Performance of the extremophilic enzyme *BglA* in the hydrolysis of two aroma glucosides in a range of model and real wines and juices.

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ABSTRACT

β-Glycosidases enhance wine aroma by releasing volatile aglycones from non-volatile glycosides. Commercial preparations contain primarily pectinases and only display β-glycosidase as a secondary activity which limits their potential. Here, the extremophilic β-glucosidase A from *Halothermothix orenii*, (*BglA*) has been compared with Rapidase® for the production of aromatic wines and in the remediation of smoke-tainted wines. Model systems as well as real juices and wines have been enriched with geranyl glucoside, typical of white varieties, and guaiacyl glucoside, commonly found in red wines exposed to oak and wines made from grapes exposed to smoke. The hydrolytic capacity of *BglA* was evaluated by measuring the released volatiles in the gas phase with Solid Phase Microextraction and GC-MS. *BglA*, despite an apparent instability at low pHs, is twice as effective in the release of volatiles in sweeter wines and in grape juices offering an excellent alternative for the early stages of the winemaking process and in the juice industry.

Keywords: Enzyme, Glucosides, Wine, Monoterpenes, Aroma, GC-MS, SPME

1 Introduction

Aroma is considered a key aspect of wine quality. Despite the identification of over 800 aroma compounds (Rapp, 1990) only a small number of them contribute substantially to the aroma of wine (Francis & Newton, 2005; Loscos, Hernández-Orte, Cacho, & Ferreira, 2009). Among the volatiles that are important to the aroma of wine there are fruity and floral monoterpenes (geraniol, linalool and a-terpineol) and volatile phenols (guaiacol and cresols), which, depending on their concentration and wine style, could affect differently the overall flavour and aroma.

Monoterpenes, formed in grapes during ripening, are crucial components of the varietal wine bouquet of Muscat and floral varieties (Sánchez Palomo, Pérez-Coello, Díaz-Maroto, González Viñas, & Cabezudo, 2006) but a major fraction is entrapped as flavourless, odourless, non-volatile glycosides, constituting an important reservoir of aroma (Skouroumounis, Massy-Westropp, Sefton, & Williams, 1995). Monoterpenes can be liberated from their glycosides by acid or enzymatic hydrolysis; but as acid hydrolysis is a slower process (Mojsov, Andronikov, Janevski, Jordeva, & Zezova, 2015; Wilkowska & Pogorzelski, 2017) and can cause rearrangements of the released aglycones, enzymes represent a useful alternative and can be added to maximize the aromatic potential of wines (González-Pombo, Fariña, Carrau, Batista-Viera, & Brena, 2014; Günata, Dugelay, Sapis, Baumes, & Bayonove 1993).

Phenolic glycosides are also formed when berries are exposed to smoke from bush fires and prescribed forest burns as the grapevines can uptake smoke constituents like guaiacols, cresols and syringols, and accumulate them in the form of glycoconjugates. However in this case, their hydrolysis leads to the release of volatile phenols (VP) giving the wine a "smoky" or "ashy" aroma/flavour (Hayasaka, Dungey, Baldock, Kennison, & Wilkinson, 2010; Mayr et al., 2014; Singh et al., 2011). In addition, breakdown of glycosides of volatile phenols in the mouth, mediated by enzymes of the oral microflora can also contribute to smoky and ashy aftertaste (Parker et al., 2012). In this case, if enzymatic hydrolysis can be performed effectively during the wine processing, phenolic glycosides can be reduced, and the release of VPs can then be minimised using different techniques (van der Hulst et al., 2019), improving the overall flavour.

The aglycone moiety in terpenyl and phenol glycosides can be linked to a β-D-glucopyranose unit or to a disaccharide (Hjelmeland & Ebeler, 2015). While β-glucosidases [E.C.3.2.1.21] are capable of cleaving the glycosidic bond between the carbohydrate moiety and the aglycone (Singh, Verma, & Kumar, 2016), the release of the aglycone from disaccharide glycosides would normally require the action of other glycosyl hydrolases. Endogenous glycosidases from the grape and the winery environment have been extensively studied for this purpose; however, they do not tolerate well the harsh physical and chemical conditions that usually characterize wine processing such as low pH, high glucose and fructose, and sulphite content. Grape and yeast glycosidases present low activity under fermentation conditions (Sánchez Palomo, Díaz-Maroto Hidalgo, González-Viñas, & Pérez-Coello, 2005), therefore commercial preparations are mainly obtained from fungi and have primarily pectinase activity, with secondary glycosidase activity. Fungal glycosidases have a weak catalytic specificity which could lead to the hydrolysis of pigment glycosides, and consequent spoiling of colours and flavours (Hu et al., 2016). In addition, glucose inhibition is a common problem among fungal β-glucosidases (Chan et al., 2016; Maicas & Mateo, 2005; Sabel, Martens, Petri, König, & Claus, 2014). Hence, the search for new enzymatic alternatives, more adapted to the wine conditions, is highly relevant.

Extremophiles, organisms very well adapted to extreme environmental conditions unbearably hostile or even lethal for other forms of life (Rampelotto, 2013), constitute a novel and alternative source of enzymes for industrial application. Extremozymes are generally more capable to withstand industrial processes in comparison with their mesophilic counterparts (Elleuche, Schröder, Sahm, & Antranikian, 2014). Among extremophiles, enzymes from halophilic microorganisms tolerate very high salinity, which normally leads to denaturation, aggregation, and precipitation of most other proteins. Genomic and structural analyses have established that halophilic enzymes have a higher pro-ratio of acidic amino acids versus hydrophobic ones and altered hydrophobicity compared to mesophilic enzymes, which enhance solubility and promote function in low water activity conditions (DasSarma & DasSarma, 2015). Adaptation to solvents follows the same principle as adaptation to salt, and thus, halophilic enzymes may be a valid option for biocatalytic processes performed in water/solvent environments like wines (Alsafadi & Paradisi, 2013).

Based on this hypothesis, the extremophilic organism *Halothemothrix orenii* was selected as a source of a β-glucosidase for possible application in the wine industry. *Halothemothrix orenii* is a true halophilic and thermophilic bacterium whose unique enzymes are described to have broad pH stability and ability to deal with high temperatures and a wide range of salt concentrations (Bhattacharya & Pletschke, 2014). In this work we evaluated the hydrolytic performance of the β-glucosidase *BglA* described by Kori et al. (Kori, Hofmann, & Patel, 2011) with two glucosides relevant to floral wine aroma and smoke-taint affected wines and compared it with a commercial preparation (Rapidase® Revelation Aroma).

2 Materials and methods

2.1 Chemicals

Water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Luria Bertani Broth, Miller and LB Agar, Miller were purchased from Fisher BioReagents™, Imidazole 99 % was purchased from Alfa Aesar (Fisher Scientific, Bishop Meadow Road, Loughborough, UK). Citric acid monohydrate, potassium L-tartrate monobasic, D-(+)-glucose 99.5

%, D-(-)-fructose European Pharmacopoeia 98% and DL-malic acid ≥ 98 % (capillary GC) were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Hepes ≥ 99.5 % (titration), sodium chloride, acetone Suprasolv® ECD, ethanol for liquid chromatography LiChrosolv®, tartaric acid and *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) were obtained from Merck Pty Ltd (Kilsyth, Victoria, Australia). Rapidase® Revelation Aroma enzymatic preparation was purchased from Vintessential Laboratories (Dromana, Victoria, Australia). Geranyl glucoside, guaiacyl glucoside, d_7-geraniol and d_3-guaiacol were synthesised in-house (Hayasaka et al., 2010; Parker et al., 2012; Pedersen, Capone, Skouroumounis, Pollnitz, & Sefton, 2003; Pollnitz, Pardon, Sykes, & Sefton, 2004).

2.2 *Microbial strains*

The halothermophilic microorganism *Halothermothrix orenii* H 168 was the source of the native β-glucosidase family 1 *BglA*. The constructed vector (BglA-pET45b) was kindly provided to us by Prof. J. Siegel at UC Davis. *E. coli* BL21 (DE3) was the laboratory strain chosen for the heterologous expression.

2.3 *Enzyme expression, purification and lyophilisation*

Cells of *E. coli* BL21 (DE3) harbouring the recombinant plasmid were grown at 37 ºC in Luria-Bertani medium supplemented with ampicillin (0.1 mg/mL). When the OD_{600} was between 0.6-0.8, isopropyl β-D-1-thiogalactopyranoside was added as inductor for the overexpression of the enzyme and the culture left at 30 ºC overnight. Cells were harvested at 4500 G, 4 °C, 20 min and the pellet stored at -20 °C until purification.

The cell pellet was resuspended in buffer (HEPES (50 mM), sodium chloride (150 mM), imidazole (10 mM), pH 7.5) and cells were broken by sonication (6 min cycle, 5s on, 5s off, 50 % amplification). The lysate was collected by centrifugation at 14500 G, 1 h, 4º C, and the pellet was discarded.

The supernatant was then filtered through Millex® PVDF 0.45 µm filter before loading it onto a HisTrap IMAC column previously loaded with $NiSO_4$ 0.1 M and washed with loading buffer (HEPES

(50 mM), sodium chloride (150 mM), imidazole (10 mM), pH 7.5). The column was washed with loading buffer until a plateau in the UV_{280} absorbance was reached. Low affinity binding proteins were eluted using a step gradient 10 % elution buffer and the protein of interest was eluted using 100 % elution buffer (HEPES (50 mM), sodium chloride (150 mM), imidazole (300 mM), pH 7.5). The enzyme was dialysed overnight, flash frozen in liquid nitrogen and freeze dried overnight. (Labconco 8 Port Manifold on Consolo Freeze Dryer).

2.4 Protein quantification and SDS-PAGE

Bradford Protein Assay was used for protein quantification using bovine serum albumin as standard. Sodium dodecyl sulphate electrophoresis was performed to assess protein purity. Image Studio Software (version 4.0) was used to quantify the size of the bands corresponding to the proteins of interest.

2.5 Activity test

β-glucosidase activity was determined spectrophotometrically by adding 10 µL of the suitable enzyme dilution and 290 µL of 10 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) in buffer HEPES 50 mM, pH 7.4 at 25 ºC. The specific activity (U/mg) was expressed as µmol of product formed per minute per milligram of protein.

2.6 Model wines and juices

Two different model wines were selected in representation of a completely sugar dry wine and a table wine with sugar concentrations typical for Australian commercial wines (Godden, Wilkes, & Johnson, 2015). Model wine 1 (MW1) consisted of saturated potassium hydrogen tartrate with 10 % (v/v) ethanol, pH 3.5. Model wine 2 (MW2) consisted of saturated potassium hydrogen tartrate with 10% (v/v) ethanol, 6 g/L glucose, 6 g/L fructose, pH 3.5.

Model juice (MJ) was prepared using water, 100 g/L glucose, 100 g/L fructose, 0. 2 g/L citric acid, 3 g/L malic acid, 2.5 g/L tartaric acid, pH 3.7. pH was adjusted with tartaric acid 1M in all cases.

2.7 *Real wines and juices*

Two commercially available wines, one white (WW) and one red (RW), and a Chardonnay grape juice (WJ) produced in-house were used. A 2017 Chardonnay from Riverina, Australia with an alcohol content of 12.2% v/v, 4.9 g/L glucose and fructose, titratable acid 6.4 g/L and pH 3.35, a 2016 Shiraz from South Eastern Australia with an alcohol content of 13.9% v/v, 5.8 g/L glucose and fructose, titratable acid 6.2 g/L and pH 3.66 and a Chardonnay juice with total soluble solids 22.6 ºBrix (~20 % total sugar content), 52 mg/L SO_2 and pH 3.5. Chardonnay and Shiraz grape varieties were chosen due to their low monoterpene content.

2.8 *Enzymatic treatment*

In separate 20 mL SPME vials, 3 mL of MW1, MW2, MJ, WW, RW and WJ were spiked with 5 µg of geranyl glucoside and 5 µg of guaiacyl glucoside. The amount added to each sample of Rapidase® or *BglA* was 0.01 mg/mL. The samples were left shaking at 22 ºC over different incubation periods to allow enzymatic hydrolysis. The reaction was stopped by adding 2 mL of saturated $CaCl_2$. Internal standards, d_7-geraniol and d_3-guaiacol, were added (2 µg) and the liberated aglycones were analysed using SPME-GCMS. All experiments were carried out in triplicate.

Geraniol and guaiacol calibration curves with a linear range between 0.02-5 µg were performed for each matrix.

2.9 *GC-MS analysis of volatiles*

A Gerstel autosampler (MPS) (Lasersan Australasia Pty Ltd, Robina, Queensland, Australia) was fitted with a 2 cm DVB/CAR/PDMS fibre assembly (Supelco, Bellefonte, PA) to sample the headspace above the stirred sample for 20 min at 35 ºC, immediately prior to instrumental analysis. Analyses were carried out with an Agilent 6890A gas chromatograph and an Agilent 5973 mass selective detector (Agilent Technologies, Forest Hill, Australia) fitted with a Gerstel autosampler (MPS). The sample was injected in splitless mode. The splitter, at 58:1, was opened after 60 s. The injection liner was a Supelco injection sleeve made of 0.75 mm i.d. deactivated borosilicate glass.

The gas chromatograph was fitted with a 30 m x 0.25 mm Agilent J&W DB-35ms Ultra Inert column, 0.25 µm film thickness. The carrier gas was helium, linear velocity was 36 cm/s, and flow rate was 1 mL/min. The oven temperature, was held at 40 ºC for 1 min, increased to 240 ºC at a 5 ºC/min rate, and held at this temperature for 2 min. The injector temperature was 220 ºC, and the transfer line was held at 240 ºC. Positive electron ionisation mass spectra at eV were recorded in SIM mode with *m/z* 69, 81, 93, 99, 109, 121, 123, 124, 127, 128, 136, 154, and 161 with dwell 25 ms (See section 1 of the supplementary information for geraniol and guaiacol quantifiers and qualifiers for identification with MS).

Mass Hunter software (version B.09.00 Agilent) was used for the quantitative analysis.

The hydrolysis percentages were calculated using the following equations:

$$\%\ geraniol\ release = \left(\left(\frac{amount\ of\ free\ geraniol\ detected}{amount\ of\ geranyl\ glucoside\ added}\right)\frac{316}{154}\right) \times 100$$

$$\%\ guaiacol\ release = \left(\left(\frac{amount\ of\ free\ guaiacol\ detected}{amount\ of\ guaiacyl\ glucoside\ added}\right)\frac{286}{124}\right) \times 100$$

2.10 Data analysis

For the experiments in model wines (MW1, MW2) and model juice (MJ) two-way analyses of variance (ANOVA) (GraphPad Prism 8, San Diego, California, USA) were carried out to assess the effects of enzyme and incubation period on the hydrolysis of glycosides. For the experiments in real wines (WW, RW) and real juice (WJ) a paired t-test was run to assess the effect of the enzyme. Significant difference values were calculated in all cases (****$\rho \leq 0.0001$; ***$\rho \leq 0.001$; **$\rho \leq 0.01$, *$\rho < 0.05$).

3 Results and discussion

3.1 Protein expression, purification, and lyophilisation.

BglA was expressed with an average yield of 53 mg protein/L of culture. Estimation by quantification analysis (using Li-cor Odyssey Fc scanner and software Image Studio version 4.0) suggested that 13 % of the crude extract corresponded to *BglA* (Fig 1). The activity of the crude extract was found to be 2.1 U/mg of total proteins. The enzyme was then purified by metal affinity chromatography, to better asses its hydrolytic capacity, and SDS-PAGE was done to assess its purity. For Rapidase®, estimation by quantification suggests that 10 % of the commercial preparation would correspond to β-glucosidases (Fig 1).

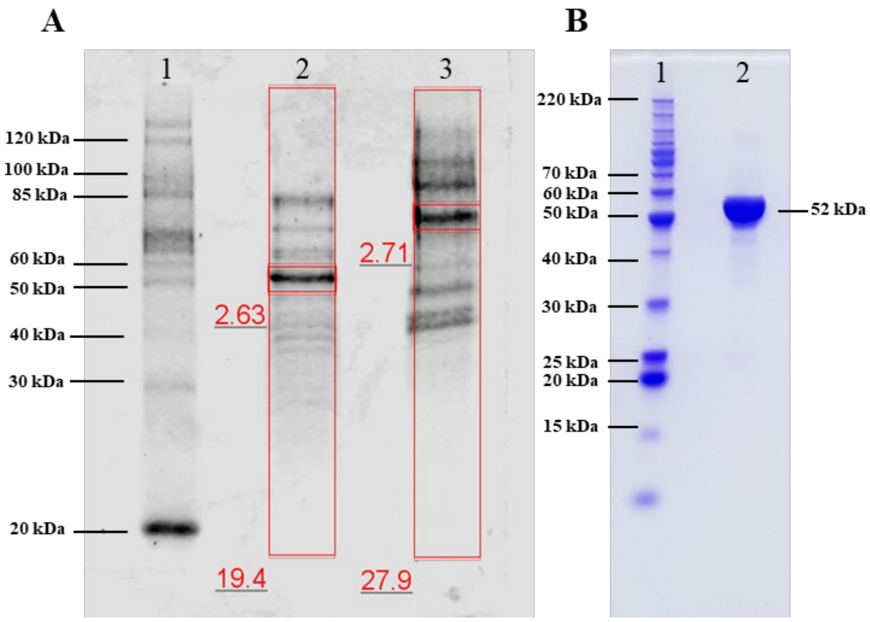


Figure 1. A. Quantification of the bands corresponding to *BglA* in the crude extract and to the β-glucosidase in the Rapidase® preparation are indicated in red, the signal is expressed in relative fluorescence units (RFU) using Li-cor Odyssey Fc scanner and software Image Studio version 4.0), (1) ThermoFisher Scientific PageRuler™ Unstained Protein Ladder, (2) *BglA* in the crude extract (5 µg) (3) β-glucosidases in Rapidase® (5 µg). B. SDS-PAGE after *BglA* purification (1) Invitrogen™ BenchMar™ Protein Ladder (2) Pure *BglA* (5 µg).

Following dialysis, *BglA* was lyophilized and stored at 4 ºC until needed. An activity assay under standard conditions was performed before and after lyophilisation confirming enzymatic stability with a specific activity of 5.5 U/mg of protein. Rapidase® was used directly from the commercial packaging with no further treatment. The specific β-glucosidase activity of the commercial preparation was calculated as 0.16 U/mg of protein.

3.2 BglA preliminary assessment in media mimicking wine conditions

As a first assessment of the suitability of *BglA*, a general characterization of the enzyme activity and stability under different conditions and in buffers mimicking different stages of wine processing was carried out. The enzyme retained 32 % and 90 % of activity when tested in the presence of 5 % (w/v) glucose and fructose respectively, and 80 % in the presence of 10 % (v/v) ethanol (See section 2 of the supplementary information). However, prolonged incubation of the enzyme in the different conditions, did not affect the enzyme structural integrity and a 100 % recovered activity was observed in all cases (See section 3 of the supplementary information).

3.3 Enzymatic stability

Stability assays of *BglA* and Rapidase® were also carried out in more complex media; two model wine systems (MW1 and MW2) and a model juice (MJ) were selected to mimic operational conditions. In addition, two real wine matrices (a white, WW, and a red, RW) and a white grape juice (WJ) were included in the screening to assess the performance and longevity of both enzymes in real complex matrices. The appropriate amount of lyophilised enzyme was dissolved in the different systems and incubated for varying periods of time at 22 ºC. An activity test was performed at suitable intervals (1 h, 3 h, 24 h, and 120 h) to assess how the chemical conditions of the matrix (pH, ethanol and sugars) affect the stability of the enzymes.

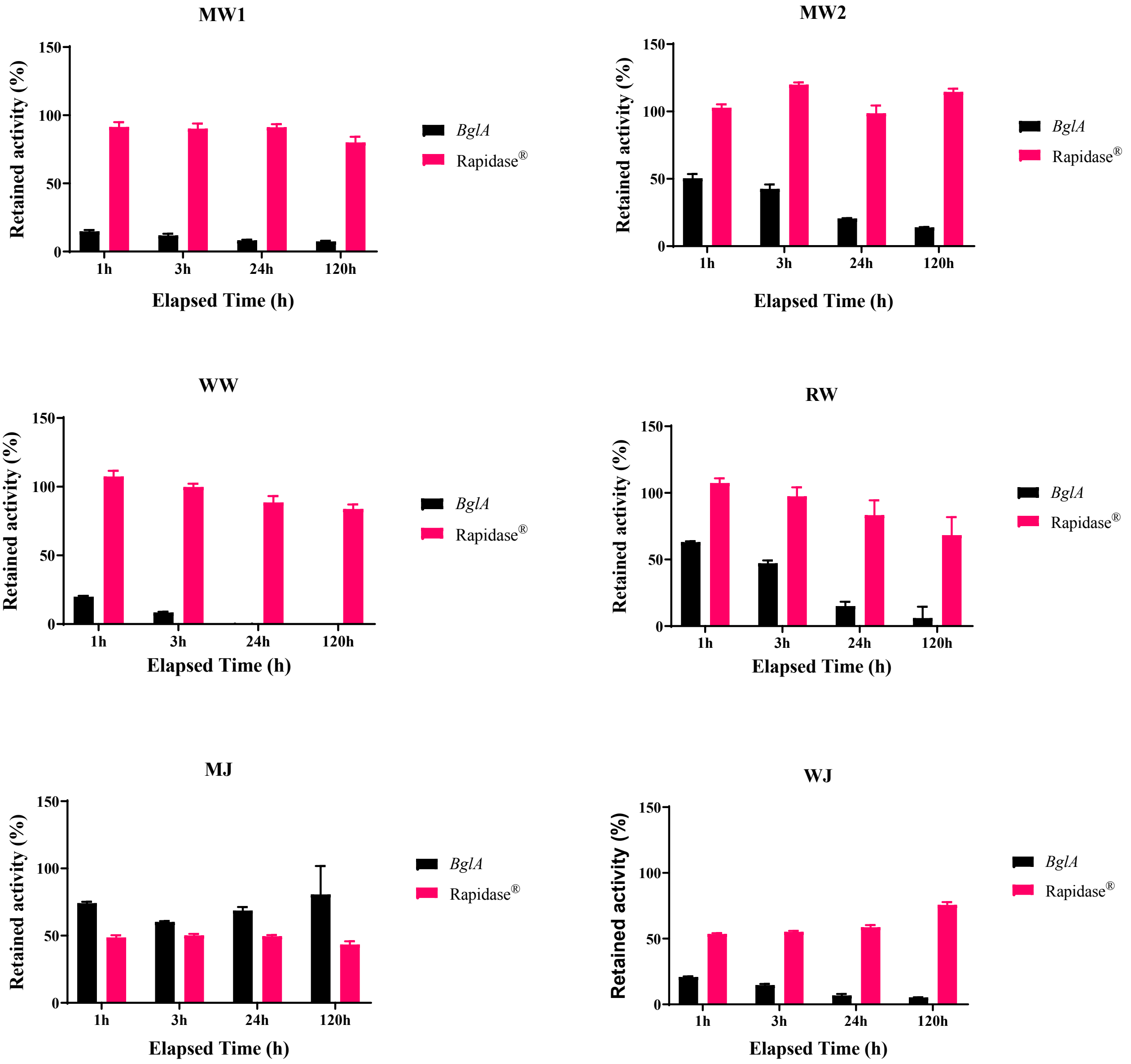


Figure 2. *BglA* and Rapidase® stability assays in Model wine 1 (MW1), Model wine 2 (MW2), White wine (WW), Red wine (RW), Model juice (MJ) and White grape juice (WJ) incubated at 22 ºC during 1 h, 3 h, 24 h and 120 h. Each data point is an average of 3 measurements.

As shown in Figure 2 commercial Rapidase® shows a better stability when incubated in MW1 (10 % ethanol, no sugar, pH 3.5), MW2 (10 % ethanol, 12 g/L glu+fru, pH 3.5), WW (12.2 % ethanol, 4.9 g/L glu+fru, pH 3.35) and RW (13.9 % ethanol, 5.8 g/L glu+fru, pH 3.66), retaining over 60 % activity after 5 days of incubation. *BglA* retains 7 % of activity after 5 days incubation in MW1 and no activity after 24 h in WW. However, after 5 days incubation in MW2, *BglA* shows a retained activity of 14 %, a two-fold increase with respect to MW1. As the sugar content is the only difference between these two model matrices, it appears that fructose and glucose have a protective effect towards *BglA* stability. The stability of *BglA* improves when incubated in RW, retaining 15 % of activity, which is linked to the difference in pH between the two systems; 3.35 for WW (white) and 3.66 for RW (red). On the contrary, Rapidase® shows the opposite behaviour with a 15 % drop in activity when incubated for 5 days in RW in comparison with WW and this could be caused by the higher glucose and fructose content in the red wine (5.8 g/L for the red wine and 4.9 g/L for the white wine) which negatively impacts the stability of the commercial preparation.

However, in the model juice MJ (no ethanol, 200 g/L of glu+fruc, pH of 3.7) *BglA* is considerably more stable, retaining around 45% of activity after 120 h incubation. In the same matrix, Rapidase® stability suffers in comparison with its performance in real and model wines, where the pH and the sugar content are significantly lower, and it compares poorly with *BglA*. In white grape juice (WJ), Rapidase® outperforms *BglA*. WJ has less sugar content and lower pH than MJ which clearly impacts *BglA* stability.

3.4 Analytical determination of volatiles released upon enzymatic hydrolysis of glucosides

The hydrolytic capacity of *BglA* and Rapidase® was evaluated with geranyl and guaiacyl glucoside by measuring the release of the free volatiles in the gas phase with SPME-GCMS. To keep the assessment consistent with the stability tests, the catalytic efficiency was also assessed in model systems and real wines as opposed to simpler buffer solutions. The recommended dosage of Rapidase® for white wines is 1 mg of lyophilised powder per hectolitre of wine, and for red wines 2 mg/hl. However, *BglA* has been used as a purified preparation in all the assays to better assess its performance. To have consistency among all systems, the effective enzyme quantity has been determined by Bio-Rad protein assay, and the powders weighed to achieve 0.01 mg of protein per mL of matrix in all tests.

3.4.1 Release of volatiles from glucosides in model and real wines

Interestingly, despite a lower stability determined for *BglA* (Fig 1), the catalytic efficiency of this enzyme in MW1 equals that of Rapidase® in the release of geraniol with no significative differences (Table 1). The release of guaiacol by *BglA* is, on the other hand, significantly better after 5 days (97 %) in comparison with Rapidase® (75 %). The observed drop in the hydrolysed substrate after 8 days incubation is a known artefact due to the rearrangement of the terpenes under acidic conditions (Hampel, Robinson, Johnson, & Ebeler, 2014; Skouroumounis & Sefton, 2000).

When the catalytic performance was assessed in MW2, Rapidase® hydrolytic capacity was diminished in comparison with MW1. The difference between MW1 and MW2 is once again the sugar content. It is known that glucose is a common inhibitor for many β-glucosidases (De Giuseppe et al., 2014) and a content of 6 g/L seems to affect the activity of the commercial preparation. The formation of geraniol is complete after 24 h incubation in samples containing *BglA*, however in the case of Rapidase® 5 days are required to reach complete hydrolysis, compared with 24 h required in MW1. The guaiacol formed in samples containing *BglA* is 62 % after 5 d incubation while with Rapidase® the release of guaiacol after the same incubation period is 6 times lower (10 %). The results are in line with those obtained in the stability assays. The performance of Rapidase® is affected by sugars; probably glucose is causing inhibition of the enzyme. On the other hand, *BglA* tolerates very well high sugar contents.

MW1

Substrate	**Enzyme**	**Time *****^{a}	**Geraniol released (µg)**	**% Hydrolysis**
Geranyl glucoside	*BglA*	24h	2.33 ± 0.09	96
		5d	2.51 ± 0.11	≥ 99
		8d	2.19 ± 0.05	90
	Rapidase®	24h	2.42 ± 0.03	99
		5d	2.65 ± 0.15	≥ 99
		8d	2.52 ± 0.11	≥ 99
Substrate	**Enzyme** *^{b}	**Time ******^{b}	**Guaiaciol released (µg)**	**% Hydrolysis**
Guaiacyl glucoside	*BglA*	24h	1.57 ± 0.07	72
		5d	2.12 ± 0.010	97
		8d	1.20 ± 0.27	55
	Rapidase®	24h	0.64 ± 0.04	29
		5d	1.64 ± 0.12	75

			8d	1.39 ± 0.07	64
MW2	**Substrate**	**Enzyme ***^{a}**	**Time ****^{a}**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	*BglA*	24h	2.49 ± 0.08	≥ 99
			5d	2.79 ± 0.07	≥ 99
		Rapidase®	24h	1.33 ± 0.10	55
			5d	2.70 ± 0.10	≥ 99
	Substrate	**Enzyme ****^{b}**	**Time ****^{b}**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	*BglA*	24h	0.77 ± 0.04	35
			5d	1.36 ± 0.03	62
		Rapidase®	24h	0.04 ± 0.00	2
			5d	0.23 ± 0.02	10

Table 1. *BglA* and Rapidase® release of geraniol and guaiacol over 24 h, 5 d and 8 d in Model Wine 1 and over 24 h and 5 d in Model Wine 2. ****$\rho \leq 0.0001$; ***$\rho \leq 0.001$; *$\rho < 0.05$. a=geraniol, b=guaiacol.

In comparison with model wines, real wines constitute a highly complex matrix. Without a doubt, underpinning the specific element which either inhibits or destabilises an enzyme is challenging. Potentially, any physical and chemical characteristic of wine is at play: interactions with other molecules, inhibition by sulphur dioxide, rearrangements between components, low pH, sugar content, phenolic glycosides, etc. (Plank et al., 1993). In all cases, hydrolysis was slower and that is reflected in the results.

Rapidase® shows improved activity in WW (Table 2), while the hydrolytic capacity of *BglA* is very limited. On the other hand, after 5 days incubation in RW, *BglA* releases over 30 % geraniol and over 3 % guaiacol. This improvement of the performance of *BglA* in red wine is probably related to a 0.31 pH units difference and 0.9 g/L sugars between white wine and red wine.

WW	**Substrate**	**Enzyme ****^{a}**	**Time**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	BglA	5d	0.01 ± 0.01	0
		Rapidase®	5d	1.94 ± 0.02	80
	Substrate	**Enzyme ***^{b}**	**Time**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	BglA	5d	0.00	0
		Rapidase®	5d	0.24 ± 0.00	11
RW	**Substrate**	**Enzyme **^{a}**	**Time**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	BglA	5d	0.75 ± 0.06	31
		Rapidase®	5d	2.00 ± 0.07	82
	Substrate	**Enzyme *^{b}**	**Time**	**Guaiacol released (µg)**	**% Hydrolysis**

Guaiacyl glucoside	BglA	5d	0.07 ± 0.00	3
	Rapidase®	5d	0.23 ± 0.03	11

Table 2. *BglA* and Rapidase® release of geraniol and guaiacol over 5d in White Wine (WW) and Red Wine (RW). ****$\rho \leq 0.0001$; ***$\rho \leq 0.001$; **$\rho \leq = 0.01$; *$\rho < 0.05$. a=geraniol, b=guaiacol.

3.4.2 Glycosides release in model and real juice

Results in model juice (MJ) (Table 3) highlight an outstanding performance of *BglA* in comparison with the commercial preparation. While Rapidase® hydrolysis capacity is below 6 % for both compounds, the percentage of glycosides hydrolysed by *BglA* is over 60 % for geraniol and over 25 % for guaiacol after 5 days incubation, reaching 45 % after 8 days.

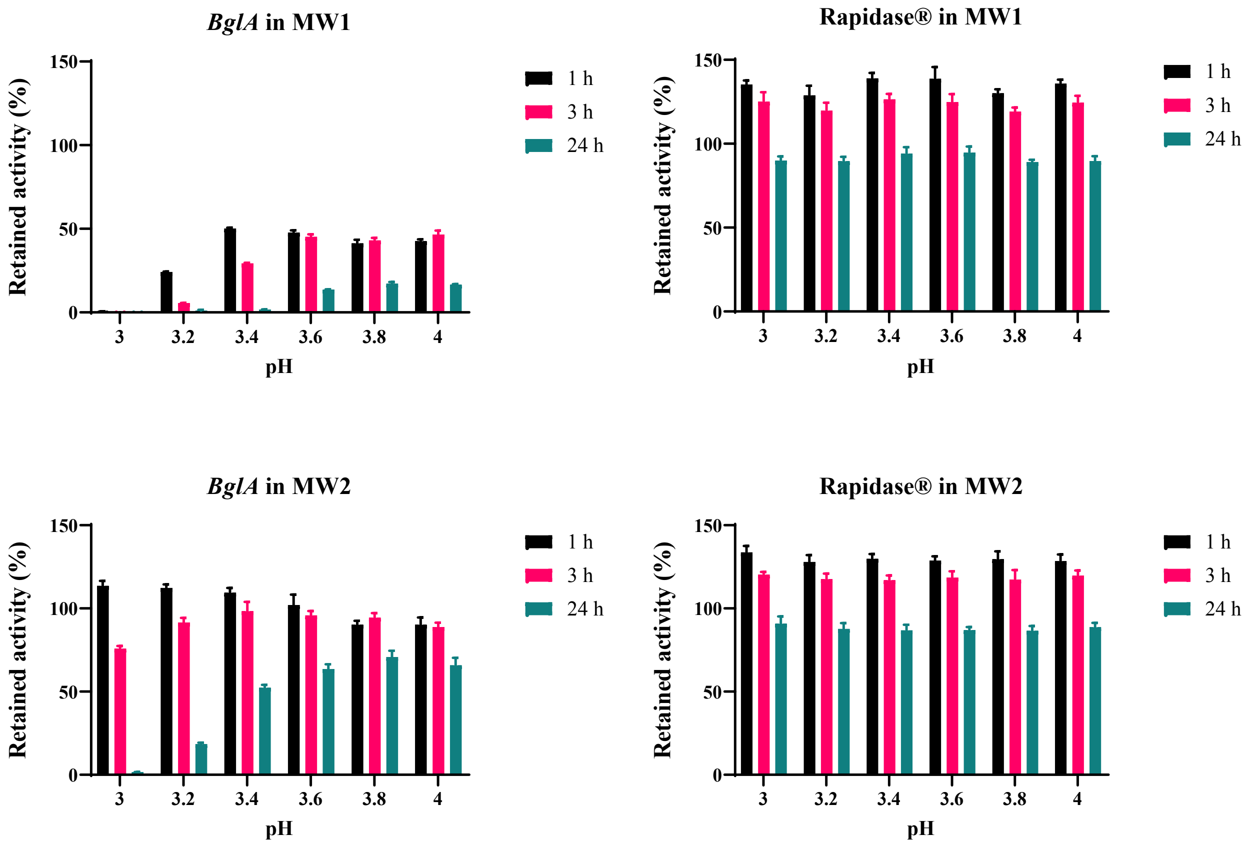
In the case of grape juice (WJ), after 5 days incubation *BglA* continues to show significantly better hydrolysis percentage for geraniol: 10 % against 6 % of Rapidase®. The amount of guaiacol liberated by *BglA* is also slightlyhigher (2 %) than the one released by Rapidase® (1 %).

MJ	**Substrate**	**Enzyme** ****^{a}	**Time** **^{a}	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	*BglA*	24h	0.94 ± 0.04	39
			5d	1.54 ± 0.04	63
			8d	1.15 ± 0.21	47
		Rapidase®	24h	0.03 ± 0.01	1
			5d	0.07 ± 0.02	3
			8d	0.08 ± 0.02	3
	Substrate	**Enzyme** ****^{b}	**Time** ****^{b}	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	*BglA*	24h	0.00	0
			5d	0.55 ± 0.11	25
			8d	0.98 ± 0.01	45
		Rapidase®	24h	0.00	0
			5d	0.04 ± 0.00	2
			8d	0.12 ± 0.04	5
WJ	**Substrate**	**Enzyme** *^{a}	**Time**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	*BglA*	5d	0.26 ± 0.01	10
		Rapidase®	5d	0.14 ± 0.01	6
	Substrate	**Enzyme**	**Time**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	*BglA*	5d	0.03 ± 0.01	2
		Rapidase®	5d	0.02 ± 0.01	1

Table 3. *BglA* and Rapidase® release of geraniol and guaiacol over 24 h, 5 d and 8 d in Model juice (MJ) and over 5 d in real White Juice (WJ). ****$\rho \leq 0.0001$; ***$\rho \leq 0.001$; **$\rho \leq = 0.01$, *$\rho < 0.05$. a=geraniol, b=guaiacol.

3.5 Detailed pH stability assay in MW1, MW2 and MJ

The results above show that *BglA* loses stability between pH 3 and 4. To further narrow the pH fork causing it, a more accurate stability assay of *BglA* and Rapidase® was carried out with 0.2 pH intervals between pH 3 and 4 in MW1, MW2 and MJ at 22 ºC. Retained activity was measured after 1 h, 3 h, 24 h and 120 h, same intervals as in the enzyme stability experiment in different matrices summarised in Figure 2. Unfortunately, measures after 120 h incubation were no longer reliable, probably due to sample concentration by water loss (results not shown).



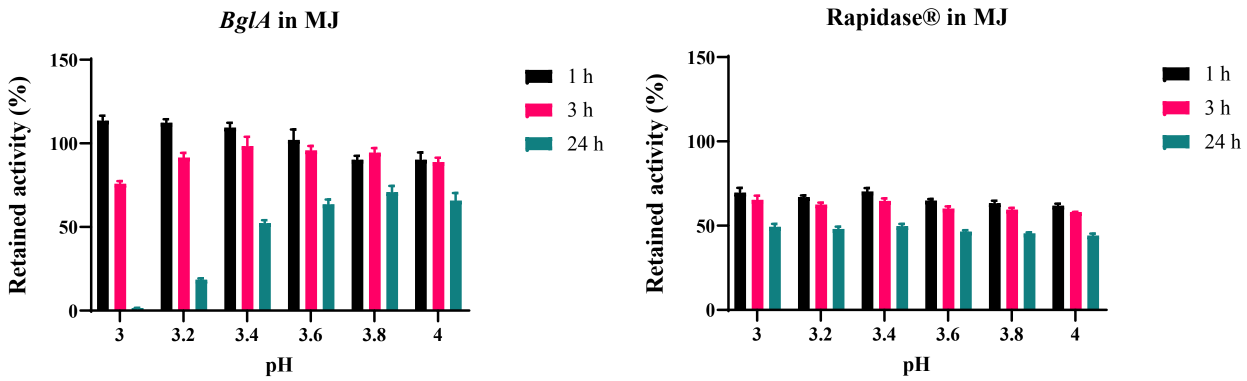


Figure 3. BglA and Rapidase® pH stability assays in Model wine 1 (MW1), Model wine 2 (MW2) incubated at 22 ºC during 1 h, 3 h and 24 h. Each data set is an average of 3 measurements.

BglA loses virtually all activity within 24h of incubation in MW1 and MW2 at a pH lower than 3.6. However, in MJ at pH 3.2, the enzyme still retains 20 % of its activity after the same incubation time. The experiments clearly show that the more sugar the matrix contains, the higher activity *BglA* retains, at all pHs. Rapidase® is clearly independent on pH and the preparation is equally stable between 3 and 4, however, the sugar content present in MJ reduces its activity by almost 50 % very rapidly. These results confirm once again the suitability of *BglA* for matrices with high content of sugars, for example during the maceration or other early stages of the winemaking, previous to the fermentation. Certainly, *BglA* displays great potential for its application in juices. In this work only grape juice has been tested but the results in model juice suggest that any other fruit juice would be a suitable matrix for *BglA*, especially those having a pH over 3.5, like some apple, orange or lemon juices (Yan et al., 2018).

Finally, it is worth to highlight that amounts of freeze-dried protein (mg), and not specific activities (U/mg) have been compared in this study. Due to the lower specific activity of Rapidase® under the same standard conditions, higher amount of freeze-dried preparation of Rapidase® would be required to achieve the same results as *BglA*. Clearly Rapidase® is stable, at least at low sugar content, and when the results are normalised per U of activity its performance is higher, however, from an industrial cost-effective point of view *BglA*, offers both as a crude preparation and in its purified form, 13-fold and 34-fold higher activity than the commercial preparation, which results in less quantity of catalyst needed during the wine-making process. In addition, the use of a purified catalyst eliminates the risk of side activities which are always possible in crude preparations and may limit in fact the quantity that can be added to the fermentation process (Sieiro, Villa, Da Silva, García-Fraga, & Vilanova, 2014)

4 Conclusions

β-Glucosidases are used in the wine industry to enhance the aroma of wines and have been proposed to remediate smoke taint defects. The hydrolytic capacity of *BglA* for geraniol glucoside and guaiacol glucoside was significantly better than the commercial preparation in all the tested matrices with high sugar content, where the performance of Rapidase® decreases considerably. In fact, *BglA* high activity in the presence of glucose, outperforms also other reported fungal β-glucosidase such as the one *W. anomalus*, which retains only 25 % of activity in the presence of 4 % (w/v) glucose (Sabel et al., 2014), or that from a *A. niger* which retains 64 % of activity when 1 g/L glucose (0.1 % w/v) is added to the reaction but only 2 % when 100 g/L glucose (10 % w/v) is used (Martino et al., 2000). *BglA* is also stable and active in the presence of ethanol as it can be observed from the results in model wines. On the other hand, the activity of *BglA* is very pH dependent and in matrices with a pH below 3.5, like real white wine, the enzyme is not able of hydrolysing glycosides. Future work on enzyme immobilization will be carried out to compare the enzymatic stability at low pH and try to improve it.

Great tolerance to sugar content along with improved performance over a broad pH range makes *BglA* an excellent candidate for aroma amelioration and mitigation of smoke taint in grape juices and wines, especially during the early stages of the winemaking process when the sugar content and the pH range is higher than in fermented wines. Future work will also include testing the enzyme in other model and real wines as well as a sensory evaluation of treated wines.

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6 Abbreviations used

ANOVA, analysis of variance; CIS, cooled inlet system; DVB/CAR/PDMS divinylbenzene/carboxen/polydimethylsiloxane, *E.coli*, *Escherichia coli*; GC, gas chromatography; g, gram; hl, hectolitre; h, hours; IPTG, Isopropil-β-D-1-tiogalactopyranoside; MW1, model wine 1; MW2, model wine 2; MJ, model juice; MPS, multipurpose sampler LB, Luria-Bertani; multipurpose sampler; MS, mass spectrometry; µg, microgram; OD, optical density; *p*NPG, paranitrophenol-β-D-Glucopyranoside; RW, red wine; rpm, revolutions per minute; s, seconds; SIM, selected ion monitoring; SPME-GCMS, solid-phase microextraction, gas chromatography mass spectrometry; VP, volatile phenols; v/v, volume volume; w/v, weight per volume; WJ, white juice; WW, white wine.

7 Conflict of interest

The authors declare no conflict of interest in publishing this work.

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