Assessment of *β*-D-Glucosidase Activity from Two Typical Strains of the Lactic Acid Bacteria, *Oenococcus oeni*, in China

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β-D-glucosidase (βG) is one of the most interesting glycosidases for the hydrolysis of glycoconjugated precursors to release active aromatic compounds in musts and wines. *Oenococcus oeni* strains SD-2a and 31MBR are widely used in Chinese wines to reduce the acidity. In the present study, the βG activity of the two strains was localised and partially characterised using synthetic substrate. The activity occurred in whole cells, sonication supernatants and debris, but not in the culture supernatants for both strains. Whole cells of strain SD-2a possessed greater βG activity, while strain 31MBR showed higher enzyme activity in the sonication supernatants. Strain 31MBR exhibited higher total enzyme activity than strain SD-2a. The optimum temperatures for βG from the two strains were 40ºC at pH 3.5 and 50ºC at pH 5.0, respectively. Ethanol at low concentrations had a positive effect on βG activity in both strains, while a wine-like pH (3.5) decreased the enzyme activity to a great extent. Whole cells of strain SD-2a showed the highest activity among all samples tested under wine-like conditions. Thus, strain SD-2a proved to have potential for aroma improvement in winemaking.

INTRODUCTION

The aroma and flavour compounds present in wine are derived mainly from grape berries, although many of these volatile compounds are also produced during fermentation, such as monoterpenes, C_{13}-norisoprenoids, benzene derivatives, aliphatic alcohols and phenols (Maicas *et al*., 1999; Mateo & Jimenez, 2000; Bartowsky *et al*., 2002; D'Incecco *et al*., 2004; Michlmayr *et al*., 2010a). It is well known that a significant part of flavour remains as odourless glycosylated precursors in newly made wine that are not hydrolysed during ethanol fermentation. These odourless precursors containing aroma aglycones are not directly accessible to the olfactory mucosa, but may greatly affect wine quality after hydrolysis (Williams *et al*., 1995; McMahon *et al*., 1999). The hydrolysis of odourless precursors could be achieved through chemical or enzymatic treatment during winemaking. However, the acid hydrolysis occurs quite slowly, and may produce undesirable and unpredictable flavours. Alternatively, enzymatic hydrolysis is a favourable method to enhance the natural aroma spectrum of wine without detrimental effects on the final quality (Spagna *et al*., 1998; McMahon *et al*., 1999; Mateo & Jimenez, 2000; Michlmayr *et al*., 2010a).

β-D-glucosidase (βG) is one of the most interesting glycosidases to hydrolyse glycosylated precursors, releasing active aroma and flavour compounds (Spagna *et al*., 1998;

Barbagallo *et al*., 2002; Palmeri & Spagna, 2007). Recently, an increased interest in the sources of βG has been focused on lactic acid bacteria, especially *Oenococcus oeni*, the main bacterial species that conducts malolactic fermentation (MLF) in winemaking, as βG activity from grape and yeasts is limited in winemaking (Maicas & Mateo, 2005; Palmeri & Spagna, 2007; Saguir *et al*., 2009; Michlmayr *et al*., 2010b). Numerous investigations have been conducted of *O. oeni* strains, providing evidence of the potential βG activity for flavour enhancement in wines (Grimaldi *et al*., 2000; Boido *et al*., 2002; Mansfield *et al*., 2002; Ugliano *et al*., 2003; Barbagallo *et al*., 2004b; D'Incecco *et al*., 2004; Grimaldi *et al*., 2005a; Bloem *et al*., 2008; Michlmayr *et al*., 2010a; Gagné *et al*., 2011). It has been reported that possession of glycosidic activities was widespread and strain-dependent in these strains commonly used for MLF in winemaking (Grimaldi *et al*., 2005b). Yet among all of this research there is not much about the enzyme localisation and its characterisations under oenological conditions.

Recently, the consumption of wine, especially red wine, has shown an increasing trend in China. However, wines made in China are usually characterised by low pH and a bland flavour due to the climate and cultivars. Thus MLF is necessary and the selection of starter cultures possessing important oenological characteristics is significant for the wine industry in China. *Oenococcus oeni* strains SD-2a and

31MBR have been isolated and widely used in winemaking in China. Up until now, little information has been available on the βG activity of both strains.

The aim of the present study was to localise βG activity within *O. oeni* strains SD-2a and 31MBR and partially characterise the enzyme under different physicochemical conditions.

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

Sample preparation
Bacterial growth was monitored by measuring OD_{600nm} until the end of the exponential growth phase (about 80 h and 40 h for strains SD-2a and 31MBR, respectively). Cultures of 1 mL were centrifuged at 5000 x g for 20 min at 4°C to collect supernatants and whole cells. Whole cells were washed twice with 1 mL 150 mmol/L NaCl, then resuspended in 1 mL cold aseptic distilled water for the enzyme assay. The supernatants and washes were used directly to determine βG activity. For the determination of intracellular enzyme activity, whole cells that had been washed were resuspended in 1 mL PBS 1 × buffer (140 mmol/L NaCl; 2.7 mmol/L KCl; 10 mmol/L Na_2HPO_4; 1.8 mmol/L KH_2PO_4; pH 7.4), sonicated for 20 min in an ice bath using a sonicator (Hielscher GmbH, Germany) at 100 w and centrifuged at 14 000 x g for 15 min at 4°C. The resulting debris was washed twice with 1 mL 150 mmol/L NaCl and resuspended in 1 mL cold aseptic distilled water for the enzyme assay. The corresponding supernatants and washes were used directly to determine enzyme activity. The enzyme assay was also conducted on disrupted lysate (solution of whole cells treated with sonication) containing intracellular βG, either soluble or attached to the ruptures.

Enzyme assay
βG activity was determined spectrophotometrically using *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG, Sigma cat no. N7006) as the substrate according to a method described previously with some modifications (Barbagallo *et al.*, 2004b). To yield a final absorbance value of between 0.2 and 1.0 at 400 nm, 200 μL of the sample was diluted with distilled water to 500 μL. Reaction mixtures were made by the introduction of each diluted sample into 500 μL 2 × citrate-phosphate/*p*-NPG solution with a final pH of 5.0 and a substrate concentration of 5 mmol/L. Incubation at 30°C for 1 h was followed by colour development. In order to minimise the spontaneous hydrolysis of the substrate under alkaline conditions, which are necessary for colour development, the mixtures were first centrifuged at 14 000 x g for 15 min at 4°C to remove cells or debris, after which

the supernatants were transferred immediately into 2 mL of 1 mol/L Na_2CO_3 to stop the reaction and allow the development of the yellow colour of *p*-nitrophenolate ion. A blank was prepared using distilled water instead of samples, but otherwise treated in the same manner. The absorbance of the yellow *p*-nitrophenolate ion was measured at 400 nm with a 1 cm cell in a Beckman DU-800 spectrophotometer. The concentration of liberated *p*-nitrophenol (*p*-NP) was calculated from a calibration curve prepared from a series of standard *p*-NP solutions containing 0 to 100 μmol/L *p*-NP (ε_{400} = 6000 L/mol/cm). One unit of enzyme activity was defined as μmol of *p*-NP released per min per gram of cell dry weight. All assays were performed in quadruplicate for both strains.

Assessment of enzyme activity under different physicochemical conditions
Disrupted lysate of both strains SD-2a and 31MBR, as well as whole cells of strain SD-2a, were used to study the influences of temperature, ethanol and pH on βG activity. For the influence of temperature at different pH, assay mixtures at pH 3.5 (a wine-like pH) or 5.0 were incubated at temperatures ranging from 20 to 70°C at intervals of 10°C for 1 h. For the influence of ethanol at different pH, assay mixtures at pH 3.5 or 5.0, with a series of final ethanol concentrations from 0% to 32% at intervals of 4% (v/v), were incubated at 30°C for 1 h.

Statistical analysis
The mean values of those replicated counts were subjected to analysis of variance using the statistical software SAS (SAS Institute, Cary, NC, USA) at a 1% level of significance.

RESULTS
Enzyme localization
βG activity from different parts of strains SD-2a and 31MBR varied greatly, as shown in Table 1. Fairly low enzyme activity was detected in the culture supernatants and whole cell washes of both strains. After the sonication treatment, both the supernatants and the debris of the two strains displayed obvious activity, with the activity higher in the supernatants than in the debris, especially for strain 31MBR. This may demonstrate that the βG from the two strains that was not secreted outside the cells was mainly intracellular in form and that some was adhered on the debris after the sonication treatment. In particular, great enzyme activity was detected on whole cells for strain SD-2a, much higher than that from the sonication supernatants or the debris, and almost equivalent to that observed in the disrupted lysate. For strain 31MBR, on the other hand, the highest enzyme activity was observed in the sonication supernatants instead of the whole cells, which could be attributed to the release of βG after the sonication treatment. This result is in good agreement with the findings reported by Barbagallo *et al.* (2004b). Moreover, strain 31MBR possessed higher total βG activity (disrupted lysate activity) than strain SD-2a.

Enzyme characterisations under different conditions
As shown in Fig. 1, the influences of temperature, ethanol and pH on βG activity were conducted on disrupted lysate

TABLE 1
β-D-glucosidase activities by location of enzyme activity for *Oenococcus oeni* strains.

Oenococcus oeni strains	CS	WCW	WC	SS	DW	D	DL
SD-2a	0.05 d	0.05 d	7.67 a	4.35 b	0.03 d	2.75 c	7.48 a
31MBR	0.04 c	0.07 c	1.38 c	18.37 b	0.09 c	1.93 c	22.27 a

CS, culture supernatants; WCW, whole cell washes; WC, whole cells; SS, sonication supernatants; DW, debris washes; D, debris; DL, disrupted lysate. Values are the averages of quadruplicate replications. Different letters within columns indicate significance at $P < 0.01$. Means with the same letter are not significantly different. Activity is expressed as *μ*mol of *p*-NP liberated per min per gram of cell dry weight.
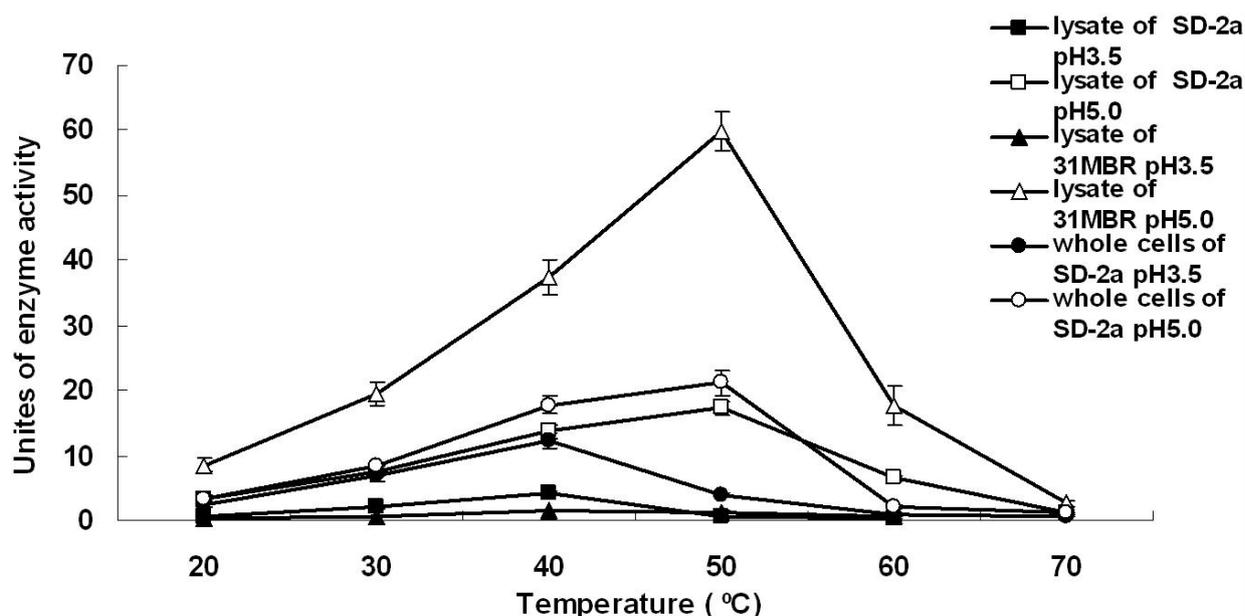
FIGURE 1A
In the present study, attention was focused on *O. oeni* strains SD-2a and 31MBR. Both strains are widely used in winemaking in China

of both strains as well as on whole cells of strain SD-2a, since these three samples displayed high activity in the enzyme location assay. The optimum temperature of *β*G activity from all the samples was 40ºC at pH 3.5 and 50ºC at pH 5.0 (Fig. 1A). The influence of ethanol showed similar trends in three samples (Fig. 1B). The samples tested showed increased or retained high enzyme activity at low ethanol concentrations, and the enzyme activity decreased at high concentrations. Lysate of strain 31MBR showed high activity between ethanol concentrations of 16% to 20% at pH 5.0. Such high ethanol tolerance for *β*G from *O. oeni* strains has not been reported, particularly at an ethanol concentration of 32%, yet 20% of maximal activity remained. At pH 3.5, whole cells of strain SD-2a still retained high activity at an ethanol concentration of 8%. As to pH, the samples showed higher activity at pH 5.0 than that at pH 3.5 in all treatments (Fig. 1A and B), and the optimum temperature (Fig. 1A) and ethanol tolerance (Fig. 1B) of the enzyme decreased at pH 3.5 compared with that at pH 5.0. This could be explained by previous reports that the optimum pH of *β*G was around 5.0 (Spagna *et al*., 1998; Grimaldi *et al*., 2000; Barbagallo *et al*., 2004a; González-Pombo *et al*., 2008; Michlmayr *et al*., 2010a), notwithstanding that an optimum pH of 3.8 has also been reported (Grimaldi *et al*., 2005b). In addition, at a wine-

like pH of 3.5, whole cells of strain SD-2a had the highest activity under temperatures of 30, 40 and 50°C (Fig. 1A) and ethanol concentrations of 0%, 4% and 8% (Fig. 1B) in all the samples tested.

DISCUSSION
In the present study, attention was focused on *O. oeni* strains SD-2a and 31MBR. Both strains are widely used in winemaking in China and possess important oenological characteristics, particularly being able to perform MLF effectively under winery conditions. Great physiological differences were observed between the two strains when cultured in ATB medium. It took strain SD-2a 80 h to reach the end of the exponential growth phase, while strain 31MBR needed only 40 h to reach the same stage. In addition, strain SD-2a was more fastidious than strain 31MBR about culture conditions. In order to assess the profile of *β*G from strains SD-2a and 31MBR, synthetic substrate *p*-nitrophenyl *β*-D-glucopyranoside was used in the study. However, some authors have pointed out that glycosidase assay depends to a large extent on the chemical structures of the substrate, and natural aroma precursors were recommended for an adequate evaluation of the glycosidases potential of the *O. oeni* strains (Gagné *et al*., 2011).
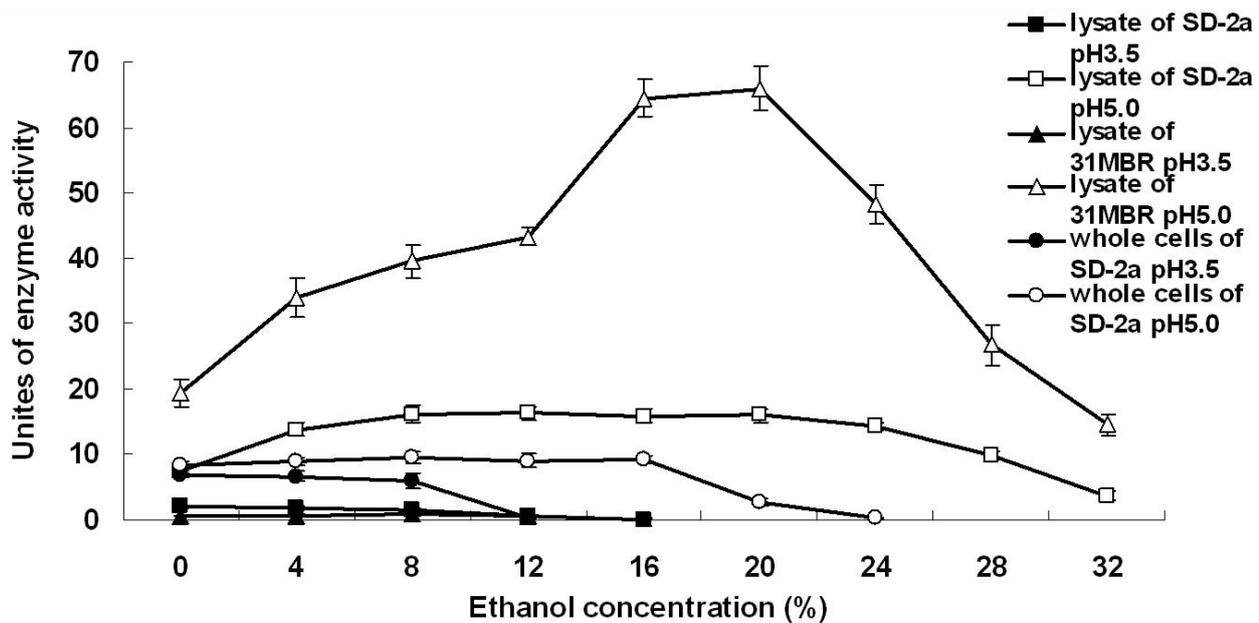
FIGURE 1B
In the present study, attention was focused on *O. oeni* strains SD-2a and 31MBR. Both strains are widely used in winemaking in China

Different *β*G activities were observed between *O. oeni* strains SD-2a and 31MBR in the present study. This confirms the previous reports that glycosidic activity was widespread and strain dependent among *O. oeni* strains (Mansfield *et al*., 2002; Grimaldi et al., 2005b). The result of intracellular enzyme activity for both strains is in good agreement with the findings reported on other *O. oeni* strains (Barbagallo *et al*., 2004b). However, extracellular *β*G activity in *O. oeni* strains has also been observed (Mansfield *et al*., 2002). This seems to suggest that the location of *β*G in *O. oeni* strains is variable and strain dependent. However, it should be noted that considerable enzyme activity was detected on whole cells of strain SD-2a. Similar results, namely that whole cells of *O. oeni* strains possess high *β*G activity, have been reported (Grimaldi *et al*., 2000, 2005b; Michlmayr *et al*., 2010a). This could be explained by the presence of intracellular phospho-*β*-glucosidases and the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). PEP-PTS is a bacterial transport system that allows bacterial cells to grow on various carbon sources, including *β*-glucosides (Deutscher *et al*., 2006). Capaldo *et al*. (2011) proposed that *O. oeni* was able to take up *β*-glucosides via a PEP-PTS involving phosphorylation and the subsequent hydrolysis of the phosphorylated glucosides in the cytoplasm through the action of phospho-*β*-glucosidases. The related genes bglA, B, C and D, encoding PEP-PTS components EIIC, EIIA, EIIB and phospho-*β*-glucosidase, have been studied (Capaldo *et al*., 2011). Low enzyme activity of whole cells for 31MBR might be due to the lack of PEP-PTS. Thus it may be indicated that strain SD-2a is preferred to strain 31MBR for aroma enhancement in winemaking, since whole cells with great *β*G activity could be utilised directly in application. Strain 31MBR, on the other hand, may be preferable for *β*G production due to the high total enzyme activity.

Based on the results of enzyme localisation that *β*G was the intracellular form for strains SD-2a and 31MBR,

and that whole cells of strain SD-2a possessed high activity, disrupted lysate of both strains and whole cells of strain SD-2a were used to discover the influences of different physicochemical factors on *β*G activity. Temperature showed a similar influence on *β*G from all samples. At pH 3.5, an optimum temperature of 40ºC was observed. This means that, in winemaking practice, the temperature, which usually is controlled at about 25ºC or lower, would have a negative effect on the enzyme activity. As for the influence of ethanol, some authors have reported that *β*G is a fairly stable enzyme in ethanol solvent (Spagna *et al*., 1998; Barbagallo *et al*., 2002; Palmeri & Spagna, 2007), while others have suggested that ethanol is the cause of the inhibition of glycosidases (Winterhalter & Skouroumounis, 1997; Grimaldi *et al*., 2000; Spagna *et al*., 2002; Barbagallo *et al*., 2004b).

In this study, *β*G activity was enhanced at low ethanol concentrations and inhibited at high concentrations. This is coincident with previous reports that ethanol at concentrations of 4%, 8% and 10% led to an enhancement of *β*G activity, while enzyme activity decreased sharply at even higher concentrations (Grimaldi *et al*., 2000; Barbagallo *et al*., 2004a, 2004b; Grimaldi *et al*., 2005b; González-Pombo *et al*., 2008). The enhancement behaviour of ethanol at low concentrations could be ascribed to the glycosyl transferase activity of *β*G (Pemberton *et al*., 1980; Barbagallo *et al*., 2004b). Ethanol increases reaction rates by acting as an acceptor of a key glycosyl intermediate. At high ethanol concentrations, the enzyme activity was most likely negatively affected by protein denaturation. It was also noticed that whole cell enzyme had a lower ethanol tolerance (16%) than that of the lysate (24%) for strain SD-2a at pH 5.0. This negative influence of ethanol at high concentrations on *β*G inside the cells might be achieved by its action on the cell membrane. Ethanol might have altered membrane permeability, resulting in difficult access between intracellular *β*G and the substrate (Grimaldi *et al*., 2000;

Barbagallo *et al.*, 2004b). pH generally is an important factor affecting enzyme activity; in fact, low pH is the main inhibitor of *β*G activity (Grimaldi *et al.*, 2000; Barbagallo *et al.*, 2004b). In the present experiment, *β*G activity from all samples decreased sharply at a pH of 3.5, compared with that at a pH of 5.0 in all the treatments. This may suggest that the *β*G activity from SD-2a and 31MBR could be negatively affected in winemaking (with a pH of around 3.5). However, at a wine-like pH of 3.5, whole cells of strain SD-2a still exhibited considerable activity, much higher than those from the lysate of strain SD-2a under different temperatures and ethanol concentrations. Therefore, strain SD-2a proves promising for flavour enhancement under oenological conditions and seems to be more suitable for the practice of winemaking.

CONCLUSIONS

*β*G was mainly intracellular form for *O. oeni* strains SD-2a and 31MBR. Whole cells of strain SD-2a showed considerable enzyme activity, while strain 31MBR possessed higher total activity. Ethanol at low concentrations enhanced *β*G activity in both strains, but the activity was greatly inhibited under the wine-like pH of 3.5. Strain SD-2a, whole cells of which showed great enzyme activity under wine-like conditions, proved to have potential for aroma enhancement in oenological application. However, further study, using natural aroma precursors instead of synthetic substrates, is still needed for an adequate evaluation of the *β*G potential of strains SD-2a and 31MBR.

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