substrates; partial characterisation

In addition to the initial impact that wine colour has on the perception of wine quality, it is aroma and flavour that have the greatest impact on consumers. The formation of wine aroma is a complex process, with a combination of many factors contributing and interacting throughout grape growth, maturation and winemaking (Bartowsky *et al.* 2011). 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 the wine sourness via the metabolism of L-malic acid, MLF can also lead to flavour modification and quality improvement (Toit *et al.* 2010; Gagne *et al*. 2011; Sumby *et al*. 2013). *Oenococcus oeni* is the main lactic acid bacterium to conduct MLF in virtually all red wines and an increasing number of white wines (Bartowsky *et al.* 2011; Olguin *et al.* 2011). During MLF, *O. oeni* strains can influence the aroma and flavour of wines by the production of volatile metabolites and the modification of aroma precursors derived from grapes and yeasts. It has been increasingly recognised that *O. oeni* exhibits a broad range of secondary metabolic activities during MLF, especially hydrolysis of glycosides, which plays a primary role in the expression of flavour characteristics of grape and wine (Mesas *et al*. 2012; Michlmayr *et al.* 2012a; Zapparoli *et al.* 2012; Dong *et al.* 2014).

β-D-Glucosidase (βG) is an important glycosidase to hydrolyse glycosylated aroma precursors, releasing active aroma and flavour compounds. Endogenous βG of grapes and fungal βG 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 glycosidase from lactic acid bacteria, especially *O. oeni* strains, for aroma release during winemaking is of great interest. Over the past decades, numerous investigations have been conducted, providing evidence of the potential βG activity of *O. oeni* strains for flavour enhancement in

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wines (Michlmayr *et al.* 2010a; Gagne *et al.* 2011; Fatima *et al.* 2012). Diverse *O. oeni* strains were tested with synthetic substrate to determine their capacity to release volatile compounds (McMahon *et al.* 1999; Grimaldi *et al.* 2000, 2005a; Gagne *et al.* 2011; Michlmayr *et al.* 2012a). It has been reported that the possession of glycosidic activities is widespread and strain dependent among *O. oeni* strains (Grimaldi *et al.* 2005a; Saguir *et al.* 2009; Michlmayr *et al.* 2012a). Moreover, some authors pointed out that during MLF, the final concentration of glycoside-derived volatile metabolites depended not only on the actual hydrolytic ability of individual strains, but also on the chemical structure of substrate (Boido *et al.* 2002; Ugliano *et al.* 2006). Thus assaying the βG activity with natural aroma precursors was necessary for an adequate evaluation of the glycosidase potential of *O. oeni* strains (Gagne *et al.* 2011). Related work of assaying the βG activity of *O. oeni* strains with natural glycosylated compounds isolated from grapes/wine and oak wood has also been reported (McMahon *et al.* 1999; Mansfield *et al.* 2002; D'Incecco *et al.* 2004; Ugliano *et al.* 2006; Bloem *et al.* 2008; Gagne *et al.* 2011).

O. oeni SD-2a and 31MBR are two important strains widely used during winemaking in China. SD-2a is a patent strain screened from spontaneous MLF wines of Yantai (Shandong Province, China), while 31MBR is a commercial strain with an excellent performance during MLF. The profile of βG activity from the two strains, including enzyme localisation and partial characterisation, has been reported (Li *et al.* 2012a, b). It showed that βG of the two strains was mainly an intracellular form, and intact cells of both strains also possessed βG activity. Intact cells possessing βG activity are of great importance for winemaking, since it is associated with aroma precursors directly during winemaking. However, up to now, not much information has been available about the βG activity of *O. oeni* SD-2a and 31MBR intact cells. In the present study, the βG activity of intact cells of the two strains was evaluated with synthetic and natural substrates, also its partial characterisation was investigated.

MATERIAL 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 acidic tomato broth (ATB) medium 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 about 1.90 (the late exponential growth phase).

Enzyme assay with synthetic substrate. The procedure described previously with modifications was used to determine βG activity of SD-2a and 31MBR (Mansfield *et al.* 2002). Bacterial growth was monitored by measuring the OD_{600nm} until the end of the exponential growth phase (about 80 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-sterilised ATB medium or ATB medium without glucose containing 1 mM of the substrate *p*-nitrophenyl-β-D-glucopyranoside (Sigma, Shanghai, China). Reaction tubes were incubated at 25°C for 48 hours. After incubation, the cultures were centrifuged at 10000 *g* for 10 min at 4°C 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 (Beckman DU-800 spectrophotometer; Beckman, California, USA) at 400 nm. A series of standards were prepared that contained 0 to 200 μM *p*-NP with intervals of 40 μM (ε_{400} = 18 000 l/mol/cm). A blank (medium supernatant with substrate) was prepared and subtracted from experimental absorbance readings. Dry cell weight was determined by filtering 50 ml of cultures on preweighed membrane filter (0.45 μm; Pall Gelman Laboratory, Ann Arbor, USA). Filters were then placed in tared aluminium pans, dried overnight at more than 60°C, and reweighed. Dry cell weight from 10 ml cultures was obtained from dry cell weight of 50 ml cultures. One unit of enzyme activity was defined as μM of *p*-NP liberated per hour per gram of dry cell weight.

Preparation of aroma precursor extract. The precursors were extracted from the grape variety Chardonnay cultivated in Shaanxi province, China. The procedure was based on the method described by Ibarz *et al.* (2006) with slight modifications. Briefly, 500 g grapes were homogenised and centrifuged (4500 *g*, 15 min, 5°C) to get must and skins. The mashes of skins were suspended in 380 ml of a buffer solution (0.1 M Na_2HPO_4/NaH_2PO_4) at pH 7.0

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and 13% (v/v) ethanol and allowed to macerate in the dark (36 h, 20°C, nitrogen atmosphere) to extract the precursors. This solution was centrifuged (4500 *g*, 15 min, 5°C), filtered through filter paper, then ethanol was removed under vacuum to get a "macerate" (ca. 260 ml). The must (ca. 300 ml) and the macerate were percolated through two LiChrolut EN (1300 mg; Merck, Darmstadt, Germany) resin beds (previously preconditioned with 32 ml of dichloromethane, 32 ml of methanol, and 65 ml of water). In both cases the column was washed with 26 ml of water, and then with 40 ml of a pentane/dichloromethane (2 : 1; v/v) mixture. The retained precursors were finally eluted with 50 ml of an ethyl acetate/methanol (9 : 1 v/v) mixture. The two extracts were mixed, evaporated under vacuum to dryness. The residue was dissolved in 10 ml citrate-phosphate buffer (pH 5.0) and stored at –20°C.

Enzyme assay with natural substrate. Cell pellets from 20 ml cultures obtained by the method described above were resuspended in 20 ml of 100 mM citrate-phosphate buffer (pH 5.0) and added to 1 ml glycoside stock solution (equivalent to an aliquot of glycosides from 50 g grapes). After 48 h incubation at 25°C, liberated glycosyl-glucose was measured spectrophotometrically at 340 nm using an enzymatic glucose assay (Megazyme, Wicklow, Ireland). A substrate blank (buffer and glycosides) and sample blanks (cell pellets and buffer) were prepared. A positive control (glycosides in pH 2.5 phosphate citrate buffer and cooked in boiling water for 2 h) was also prepared. One unit of enzyme activity was defined as μM of glycosyl–glucose liberated per hour per gram of dry cell weight. All assays were performed in duplicate and were repeated twice.

Release of volatile compounds from precursors. The volatile aglycones released from aroma precursors by SD-2a and 31MBR were monitored by GC/MS. 15 ml of samples, to which 2,5-dimethyl phenol was added as an internal standard (60 μg/l final concentration), was passed through a 50 mg LiChrolut EN cartridge previously preconditioned (2 ml of dichloromethane, 2 ml of methanol, and 2 ml of a 12% ethanol solution). The sorbent was washed with 5 ml of 40% (v/v) methanol solution and dried by letting air pass through (0.6 bar, 10 min). Analytes were recovered by elution with 600 μl of dichloromethane. The GC-MS conditions and analysis followed the method described by Jiang (2010). Analyses were carried out in duplicate.

Glucose consumption assay. The assay of glucose consumption by SD-2a and 31MBR was conducted by the same method of enzyme assay with natural substrate described above, except for adding glucose (final concentration 50 μM/ml) instead of natural substrate.

Partial characterisation assessment. For the influence of temperature on βG activity of intact cells, reaction tubes were incubated at 5–55°C at intervals of 10°C for 48 hours. For the influence of pH, ethanol, and glucose on enzyme activity in intact cells, 10 ml of ATB medium with 1 mM of substrate was adjusted to pH 3.0, 3.5, 4.0, 4.5, and 5.0, ethanol concentration of 0–28% at intervals of 4%, and glucose concentration of 0–60 g/l at intervals of 10 g/l. Otherwise it was the same as the described assay with synthetic substrate. All assays were performed in duplicate and were repeated at least twice.

Statistical analysis. The mean values of those replicated counts were subjected to analysis of variance using the SAS statistical software (SAS Institute, Cary, USA) at the 5% level of significance.

RESULTS AND DISCUSSION

As shown in Table 1, the intact cells of both SD-2a and 31MBR displayed βG activity against synthetic substrate, with SD-2a showing higher activity than 31MBR. This is consistent with the results of βG localisation in the two strains (Li *et al.* 2012b). The βG activity of intact cells was also evaluated with natural substrate. The concentration of glycosyl–glucose released from natural substrate by SD-2a and 31MBR, with higher value for 31MBR (Figure 1). This indicates that 31MBR intact cells possessed higher βG activity with natural substrate (Table 1). This is contrary to the result obtained with synthetic substrate. Table 2 shows the aglycones released from natural substrate by the two strains. Thirteen kinds

Table1. β-D-Glucosidase activity of SD-2a and 31MBR intact cells with synthetic and natural substrate

Oenococcus oeni strains	β-D-Glucosidase activity	
	synthetic substrate	natural substrate
SD-2a	15.30 ± 0.13[a]	1.60 ± 0.03[a]
31MBR	10.86 ± 0.11[b]	2.11 ± 0.02[b]

Values are the average of quadruplicate replications. Different letters within columns indicate significance at $P < 0.05$; activity with synthetic substrate is expressed as μM of *p*-NP liberated/h/g of dry cell weight; activity with natural substrate is expressed as μM of glycosyl–glucose liberated/h/g of dry cell weight

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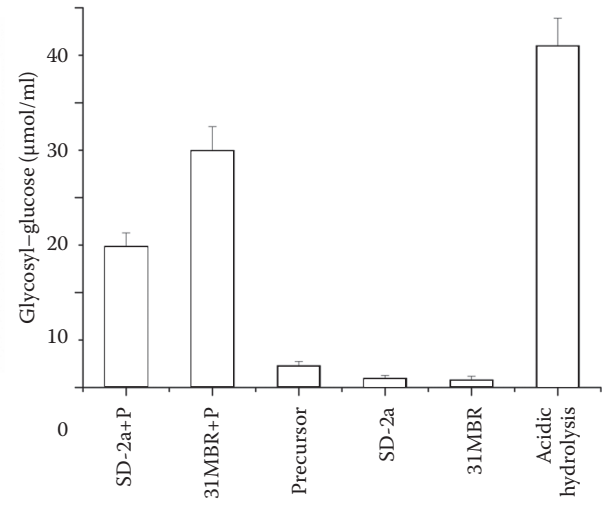
Table 2. Concentration of aglycones released from natural substrates by SD-2a and 31MBR in citrate-phosphate buffer (pH 5.0) after 48-h incubation

Aglycons	Concentration (μg/l)	
	SD-2a	31MBR
Alpha-terpineol	4.40 ± 0.08	ND
Limonene	50.71 ± 0.16	ND
Citronellol	25.00 ± 0.18	14.80 ± 0.10
Linalool	2.32 ± 0.05	ND
Nerol	1.04 ± 0.03	ND
Geraniol	1.51 ± 0.02	ND
3-Hydroxydamascone	50.84 ± 0.12	30.73 ± 0.15
Vitispirane	ND	6.02 ± 0.07
Benzyl alcohol	45.40 ± 0.21	27.50 ± 0.24
Vanillin	35.61 ± 0.18	ND
4-Hydroxybenzoic acid	38.32 ± 0.26	19.04 ± 0.16
Tyrosol	53.60 ± 0.13	30.06 ± 0.11
Methyl vanillate	18.20 ± 0.23	10.62 ± 0.09
Hexanoic acid	20.14 ± 0.15	8.70 ± 0.08
Total	347.09	147.47

The data is mean value of duplicate samples (maximum SD ± 10%); ND – not determined

of volatile compounds were detected for SD-2a, while eight for 31MBR. All these aglycones were previously identified in Chardonnay juice and wine (Sefton *et al.* 1993; D'Incecco *et al.* 2004; Jiang *et al.* 2010). All volatile compounds, except for vitispirane, showed a higher concentration in SD-2a sample (with the total concentration of 347.09 μg/l) than in 31MBR sample (with the total concentration of 147.47 μg/l). These indicate that more aroma precursors were hydrolysed by SD-2a. This is consistent with the result of enzyme assay with synthetic substrate, but contrary to that calculated by released glycosyl–glucose. In order to clarify this difference, glucose consumption assay was conducted subsequently; swith the same conditions for the enzyme assay with natural substrate, SD-2a consumed more glucose than 31MBR (Figure 2). Thus it could be explained that actually more glycosyl-glucose was released by SD-2a during the enzyme assay with natural substrate, but at the same time much more glucose was metabolised by SD-2a, so finally more glycosyl-glucose was detected for 31MBR. Thus, it followed that SD-2a intact cells possess higher βG activity than 31MBR intact cells with either synthetic or natural substrate.

The influence of abiotic factors on βG activity of intact cells was also evaluated. Temperature showed a more significant influence on βG activity of 31MBR intact cells (Figure 3). SD-2a kept high activity within the temperature of 15–45°C, while 31MBR at 35°C. Enzyme activity decreased sharply at 45°C for both strains. Similar results show that an optimum temperature of 45°C for the βG activity of *Lactobacillus brevis* intact cells have been reported (Michlmayr *et al.* 2010). As for the influence of pH on βG activity of intact cells, the same trends were observed for the two strains (Figure 4), enzyme activity increasing with the increase of pH from 3.0 to 5.0. However, SD-2a intact cells showed much higher βG activity than 31MBR intact cells at each pH value. Similar results have also been reported (Grimaldi *et al.* 2000; Michlmayr *et al.* 2010b). Contrary to the previous reports (Mateo *et al.* 1997; Michlmayr *et al.* 2010b), glucose showed no inhibitory effect on βG activity of intact cells (Figure 5). Enzyme activity increased by about 10% of the initial activity at a glucose concentration of 60 g/l for SD-2a, while it increased by about 32% at a concentration of 5g/l and remained stable at high concentrations tested for 31MBR. As to ethanol, βG activity of intact cells showed great



SD-2a+P/31MBR+P – samples containing aroma precursor (natural substrate) and intact cells of SD-2a or 31MBR; Precursor – substrate blank containing aroma precursor (natural substrate) but not cells; SD-2a/31MBR – sample blanks containing intact cells of SD-2a or 31MBR but not substrate; Acidic hydrolysis – positive control, glycosides in pH 2.5 phosphate citrate buffer

Figure 1. Concentration of glycosyl-glucose released from natural substrate by SD-2a and 31MBR in citrate–phosphate buffer (pH 5.0) after 48-h incubation

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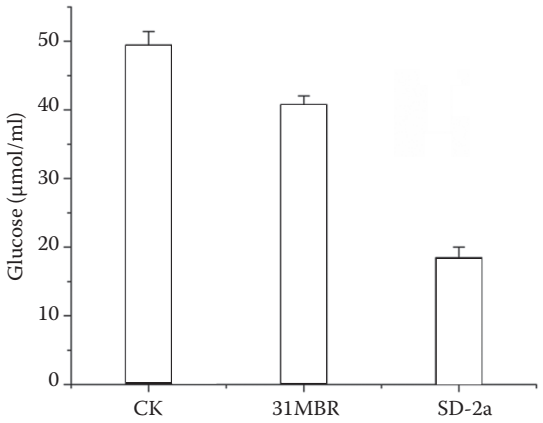

Figure 2. Concentration of glucose after consumption by SD-2a and 31MBR

CK – containing glucose but not cells; SD-2a/31MBR – samples containing glucose and intact cells of SD-2a or 31MBR

tolerance to it (Figure 6). SD-2a maintained high activity at an ethanol concentration of 0~16% and at a concentration of 24% still 65% of maximal activity remained. 31MBR exhibited high activity at a concentration of 16~24%, and the highest activity at 20%. Interestingly, ethanol at a concentration of 16~20% displayed a great promoting effect on βG activity of intact cells for 31MBR.

In the present study, attention was focused on βG activity of *O. oeni* SD-2a and 31MBR intact cells. Both strains widely used in China possess important oenological characteristics, particularly, being able to perform MLF effectively in winery conditions. The βG activity of intact cells is significant for winemaking, since it is intact cells that act directly with glycosides in winemaking. A previous study reported that the whole cells of SD-2a and 31MBR possessed βG activity (Li *et al.* 2012b). While in the present study, the βG activity of intact cells of both strains was further investigated with synthetic substrate in a growth medium and with natural substrate in a modified system. Investigating the βG activity of intact cells in a growth medium and with natural substrate is significant, since it is closer to the actual MLF situation.

The enzyme assay with synthetic substrate showed that SD-2a intact cells possessed higher activity than 31MBR intact cells. The βG activity of intact cells has also been reported in other *O. oeni* strains, as well as amongst *Lactobacilli* spp. and *Pediococci* spp. (Guilloux-Benatier *et al.* 1993; Grimaldi *et al.* 2000, 2005b; Michlmayr *et al.* 2012b). Interestingly, a previous study reported that βG of SD-2a and 31MBR is an intracellular form and 31MBR possesses higher intracellular activity than SD-2a (Li *et al.* 2012b). The difference could be attributed to the presence of the phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS). Capaldo *et al.* (2011) pointed out that *O. oeni* were able to take up β-glucosides via the PEP-PTS, which involves phosphorylation and subsequent hydrolysis of the phosphorylated glucosides through the action of phospho-β-glucosidases (Capaldo *et al.* 2011). This could also well explain the results from other authors that intact cells of the bacteria showed high βG activity while low or no intracellular and extracellular activities were observed (McMahon *et al.* 1999; Michlmayr *et al.* 2010b).

In order to further confirm and adequately evaluate the βG activity of SD-2a and 31MBR intact cells, natural substrate extracted from Chardonnay grapes was also used to perform the enzyme assay. Interestingly, opposite results were observed for enzyme assay with natural substrate (more glycosyl-glucose released for 31MBR, while more aglycones released for SD-2a) and the result of glucose consumption as-

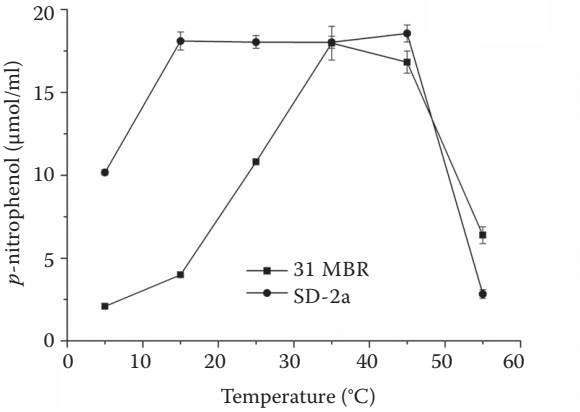

Figure 3. Influence of temperature on the β-D-glucosidase activity of SD-2a and 31MBR intact cells

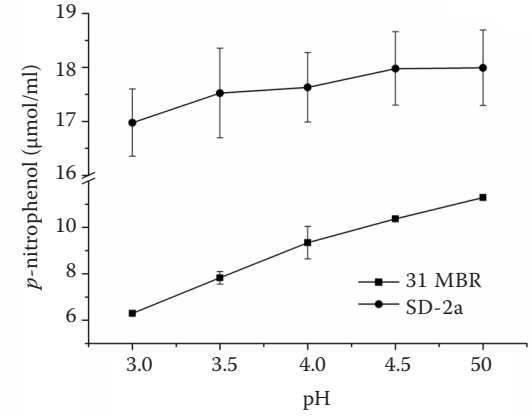

Figure 4. Influence of pH on the β-D-glucosidase activity of SD-2a and 31MBR intact cells

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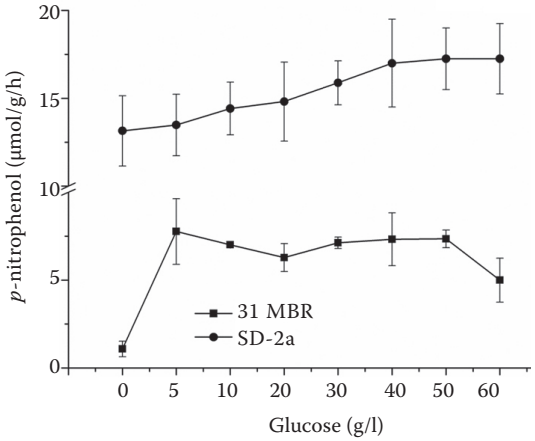

Figure 5. Influence of glucose on the β-D-glucosidase activity of SD-2a and 31MBR intact cells

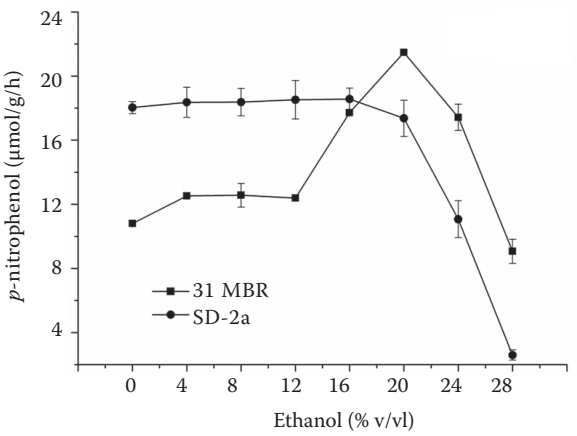

Figure 6. Influence of ethanol on the β-D-glucosidase activity of SD-2a and 31MBR intact cellsintact cells substrate

say well explained this difference. The difference in the behaviour of the two strains on natural substrate, such as the amount and variety of aglycones released, may be due to grape cultivar and individual difference between the strains. Different grape cultivars have different pools of mono- and disaccharides, also different aglycone pools. Monosaccharide glycoside hydrolysis can occur directly by βG. Disaccharide hydrolysis consists of two steps, wherein the terminal sugar is first separated by a hydrolase (α-L-arabinofuranosidase, α-L-rhamnosidase, β-apiosidase), and then glucose from the aglycone by βG (Mansfield *et al.* 2002). In addition, βG from a single organism may display selectivity to specific aglycones (Mansfield *et al.* 2002). Thus, SD-2a may possess higher total hydrolase activity than 31MBR.

An important question in considering the suitability of βG from *O. oeni* for use in oenology is the response of βG activity to temperature, pH, ethanol and sugar encountered in winemaking. βG of 31MBR intact cells exhibited more sensitivity to temperature than that of SD-2a. SD-2a intact cells showed high enzyme activity in a large range of temperatures (15~45°C), while 31MBR in a small range of temperatures (35~45°C). During winemaking, the temperature, usually controlled at about 25°C, would inhibit the βG activity of intact cells for 31MBR. The βG activity of both strains decreasing sharply at 45°C may be due to the damage caused by high temperature to intact cells, since an optimum temperature of 50°C has been observed for crude βG of the two strains (Li *et al.* 2012b). Usually, pH is known as an important factor to affect the enzyme activity, especially low pH is considered as an inhibitor of βG activity (Grimaldi *et al.* 2000; Michlmayr *et al.* 2010b, 2012b).

Interestingly, in the present study pH from 3.0 to 5.0 showed no significant difference in the βG activity of SD-2a intact cells, although a downward trend was observed as pH decreased. This may be ascribed to the protection of intact cells on intracellular βG against inhibition by pH. More than one hundred years ago, people found that the presence of the preferred sugar glucose represses the synthesis of some enzymes, which is necessary for the metabolism of less favourable carbon sources, such as glycosides. This phenomenon became known as carbon catabolite repression (Deutscher *et al.* 2006). However, in the present study glucose showed an enhanced effect on the βG activity of SD-2a and 31MBR intact cells. Similar results were also described by Grimaldi *et al.* (2000). As for ethanol, an enhancement of the βG activity of 31MBR intact cells was observed. Such high ethanol tolerance for the βG activity of *O. oeni* strains has not been reported before. This may be due to the protection of intact cells on intracellular βG. The activation of ethanol on βG activity has been reported (Grimaldi *et al.* 2000; Michlmayr *et al.* 2010b), although the inhibition on βG activity was also observed (Mateo *et al.* 1997). The enhancement behaviour of ethanol might be attributed to the glycosyl-transferase activity of βG, ethanol increasing reaction rates by acting as an acceptor of the key glycosyl intermediate (Barbagallo *et al.* 2004). Alternatively, a high ethanol concentration may alter the cell membrane permeability, thereby allowing easier access to intracellular enzyme and substrates (Tourdot-Maréchal *et al.* 2000).

O. oeni SD-2a and 31MBR intact cells showed βG activity against both synthetic and natural substrates, with SD-2a performing better than 31MBR. SD-2a

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intact cells possessing high βG activity under wine-like conditions could be of great interest for its utilisation in winemaking. SD-2a shows a great potential for flavour enhancement in winemaking. This study contributes to preliminary knowledge of the βG activity of SD-2a and 31MBR. It will also provide some information to aid winemakers in selecting starter cultures for wine quality improvement. Further study should be focused on further glycosidase activity of the two strains and their influence on the aroma profile during actual MLF.

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