

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

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18 **ABSTRACT**

19 β -Glycosidases enhance wine aroma by releasing volatile aglycones from non-volatile glycosides.
20 Commercial preparations contain primarily pectinases and only display β -glycosidase as a secondary
21 activity which limits their potential. Here, the extremophilic β -glucosidase A from *Halothermothix*
22 *oreni*, (*BglA*) has been compared with Rapidase[®] for the production of aromatic wines and in the
23 remediation of smoke-tainted wines. Model systems as well as real juices and wines have been
24 enriched with geranyl glucoside, typical of white varieties, and guaiacyl glucoside, commonly found
25 in red wines exposed to oak and wines made from grapes exposed to smoke. The hydrolytic capacity

26 of *BglA* was evaluated by measuring the released volatiles in the gas phase with Solid Phase
27 Microextraction and GC-MS. *BglA*, despite an apparent instability at low pHs, is twice as effective
28 in the release of volatiles in sweeter wines and in grape juices offering an excellent alternative for the
29 early stages of the winemaking process and in the juice industry.

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

31 **1 Introduction**

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

38 Monoterpenes, formed in grapes during ripening, are crucial components of the varietal wine bouquet
39 of Muscat and floral varieties (Sánchez Palomo, Pérez-Coello, Díaz-Maroto, González Viñas, &
40 Cabezudo, 2006) but a major fraction is entrapped as flavourless, odourless, non-volatile glycosides,
41 constituting an important reservoir of aroma (Skouroumounis, Massy-Westropp, Sefton, & Williams,
42 1995). Monoterpenes can be liberated from their glycosides by acid or enzymatic hydrolysis; but as
43 acid hydrolysis is a slower process (Mojsov, Andronikov, Janevski, Jordeva, & Zezova, 2015;
44 Wilkowska & Pogorzelski, 2017) and can cause rearrangements of the released aglycones, enzymes
45 represent a useful alternative and can be added to maximize the aromatic potential of wines
46 (González-Pombo, Fariña, Carrau, Batista-Viera, & Brena, 2014; Günata, Dugelay, Sapis, Baumes,
47 & Bayonove 1993).

48 Phenolic glycosides are also formed when berries are exposed to smoke from bush fires and
49 prescribed forest burns as the grapevines can uptake smoke constituents like guaiacols, cresols and

50 syringols, and accumulate them in the form of glycoconjugates. However in this case, their hydrolysis
51 leads to the release of volatile phenols (VP) giving the wine a “smoky” or “ashy” aroma/flavour
52 (Hayasaka, Dungey, Baldock, Kennison, & Wilkinson, 2010; Mayr et al., 2014; Singh et al., 2011).
53 In addition, breakdown of glycosides of volatile phenols in the mouth, mediated by enzymes of the
54 oral microflora can also contribute to smoky and ashy aftertaste (Parker et al., 2012). In this case, if
55 enzymatic hydrolysis can be performed effectively during the wine processing, phenolic glycosides
56 can be reduced, and the release of VPs can then be minimised using different techniques (van der
57 Hulst et al., 2019), improving the overall flavour.

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

74 Extremophiles, organisms very well adapted to extreme environmental conditions unbearably hostile
75 or even lethal for other forms of life (Rampelotto, 2013), constitute a novel and alternative source of

76 enzymes for industrial application. Extremozymes are generally more capable to withstand industrial
77 processes in comparison with their mesophilic counterparts (Elleuche, Schröder, Sahm, &
78 Antranikian, 2014). Among extremophiles, enzymes from halophilic microorganisms tolerate very
79 high salinity, which normally leads to denaturation, aggregation, and precipitation of most other
80 proteins. Genomic and structural analyses have established that halophilic enzymes have a higher
81 pro-ratio of acidic amino acids versus hydrophobic ones and altered hydrophobicity compared to
82 mesophilic enzymes, which enhance solubility and promote function in low water activity conditions
83 (DasSarma & DasSarma, 2015). Adaptation to solvents follows the same principle as adaptation to
84 salt, and thus, halophilic enzymes may be a valid option for biocatalytic processes performed in
85 water/solvent environments like wines (Alsafadi & Paradisi, 2013).

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

94 **2 Materials and methods**

95 *2.1 Chemicals*

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

100 %, D-(-)-fructose European Pharmacopoeia 98% and DL-malic acid \geq 98 % (capillary GC) were
101 purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Hepes \geq 99.5 % (titration),
102 sodium chloride, acetone Suprasolv® ECD, ethanol for liquid chromatography LiChrosolv®, tartaric
103 acid and *p*-nitrophenyl β -D-glucopyranoside (*p*NPG) were obtained from Merck Pty Ltd (Kilsyth,
104 Victoria, Australia). Rapidase® Revelation Aroma enzymatic preparation was purchased from
105 Vintessential Laboratories (Dromana, Victoria, Australia). Geranyl glucoside, guaiacyl glucoside, d₇-
106 geraniol and d₃-guaiacol were synthesised in-house (Hayasaka et al., 2010; Parker et al., 2012;
107 Pedersen, Capone, Skouroumounis, Pollnitz, & Sefton, 2003; Pollnitz, Pardon, Sykes, & Sefton,
108 2004).

109 2.2 Microbial strains

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

114 2.3 Enzyme expression, purification and lyophilisation

115 Cells of *E. coli* BL21 (DE3) harbouring the recombinant plasmid were grown at 37 °C in Luria-
116 Bertani medium supplemented with ampicillin (0.1 mg/mL). When the OD₆₀₀ was between 0.6-0.8,
117 isopropyl β -D-1-thiogalactopyranoside was added as inductor for the overexpression of the enzyme
118 and the culture left at 30 °C overnight. Cells were harvested at 4500 G, 4 °C, 20 min and the pellet
119 stored at -20 °C until purification.

120 The cell pellet was resuspended in buffer (HEPES (50 mM), sodium chloride (150 mM), imidazole
121 (10 mM), pH 7.5) and cells were broken by sonication (6 min cycle, 5s on, 5s off, 50 % amplification).
122 The lysate was collected by centrifugation at 14500 G, 1 h, 4° C, and the pellet was discarded.

123 The supernatant was then filtered through Millex® PVDF 0.45 μ m filter before loading it onto a
124 HisTrap IMAC column previously loaded with NiSO₄ 0.1 M and washed with loading buffer (HEPES

125 (50 mM), sodium chloride (150 mM), imidazole (10 mM), pH 7.5). The column was washed with
126 loading buffer until a plateau in the UV₂₈₀ absorbance was reached. Low affinity binding proteins
127 were eluted using a step gradient 10 % elution buffer and the protein of interest was eluted using 100
128 % elution buffer (HEPES (50 mM), sodium chloride (150 mM), imidazole (300 mM), pH 7.5). The
129 enzyme was dialysed overnight, flash frozen in liquid nitrogen and freeze dried overnight. (Labconco
130 8 Port Manifold on Consolo Freeze Dryer).

131 2.4 Protein quantification and SDS-PAGE

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

136 2.5 Activity test

137 β -glucosidase activity was determined spectrophotometrically by adding 10 μ L of the suitable
138 enzyme dilution and 290 μ L of 10 mM *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) in buffer HEPES
139 50 mM, pH 7.4 at 25 °C. The specific activity (U/mg) was expressed as μ mol of product formed per
140 minute per milligram of protein.

141 2.6 Model wines and juices

142 Two different model wines were selected in representation of a completely sugar dry wine and a table
143 wine with sugar concentrations typical for Australian commercial wines (Godden, Wilkes, &
144 Johnson, 2015). Model wine 1 (MW1) consisted of saturated potassium hydrogen tartrate with 10 %
145 (v/v) ethanol, pH 3.5. Model wine 2 (MW2) consisted of saturated potassium hydrogen tartrate with
146 10% (v/v) ethanol, 6 g/L glucose, 6 g/L fructose, pH 3.5.

147 Model juice (MJ) was prepared using water, 100 g/L glucose, 100 g/L fructose, 0.2 g/L citric acid, 3
148 g/L malic acid, 2.5 g/L tartaric acid, pH 3.7. pH was adjusted with tartaric acid 1M in all cases.

149 2.7 *Real wines and juices*

150 Two commercially available wines, one white (WW) and one red (RW), and a Chardonnay grape
151 juice (WJ) produced in-house were used. A 2017 Chardonnay from Riverina, Australia with an
152 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
153 Shiraz from South Eastern Australia with an alcohol content of 13.9% v/v, 5.8 g/L glucose and
154 fructose, titratable acid 6.2 g/L and pH 3.66 and a Chardonnay juice with total soluble solids 22.6
155 °Brix (~20 % total sugar content), 52 mg/L SO₂ and pH 3.5. Chardonnay and Shiraz grape varieties
156 were chosen due to their low monoterpene content.

157 2.8 *Enzymatic treatment*

158 In separate 20 mL SPME vials, 3 mL of MW1, MW2, MJ, WW, RW and WJ were spiked with 5 µg
159 of geranyl glucoside and 5 µg of guaiacyl glucoside. The amount added to each sample of Rapidase®
160 or *BglA* was 0.01 mg/mL. The samples were left shaking at 22 °C over different incubation periods
161 to allow enzymatic hydrolysis. The reaction was stopped by adding 2 mL of saturated CaCl₂. Internal
162 standards, d₇-geraniol and d₃-guaiacol, were added (2 µg) and the liberated aglycones were analysed
163 using SPME-GCMS. All experiments were carried out in triplicate.

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

166 2.9 *GC-MS analysis of volatiles*

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

174 The gas chromatograph was fitted with a 30 m x 0.25 mm Agilent J&W DB-35ms Ultra Inert column,
175 0.25 µm film thickness. The carrier gas was helium, linear velocity was 36 cm/s, and flow rate was 1
176 mL/min. The oven temperature, was held at 40 °C for 1 min, increased to 240 °C at a 5 °C/min rate,
177 and held at this temperature for 2 min. The injector temperature was 220 °C, and the transfer line was
178 held at 240 °C. Positive electron ionisation mass spectra at eV were recorded in SIM mode with *m/z*
179 69, 81, 93, 99, 109, 121, 123, 124, 127, 128, 136, 154, and 161 with dwell 25 ms (See section 1 of
180 the supplementary information for geraniol and guaiacol quantifiers and qualifiers for identification
181 with MS).

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

183 The hydrolysis percentages were calculated using the following equations:

$$184 \quad \% \text{ geraniol release} = \left(\left(\frac{\text{amount of free geraniol detected}}{\text{amount of geranyl glucoside added}} \right) \frac{316}{154} \right) \times 100$$

$$185 \quad \% \text{ guaiacol release} = \left(\left(\frac{\text{amount of free guaiacol detected}}{\text{amount of guaiacyl glucoside added}} \right) \frac{286}{124} \right) \times 100$$

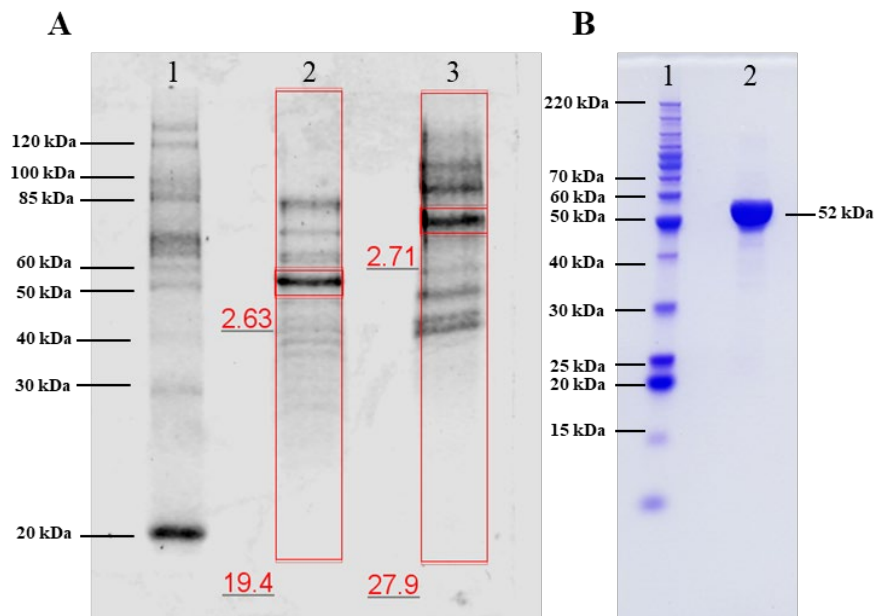
186 2.10 Data analysis

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

193 **3 Results and discussion**

194 **3.1 Protein expression, purification, and lyophilisation.**

195 *BglA* was expressed with an average yield of 53 mg protein/L of culture. Estimation by quantification
196 analysis (using Li-cor Odyssey Fc scanner and software Image Studio version 4.0) suggested that 13
197 % of the crude extract corresponded to *BglA* (Fig 1). The activity of the crude extract was found to
198 be 2.1 U/mg of total proteins. The enzyme was then purified by metal affinity chromatography, to
199 better assess its hydrolytic capacity, and SDS-PAGE was done to assess its purity. For Rapidase[®],
200 estimation by quantification suggests that 10 % of the commercial preparation would correspond to
201 β -glucosidases (Fig 1).



202

203 **Figure 1.** A. Quantification of the bands corresponding to *BglA* in the crude extract and to the β -glucosidase in the Rapidase[®]
204 preparation are indicated in red, the signal is expressed in relative fluorescence units (RFU) using Li-cor Odyssey Fc scanner and
205 software Image Studio version 4.0), (1) ThermoFisher Scientific PageRuler™ Unstained Protein Ladder, (2) *BglA* in the crude extract
206 (5 μ g) (3) β -glucosidases in Rapidase[®] (5 μ g). B. SDS-PAGE after *BglA* purification (1) Invitrogen™ BenchMar™ Protein Ladder (2)
207 Pure *BglA* (5 μ g).

208

209 Following dialysis, *BglA* was lyophilized and stored at 4 °C until needed. An activity assay under
210 standard conditions was performed before and after lyophilisation confirming enzymatic stability
211 with a specific activity of 5.5 U/mg of protein. Rapidase[®] was used directly from the commercial

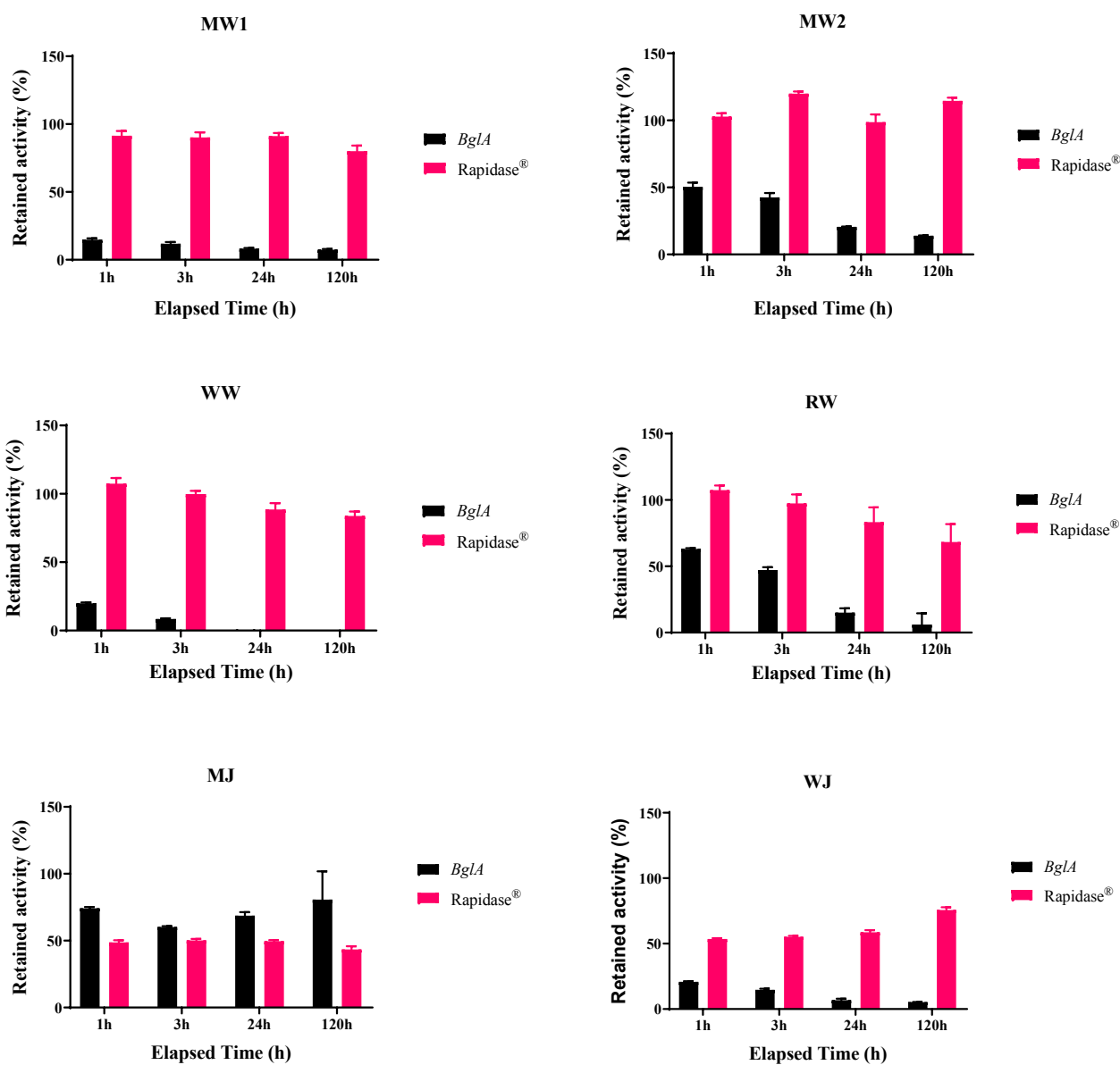
212 packaging with no further treatment. The specific β -glucosidase activity of the commercial
213 preparation was calculated as 0.16 U/mg of protein.

214 3.2 *BglA preliminary assessment in media mimicking wine conditions*

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

222 3.3 *Enzymatic stability*

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



231

232

233

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

237

238 As shown in Figure 2 commercial Rapidase® shows a better stability when incubated in MW1 (10 %
 239 ethanol, no sugar, pH 3.5), MW2 (10 % ethanol, 12 g/L glu+fru, pH 3.5), WW (12.2 % ethanol, 4.9
 240 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
 241 after 5 days of incubation. *BglA* retains 7 % of activity after 5 days incubation in MW1 and no activity
 242 after 24 h in WW. However, after 5 days incubation in MW2, *BglA* shows a retained activity of 14
 243 %, a two-fold increase with respect to MW1. As the sugar content is the only difference between

244 these two model matrices, it appears that fructose and glucose have a protective effect towards *BglA*
245 stability. The stability of *BglA* improves when incubated in RW, retaining 15 % of activity, which is
246 linked to the difference in pH between the two systems; 3.35 for WW (white) and 3.66 for RW (red).
247 On the contrary, Rapidase[®] shows the opposite behaviour with a 15 % drop in activity when incubated
248 for 5 days in RW in comparison with WW and this could be caused by the higher glucose and fructose
249 content in the red wine (5.8 g/L for the red wine and 4.9 g/L for the white wine) which negatively
250 impacts the stability of the commercial preparation.

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

257 3.4 Analytical determination of volatiles released upon enzymatic hydrolysis of glucosides

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

267 3.4.1 Release of volatiles from glucosides in model and real wines

268 Interestingly, despite a lower stability determined for *BglA* (Fig 1), the catalytic efficiency of this
 269 enzyme in MW1 equals that of Rapidase® in the release of geraniol with no significative differences
 270 (Table 1). The release of guaiacol by *BglA* is, on the other hand, significantly better after 5 days (97
 271 %) in comparison with Rapidase® (75 %). The observed drop in the hydrolysed substrate after 8 days
 272 incubation is a known artefact due to the rearrangement of the terpenes under acidic conditions
 273 (Hampel, Robinson, Johnson, & Ebeler, 2014; Skouroumounis & Sefton, 2000).

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

MW1	Substrate	Enzyme	Time ****a	Geraniol released (μg)	% Hydrolysis
	Geranyl glucoside	<i>BglA</i>	24h	2.33 \pm 0.09	96
			5d	2.51 \pm 0.11	\geq 99
			8d	2.19 \pm 0.05	90
		Rapidase®	24h	2.42 \pm 0.03	99
			5d	2.65 \pm 0.15	\geq 99
			8d	2.52 \pm 0.11	\geq 99
	Substrate	Enzyme **b	Time ****b	Guaiaciol released (μg)	% Hydrolysis
	Guaiacyl glucoside	<i>BglA</i>	24h	1.57 \pm 0.07	72
			5d	2.12 \pm 0.010	97
			8d	1.20 \pm 0.27	55
		Rapidase®	24h	0.64 \pm 0.04	29
			5d	1.64 \pm 0.12	75

MW2	Substrate	Enzyme ****a	8d	1.39 ± 0.07	64
			Time *****a	Geraniol released (µg)	% Hydrolysis
Geranyl glucoside	<i>BglA</i>		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	<i>BglA</i>		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

285

286 **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
287 Model Wine 2. ****p ≤ 0.0001; ***p ≤ 0.001; *p < 0.05. a=geraniol, b=guaiacol.

288

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

295 Rapidase® shows improved activity in WW (Table 2), while the hydrolytic capacity of *BglA* is very
296 limited. On the other hand, after 5 days incubation in RW, *BglA* releases over 30 % geraniol and over
297 3 % guaiacol. This improvement of the performance of *BglA* in red wine is probably related to a 0.31
298 pH units difference and 0.9 g/L sugars between white wine and red wine.

WW	Substrate	Enzyme *****a	Time	Geraniol released (µg)	% Hydrolysis
				Geraniol released (µg)	% Hydrolysis
Geranyl glucoside		<i>BglA</i>	5d	0.01 ± 0.01	0
			Rapidase®	5d	1.94 ± 0.02
Substrate	Enzyme ****b	Time	Guaiacol released (µg)	% Hydrolysis	
Guaiacyl glucoside		<i>BglA</i>	5d	0.00	0
			Rapidase®	5d	0.24 ± 0.00
Substrate	Enzyme ***a	Time	Geraniol released (µg)	% Hydrolysis	
Geranyl glucoside		<i>BglA</i>	5d	0.75 ± 0.06	31
			Rapidase®	5d	2.00 ± 0.07
Substrate	Enzyme **b	Time	Guaiacol released (µg)	% Hydrolysis	

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

299

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

302 3.4.2 Glycosides release in model and real juice

303 Results in model juice (MJ) (Table 3) highlight an outstanding performance of *BglA* in comparison
 304 with the commercial preparation. While Rapidase® hydrolysis capacity is below 6 % for both
 305 compounds, the percentage of glycosides hydrolysed by *BglA* is over 60 % for geraniol and over 25
 306 % for guaiacol after 5 days incubation, reaching 45 % after 8 days.

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

MJ	Substrate	Enzyme ****a	Time ***	Geraniol released (µg)	% Hydrolysis
	Geranyl glucoside	<i>BglA</i>	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	<i>BglA</i>	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	<i>BglA</i>	5d	0.26 ± 0.01	10
		Rapidase®	5d	0.14 ± 0.01	6
	Substrate	Enzyme	Time	Guaiacol released (µg)	% Hydrolysis
	Guaiacyl glucoside	<i>BglA</i>	5d	0.03 ± 0.01	2
		Rapidase®	5d	0.02 ± 0.01	1

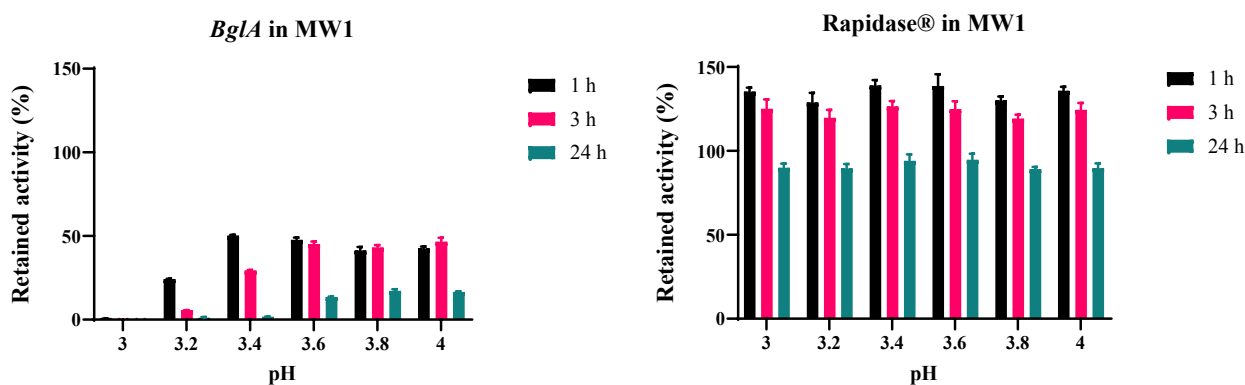
310

311 **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
 312 Juice (WJ). **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$, * $p < 0.05$. a=geraniol, b=guaiacol.

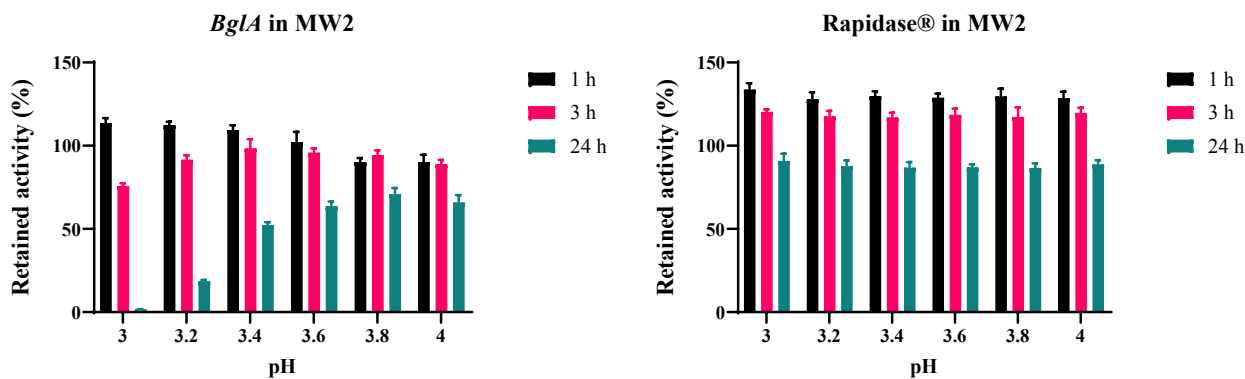
313 3.5 Detailed pH stability assay in MW1, MW2 and MJ

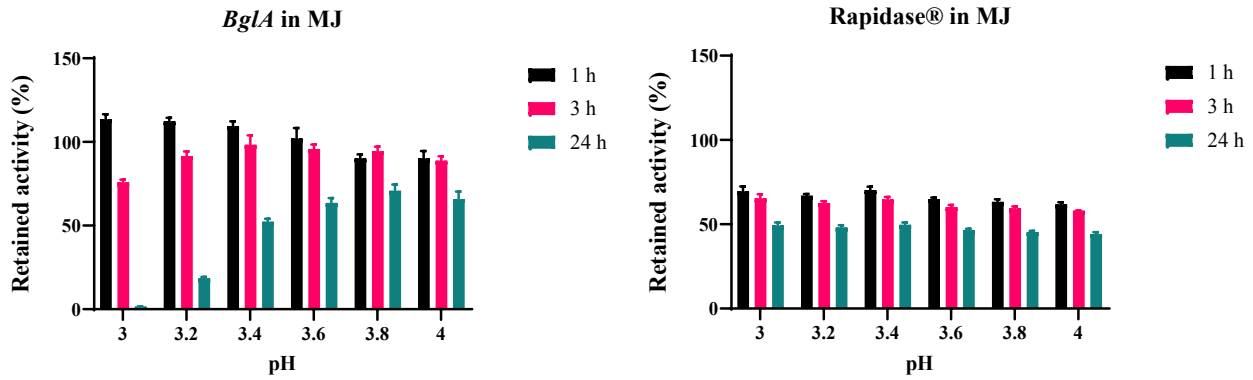
314 The results above show that *BglA* loses stability between pH 3 and 4. To further narrow the pH fork
315 causing it, a more accurate stability assay of *BglA* and Rapidase® was carried out with 0.2 pH intervals
316 between pH 3 and 4 in MW1, MW2 and MJ at 22 °C. Retained activity was measured after 1 h, 3 h,
317 24 h and 120 h, same intervals as in the enzyme stability experiment in different matrices summarised
318 in Figure 2. Unfortunately, measures after 120 h incubation were no longer reliable, probably due to
319 sample concentration by water loss (results not shown).

320



321





322

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

325

326 *BglA* loses virtually all activity within 24h of incubation in MW1 and MW2 at a pH lower than 3.6.

327 However, in MJ at pH 3.2, the enzyme still retains 20 % of its activity after the same incubation time.

328 The experiments clearly show that the more sugar the matrix contains, the higher activity *BglA* retains,

329 at all pHs. Rapidase® is clearly independent on pH and the preparation is equally stable between 3

330 and 4, however, the sugar content present in MJ reduces its activity by almost 50 % very rapidly.

331 These results confirm once again the suitability of *BglA* for matrices with high content of sugars, for

332 example during the maceration or other early stages of the winemaking, previous to the fermentation.

333 Certainly, *BglA* displays great potential for its application in juices. In this work only grape juice has

334 been tested but the results in model juice suggest that any other fruit juice would be a suitable matrix

335 for *BglA*, especially those having a pH over 3.5, like some apple, orange or lemon juices (Yan et al.,

336 2018).

337 Finally, it is worth to highlight that amounts of freeze-dried protein (mg), and not specific activities

338 (U/mg) have been compared in this study. Due to the lower specific activity of Rapidase® under the

339 same standard conditions, higher amount of freeze-dried preparation of Rapidase® would be required

340 to achieve the same results as *BglA*. Clearly Rapidase® is stable, at least at low sugar content, and

341 when the results are normalised per U of activity its performance is higher, however, from an

342 industrial cost-effective point of view *BglA*, offers both as a crude preparation and in its purified form,

343 13-fold and 34-fold higher activity than the commercial preparation, which results in less quantity of
344 catalyst needed during the wine-making process. In addition, the use of a purified catalyst eliminates
345 the risk of side activities which are always possible in crude preparations and may limit in fact the
346 quantity that can be added to the fermentation process (Sieiro, Villa, Da Silva, García-Fraga, &
347 Vilanova, 2014)

348 **4 Conclusions**

349 β -Glucosidases are used in the wine industry to enhance the aroma of wines and have been proposed
350 to remediate smoke taint defects. The hydrolytic capacity of *BglA* for geraniol glucoside and guaiacol
351 glucoside was significantly better than the commercial preparation in all the tested matrices with high
352 sugar content, where the performance of Rapidase[®] decreases considerably. In fact, *BglA* high activity
353 in the presence of glucose, outperforms also other reported fungal β -glucosidase such as the one *W.*
354 *anomalus*, which retains only 25 % of activity in the presence of 4 % (w/v) glucose (Sabel et al.,
355 2014), or that from a *A. niger* which retains 64 % of activity when 1 g/L glucose (0.1 % w/v) is added
356 to the reaction but only 2 % when 100 g/L glucose (10 % w/v) is used (Martino et al., 2000). *BglA* is
357 also stable and active in the presence of ethanol as it can be observed from the results in model wines.
358 On the other hand, the activity of *BglA* is very pH dependent and in matrices with a pH below 3.5,
359 like real white wine, the enzyme is not able of hydrolysing glycosides. Future work on enzyme
360 immobilization will be carried out to compare the enzymatic stability at low pH and try to improve
361 it.

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

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372 Australian Government.

373 **6 Abbreviations used**

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

383 **7 Conflict of interest**

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

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