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1 Identification of *Bacillus* strains producing glycosidases active on rutin and
2 grape glycosidic aroma precursors

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1

15 **Abstract**

16 Most of the glycosidic aroma precursors in grape consist of diglycosides such as rutinose.
17 Yeast glycosidases have been extensively studied but their activity is limited to
18 monoglucosidic precursors. Yet, there is no study about application of glycosidases active on
19 rutinose in grape. In this study, we screened *Bacillus* isolates for their glycosidase activities
20 on rutin and grape aroma precursors. One *Bacillus amyloliquefaciens* and one *Bacillus*
21 *licheniformis* strains produced extracellular glycosidases, active on both rutin and *para*-
22 nitrophenol- α -L-rhamnoside. The specific rutinoidic activities were 14 and 9.0 μ kat/mg
23 respectively, and supernatants were active on Muscat grape glycosidic aroma precursors. Both
24 strains were able to release monoterpenoids and benzenoids. The most released
25 monoterpenoid in grape pomace was neric acid, with 378 μ g/kg and the most released
26 benzenoid was 2-phenylethanol, with 189 μ g/kg for *B. licheniformis*. These glycosidases are
27 interesting tools to target rutinoidic glycosides in grape and can be applied as a new way of
28 valorising grape pomace.

29

30 **Keywords:** rutin; grape; glycosides; glycosidase; *Bacillus licheniformis*

31

32

33 **Introduction**

34 Fruit and vegetable transformation generates tons of by-products every year. Although by-
35 products used to be considered as “waste”, it is nowadays accepted that they are resourceful
36 and can be reused as colorants, source of proteins and dietary fibres and can hold some
37 antioxidants activities (Gowe, 2015). They are also studied for their aroma potential through
38 their high quantity of glycoside aroma precursors (Liang et al., 2020). A glycoside is a
39 molecule, generally a volatile compound or polyphenol, bound to a sugar moiety. Most of the
40 glycosides are not degraded during fruit and vegetable processes and they are part of by-
41 products. The hydrolysis of these glycosides is conducted by acid or enzymatic hydrolysis.
42 Acid hydrolysis leads to chemical transformations, like terpenic rearrangements or oxidations,
43 of the released volatile compounds (Dziadas & Jeleń, 2016) and is therefore less studied
44 nowadays.

45 Enzymatic hydrolysis is the most widespread hydrolysis tool employed to free volatile
46 compounds from glycosidic precursors. Some industrial glycosidases are AR2000 and
47 Rapidase X-press (DSM, Delft, The Netherlands), Pectinol and Rohapect C (AB Enzymes
48 GmbH, Darmstadt, Germany) and Aromase (Amano Enzyme Co., Nagoya, Japan). These
49 enzymes are already used to release aroma precursors from several matrices, such as tea
50 (Zhang et al., 2020), hop (Holt et al., 2019), strawberry (Gaborieau et al., 2018) or grape
51 (Sarry & Günata, 2004). They are also industrially employed to enhance varietal aromas in
52 wine or tea (Ni et al., 2021; Sun et al., 2018). Furthermore, the released volatiles can also be
53 extracted from the matrix. Although it is still not quite common, new sources of natural
54 flavours, produced from by-products, would be interesting for perfume industries as the
55 consumer is more and more aware of the sourcing of its cosmetic products.

56 However, most of enzymes employed today are a mixture of several enzymes produced by
57 fungi. They exhibit side activities that might release undesired aromas during the enzymatic
58 process, which impact the organoleptic properties of the produced wine (Fia et al., 2016).

59 Screening of more specific glycosidases is a strategy that can overcome the off-flavours
60 release. The matrix needs however to be well characterised as glycosidic moieties can be
61 widely different from one matrix to another. Grapes received extensive attention throughout
62 the years and are still today the most studied matrices in terms of glycosidically bound
63 volatiles. Main glycosides in grape are constituted of 6-*O*- α -L-arabinofuranosyl- β -D-
64 glucopyranosides, 6-*O*- β -D-apiofuranosyl- β -D-glucopyranosides (acuminose) and 6-*O*- α -L-
65 rhamnopyranosyl- β -D-glucopyranoside (rutinose) (Liang et al., 2020). Numerous studies
66 focused on the application of yeasts and lactic acid bacteria (LAB) glycosidases on grape
67 (Hernandez-Orte et al., 2009; Tufariello et al., 2021). These glycosidases are mainly active on
68 monoglycosides of β -D-glucose, which are not the main glycosides in grape. The use of
69 diglycoside-specific glycosidases (diglycosidases) would allow a more complete release of
70 volatile compounds.. To our knowledge, only one study dealt with the incubation of a
71 rutinoidase with grape glycosides (Minig et al., 2011). For now, studies of such glycosidases
72 were mainly focused on fungi (Koseki et al., 2018). Yet, some bacteria are known to produce
73 diglycosidases (Neher et al., 2016) and among them *Bacillus* spp. have already shown activity
74 toward rutin (Yang et al., 2012; Zhu et al., 2017), one of the main glycosidic moiety in grape.
75 Such glycosidic moiety has also been identified in other matrices like acerola (Boulanger &
76 Crouzet, 2001), nutmeg (Ananthakumar et al., 2006), passion fruit (Chassagne et al., 1998)
77 and tobacco (Cai et al., 2013) for example. Thus, strains producing glycosidases active on
78 rutinoidic moieties could be used to enhance varietal aromas from industrial food products
79 or to produce natural flavours from by-products for perfume industries.

80

81 The aim of this study was to screen *Bacillus* isolates producing glycosidases active on rutin
82 and grape glycosides. For this purpose, we screened *Bacillus* strains from our laboratory
83 collection for their ability to produce diglycosidases. We then assessed their hydrolytic
84 activity toward rutin and grape glycosides.

85

86 **Material and Methods**

87 Materials

88 The *p*-nitrophenyl (*p*NP) substrates *p*NP- β -D-gluco-pyranoside (*p*NPG), *p*NP- β -D-
89 cellobioside (*p*NPC) and *p*NP- α -L-rhamno-pyranoside (*p*NPR) and the 5-bromo,4-chloro,3-
90 indolyl- β -D-cellobioside (BCIC, X- β -D-Cellobioside) were purchased from Glycosynth
91 (Cheshire, England. Grape (var *Muscat Italia*) was purchased from local market. Solid phase
92 extraction (SPE) cartridges Isolute ENV+ (200 mg and 50 mg) were obtained from Biotage
93 (Uppsala, Sweden). Rutin was purchased from Carbosynth Ltd (United Kingdom). An
94 enzymatic preparation of pectinases and glycosidases, AR2000 was obtained from DSM
95 (Delft, The Netherlands).

96 All other chemicals used were analytical reagents.

97

98 Bacterial isolates used for the screening

99 The 20 isolates used were isolated from local market raw Vietnamese fermented pork
100 sausages, *nem chua* (laboratory collection) (Lorn et al., 2021). They were obtained by
101 successive streaks on LB agar medium with incubations for 24 h at 37°C and were positive
102 for CMCase activities using the screening method described by Guo *et al.* (2017) (Guo et al.,

103 2017). They were cultured in LB-agar medium or LB broth at 37°C under orbital agitation.
104 When specified, LB was supplemented with 1% (w/v) CMC or rutin.

105

106 DNA extraction and molecular identification by 16S rRNA gene sequencing

107 Genomic DNA was extracted using the InstaGene™ Matrix (Bio-Rad) as described
108 previously (Lorn et al., 2021). The V1-V9 regions of 16S rRNA gene were amplified using
109 primers 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R' (5'-
110 TACGGYTACCTTGTTACGACTT-3'). DNA sequencing reactions were performed by
111 Eurofins Genomics (Germany) using both forward and reverse primers (16S-SeqF, 5'-
112 AGTAGGGAATCTTCCACA-3' and 16S-SeqR, 5'-CTTGCCACCTACGTATTA-3').
113 Taxonomic identification was determined by comparing the sequence of each isolate with
114 those reported in the basic BLAST database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

115

116 Intracellular protein extraction

117 One millilitre of bacterial culture was centrifuged (10000 g, 5 min). Supernatant was collected
118 for enzymatic activities. The pellet was washed three times with sterile distilled water (1 mL).
119 500 µL of the cell suspension were then shaken twice for 35 s at 6 m.s⁻¹ with 50 mg glass
120 beads (Sigma, 100 µm diameter) in a FastPrep™ instrument (FP12, BIO101, MP Biomedicals
121 Illkirch, France). After centrifugation (10000 g, 5 min), the supernatant was collected for
122 intracellular enzymatic activities.

123

124 Screening of BCICase activity

125 Bacterial isolates were cultured in LB-CMC (1% w/v) broth for 24 h at 37°C. Ten μ L of
126 culture were dropped on LB-CMC (1% w/v) agar plate supplemented with 0.01% (w/v)
127 BCIC. Plates were incubated for 48 h at 37°C. When the aglycon was released in the medium,
128 it was air-oxidized and it turned blue (Perry et al., 2006). Thus, blue colonies were observed
129 for isolates showing positive BCICase activity.

130

131 Assay of enzymatic activities toward *Para*-nitrophenol-based activities (*p*NP- β -D-glucoside;
132 *p*NP- β -D-cellobioside; *p*NP- α -L-rhamnoside)

133 Activities toward nitrophenol-based glycosides were assayed following the protocol of
134 Deshpande *et al.* (Deshpande et al., 1984) with adapted volumes for microplate reactions (10
135 μ L of sample were incubated in 90 μ L of *p*NP glycoside solution) and with 2.77 mM of
136 *p*NPC. AR2000 was used as control. One international unit of β -glucosidase, β -
137 cellobiosidase or α -rhamnosidase activity (μ kat) was defined as the amount of enzyme that
138 produced 1 μ mol of *p*NP per seconde under the assay conditions.

139 Protein concentrations were determined according to the Bradford method (Bradford, 1976)
140 with bovine serum albumin (BSA) as standard. Specific activities toward each substrate were
141 expressed as units per milligram of protein.

142

143 Assays of rutinosidase activity

144 Assays of rutinosidase activity were conducted following the protocol established by Weiz *et*
145 *al.* (Weiz et al., 2017) with the following modifications. Briefly, 50 μ L of sample were
146 incubated with 450 μ L of 0.11% (w/v) rutin solubilized in sodium acetate buffer pH 5 and 5%
147 (w/v) DMSO at 50°C for 24 h. One volume (500 μ L) of ethyl acetate was added and mixed,

148 the tubes were then centrifuged (4200 g, 3 min). 50 μ L of supernatant were mixed with 960
149 μ L of sodium carbonate solution (50 mM, pH 10) and incubated at room temperature for 5
150 min. The absorbance was finally measured at 320 nm. One international unit of rutinoidase
151 activity (μ kat) was defined as the amount of enzyme that produced 1 μ mol of quercetin per
152 seconde under the assay conditions.

153 Extraction of grape glycosidic aroma precursors (glycosides)

154 Extraction of grape glycosides was conducted as followed: The grape juice was separated
155 from the pomace using a juice extractor. It was centrifuged (4000 g, 10 min) and filtered
156 through n°4 Whatman filter. Grape pomace (200 g) was suspended in 800 mL of phosphate
157 buffer (150 mM, pH = 7.2) and incubated at room temperature in darkness under gentle
158 agitation. After 24 h, the solution was centrifuged (4000 g, 10 min) and filtered.

159 For both matrices, Isolute ENV+ SPE cartridges (200 mg, 6 mL) were conditioned by adding
160 4 mL of methanol and then washed twice with 4 mL distilled water. Juice or pomace solution
161 (60 mL) were loaded per SPE cartridge and washed twice with 4 mL distilled water. Free
162 volatile compounds were eluted by 4 mL of a dichloromethane:pentane solution 1:2 (v/v).
163 Glycoside bound volatiles were eluted with 4 mL of methanol. The fraction was evaporated to
164 dryness under nitrogen stream and re-dissolved in 12 mL of sodium-acetate buffer (pH 5).
165 The solution was rinsed three times with the same volume of a dichloromethane-pentane
166 mixture. It was splitted into aliquots of 1.8 mL and conserved at -20°C until used.

167

168 Enzymatic hydrolysis and SPE extraction

169 Aliquots of the glycoside solution were defrosted at room temperature. 200 μ L of AR2000
170 solution (45 g/L, sodium-acetate buffer, pH 5) or of the bacterial supernatant were added and
171 the vials were incubated at 45°C under agitation for 120 h. At the end of the incubation, vials

172 were immersed in 4°C water bath to slow down the enzymatic reaction. 200 µL of 4-nonanol
173 (25 mg/kg, methanol) were added as internal standard and the solution was loaded into a
174 preconditioned ENV+ SPE cartridge (50 mg, 3 mL). Cartridges were conditioned the same
175 way as described above but volumes were divided per four. Free volatile compounds were
176 eluted with 1 mL of a dichloromethane-pentane mixture and traces of water were removed by
177 addition of anhydrous Na₂SO₄. Samples were concentrated under nitrogen steam up to 50 µL
178 before GC-MS analysis.

179

180 GC-MS chromatographic conditions

181 Measurements of released bound volatiles were performed using a gas chromatograph
182 coupled with a mass spectrometer with an Electron Impact (EI) ionization mode (GC/MS,
183 PerkinElmer, Clarus SQ 8 S, 580 series). GC was equipped with an Elite-5MS capillary
184 column (30 m length, 0.25 mm inner diameter and 0.25 µm thick film). Helium was used as
185 carrier gas at 1.0 mL/min and split ratio 1:10. The temperature of the injector was 250°C. The
186 run time was recorded in full scan mode (50-350 *m/z* range). Peaks were identified by
187 comparing their mass spectra and their linear retention index (LRI) with the NIST (National
188 Institute of Standards and Technology, USA) library. Retention indices were determined by
189 using a homologous series of *n*-alkanes (C₈–C₁₈) (**table 1**). They were quantified by
190 comparison of their chromatographic peak areas with that corresponding to the internal
191 standard (4-nonanol).

192

193 Statistical analysis

194 All experiments were carried out in three biological replicates. The data were analysed using
195 analysis of variance (one-way Anova) and Tukey's Honestly Significant Difference test

196 (HSD) or *student* test (*t*-test) (the level of significance was 5%) by the MINITAB statistical
197 software (version 19.2020.1.0).

198

199 **Results**

200 1) Screening of *Bacillus* isolates for diglycosidase activity

201 As most of grape aroma precursors are diglycosidic, the release of varietal aromas could be
202 greatly improved by using exogenous microbial diglycosidases instead of the monoglycoside-
203 specific glycosidases currently used. However, the lack of commercial diglycosides slows
204 down the research on this topic. As there is no chromophore-based rutinoid available, we
205 first screened the isolates for their diglycosidic activity toward β -D-cellobiose and then we
206 quantified the rutin deglycosylation for the isolates showing the highest activities.

207 The most employed commercial β -D-cellobiose glycosides to measure diglycosidase activity
208 are linked to methylumbelliferone or *para*-nitrophenol (Vlasenko et al., 2010). However, they
209 are not convenient for colony-based screening methods. On one hand, the detection of
210 released methylumbelliferone is fluorometric, and requires specific spectrophotometer. On the
211 other hand, the yellow-colour of nitrophenolate makes the detection difficult in the same-
212 coloured agar media. The 5-bromo,4-chloro,3-indolyl- β -D-cellobioside (BCIC, X- β -D-
213 Cellobioside) is today not common for fast screening purpose but the detection of the activity
214 is convenient because positive colonies turn blue (Perry et al., 2006). Diglycoside activities of
215 positive isolates were then quantified using *pNPC*.

216 *Bacillus* spp. are soil bacteria that are found in many traditionally fermented products
217 (Tamang et al., 2016). They are usually present in high protein food such as soybean, fish or
218 meat products and display proteolytic, cellulolytic, amylolytic and lipolytic activities (Baruzzi

219 et al., 2006; Tamang et al., 2016; Vlasenko et al., 2010). Fermented pork sausage is known to
220 be fermented not only with LAB but with *Bacillus* spp. too, which participates to the ripening
221 of the final fermented product (Baruzzi et al., 2006). Twenty bacterial isolates from fermented
222 pork sausage were subjected to BCIC screening (see **supplemental S1**). Isolates were
223 cultured for 24 h at 37°C in LB agar supplemented with 1% CMC and 0.01% BCIC (w/v).
224 They were all positives to glycosidase activity. They were identified by the mean of the
225 sequencing of 16S rRNA. They all belonged to the *Bacillus* genus: eleven were *Bacillus*
226 *subtilis*, four were *Bacillus thuringiensis*, four were *Bacillus licheniformis* and one was
227 *Bacillus amyloliquefaciens*. In order to estimate the enzymatic activity, the 20 isolates were
228 cultured for 72 h at 37°C in LB broth supplemented with 1% CMC (w/v) to induce enzyme
229 production and supernatant activities toward *pNPC* were assayed (**Figure 1**).

230 The activities ranged from 0.11 nkat/mL (strain *B. thuringiensis* 2-62e) to 1.3 nkat/mL (strain
231 *B. subtilis* 3-8a). *B. subtilis* strains generally showed highest activities, followed by the strain
232 *B. amyloliquefaciens* 1-38c and *B. licheniformis* strains. *B. thuringiensis* strains exhibited the
233 lowest activities toward *pNPC* in our study. Means were compared by the Tukey test at the
234 5% probability level for each isolate within the same species. The isolate giving the greatest
235 activity was chosen, namely *B. subtilis* 3-8a, *B. thuringiensis* 2-1a, *B. licheniformis* 4-4a and
236 *B. amyloliquefaciens* 1-38c. These isolates were used for future enzymatic activities.

237

238 2) Specific activities of selected *Bacillus* strains toward *pNPG*, *pNPR* and rutin

239 To induce diglycosidases production in the screened *Bacillus* spp., grape diglycoside moieties
240 need to be employed. Main grape diglycosides are rutinose, acuminose and 6-*O*- α -L-
241 arabinofuranosyl- β -D-glucopyranosides. The two latter glycosides are not commercially
242 available and need to be extracted from plants (Sakata et al., 2003). Production of

243 rutinoidases can be induced by adding rutin in the culture medium (Neher et al., 2016) and
244 some *Bacillus* sp. degrading rutin have already been found (Yang et al., 2012; Zhu et al.,
245 2017).

246 Thus, the four strains were cultured in LB broth supplemented with 1% rutin (w/v) for 72 h at
247 45°C. Intracellular activities were evaluated on the *p*NPG and *p*NPR substrates but they were
248 below the detection limit of the method. Specific *para*-nitrophenol (β -D-glucoside and α -L-
249 rhamnoside) glycosidases and rutinoidase extracellular activities were assayed. No
250 extracellular activities toward *para*-nitrophenol- β -D-glucoside were detected. The highest
251 results toward *p*NPR and rutin were obtained at 72 hours of culture (**table 2**).

252 Among the four strains, *B. amyloliquefaciens* and *B. licheniformis* had activities toward both
253 substrates whereas no activity was detectable for *B. thuringiensis*. *B. subtilis* was active
254 toward *p*NPR but not toward rutin. The *p*NPR activity has already been characterised in
255 *Bacillus* sp. with wide range of specific activity (Hashimoto et al., 1999; Jung et al., 2006).
256 Rutinoidase activities for *B. amyloliquefaciens* and *licheniformis* were 0.62 and 0.41 pkat/mg
257 respectively. In 1990, Sang-Joon *et al.* (Sang-Joon et al., 1990) purified an enzyme from
258 *Arthobacter* sp., which displayed 1.9 μ kat/mg specific rutinoidase activity. To our
259 knowledge, this is the only quantified bacterial specific activity to this date. Rutin
260 deglycosylation by bacteria is usually shown in relative hydrolysis compared to initial
261 substrate concentration (Zhu et al., 2017). Thus, our results could not be compared with those
262 obtained for other *Bacillus* strains. However, activities toward rutin were in the range as those
263 detected for native buckwheat flour enzymes by Weiz *et al.* (Weiz et al., 2017) who first
264 proposed the quantification technique we employed. AR2000 is a commercial mixture of
265 enzymes derived from *Aspergillus niger* containing multiple glycosidase activities. It was
266 used in this study as a control. Specific activities of the strains *B. amyloliquefaciens* and *B.*
267 *licheniformis* were similar to those obtained for AR2000. These results were in accordance

268 with previous study which showed that the specific activity of AR2000 toward α -L-
269 rhamnoside-based substrate was the lowest compared to other glycosidic moieties (Sarry &
270 Günata, 2004).

271

272 3) Activities of selected *Bacillus* strains on glycosidic aroma precursors extracted from
273 grape juice

274

275 Diglycosides constitute the main glycosides in grape and rutinosidic glycosides account for
276 one of the highest (Liang et al., 2020). *B. amyloliquefaciens* 1-38c and *B. licheniformis* 4-4a
277 supernatants were active on rutin and the two others were not. The extracellular activities of
278 the four strains toward glycosidic aroma precursors extracted from grape juice were assayed.
279 The released volatile compounds for each strain were measured in the gas phase using a GC-
280 MS and are shown in **table 3**.

281 Compounds were classified into three classes, namely monoterpenoids, benzenoids and
282 norisoprenoids. In total, the incubation with AR2000 released 4101 $\mu\text{g}/\text{kg}$ of aroma
283 precursors, 176 with *B. licheniformis*, 136 with *B. amyloliquefaciens*, 76 with *B. thuringiensis*
284 and 88 $\mu\text{g}/\text{kg}$ of aroma precursors with *B. subtilis*. Glycoside hydrolysis led by AR2000 (4.5
285 g/L) was used as control and was considered as being 100% of total enzyme hydrolysis due to
286 its various substrate activities (Palomo et al., 2005). Its hydrolysis led to higher content of
287 monoterpenoids and benzenoids than C13-norisoprenoids. These results are in accordance
288 with previous studies, which showed that Muscat grape are characterised by floral aroma from
289 monoterpenoids and low quantities of C13-norisoprenoids (Liang et al., 2020). The latter are
290 characteristics of non-floral grapes, such as Syrah, Sauvignon blanc, Pinot noir or Cabernet
291 (Tufariello et al., 2021).

292 *B. thuringiensis* and *B. subtilis* supernatants did not show any rutinoid activity in our study.
293 These strains released respectively 12 and 21 µg/kg of benzenoids. These results are greatly
294 supported by the low release of benzaldehyde and benzyl alcohol, which are similar between
295 all the strains (see supplemental table S1). Phenyl acetaldehyde was not released after
296 incubation with both *B. thuringiensis* and *B. subtilis* supernatants. 8.3 µg/kg of 2-
297 phenylethanol were detected after incubation of *B. subtilis* but this compound was not
298 detected for *B. thuringiensis*. Higher amounts of 2-phenylethanol were observed for samples
299 treated with either *B. licheniformis* or *B. amyloliquefaciens* supernatants at respectively, 80
300 and 21 µg /kg. No monoterpene compound was significantly released by any strain, thus the
301 total amount of monoterpenes remained similar between all the strains. The release of
302 monoterpenes does not seem to be supported by any enzymatic activity evaluated during our
303 study.

304

305 4) Activities of *B. licheniformis* and *B. amyloliquefaciens* on glycosidic aroma precursors
306 extracted from grape pomace

307 Because the glycosidic precursors are different between juice and pomace grape (B. Wilson,
308 C. R. Strauss, 1986), the release of aromatic glycosides from an extract of grape pomace,
309 measured in the gas phase using a GC-MS, were further characterised by incubation with the
310 supernatant of *B. licheniformis* and *B. amyloliquefaciens* (table 4).

311 In total, 4421 µg/kg of volatile glycosides were released after incubation of AR2000, and
312 respectively 906 and 235 µg/kg for supernatants of *B. licheniformis* and *B. amyloliquefaciens*.

313 Any strain could release compounds belonging to the class of norisoprenoids. 1.7 and 5 times
314 more volatile compounds were respectively released by *B. amyloliquefaciens* and *B.*
315 *licheniformis* supernatants in the grape pomace glycosidic extract compared to the grape juice

316 glycosidic extract. The main released volatile compounds after enzymatic hydrolysis by both
317 strains are reported in **Figure 2**.

318 The *B. licheniformis* supernatant led to the release of four times more total volatile
319 compounds, 7.6 times more monoterpenoids and 2.4 times more benzenoids than *B.*
320 *amyloliquefaciens*. Main released terpenoids were geraniol, nerol and neric acid whereas
321 main released benzenoids were 2-phenylethanol, benzaldehyde and homovanillyl alcohol.
322 Means of geraniol, neric acid, benzyl alcohol and 2-phenylethanol were significantly different
323 (*t-test*, $P < 0.05$). For all of them, *B. licheniformis* hydrolysed more volatile compounds than
324 the other strain. While *B. amyloliquefaciens* supernatant showed higher specific activities for
325 both rutin and *p*NPR, the supernatant activities toward rutin were higher for *B. licheniformis*
326 and were similar toward *p*NPR (**table 2**). *B. licheniformis* supernatant displayed higher
327 rutinoid activity than *B. amyloliquefaciens* and led to a higher release of volatile
328 compounds from glycosides.

329 Content of both 2-phenylethanol and neric acid were similar between *B. licheniformis* and the
330 control AR2000 (see **supplemental table S2**). If we consider that the commercial enzymatic
331 preparation AR2000 released all the glycosidic grape precursors, then this strain led to the
332 release of the total glycosidic fraction of neric acid and 2-phenylethanol of grape pomace.

333

334 **Discussion**

335 In this study, we developed an approach dedicated to the screening of the most relevant
336 bacterial glycosidases for releasing grape aroma compounds. Hence, we searched for *Bacillus*
337 spp. producing glycosidases active on rutin and assessed their activities on grape glycosides.
338 We first assessed their diglucosidase activities toward BCIC and *p*NPC and then we assessed

339 their heteroglycosidase activities toward rutin and grape glycosidic extract. We also
340 investigated the nature of the released grape glycosidic volatile compounds.

341

342 Screening of rutinoidase activity in bacteria

343 Among the four strains tested, we found that the supernatant of three of them were active on
344 *p*NPR and of two strains on both *p*NPR and rutin. Several studies focused on the screening of
345 the 6-*O*- α -L-rhamnoside- β -D-glucosidic activity in fungi (Koseki et al., 2018) but less
346 information about bacteria displaying such activity is available. Previous studies have
347 evaluated the capacity of some bacteria to hydrolyse rutin but none of the *Bacillus* tested were
348 retained (Neher et al., 2016; Westlake et al., 1959). In 2012, Yang *et al.* (Yang et al., 2012)
349 discovered that *Bacillus* sp. 52 produced an α -L-rhamnosidase that could convert rutin to
350 quercetin-3-*O*-glucoside but not to free quercetin.. Others *Bacillus* sp. producing α -L-
351 rhamnosidases active on flavonoids were identified, especially toward naringin (Hashimoto et
352 al., 2003; Mukund et al., 2014). In 2017, Zhu *et al.* (Zhu et al., 2017) found a naringinase
353 produced by a *B. amyloliquefaciens* strain that retained little activity on rutin.

354 In our study, both *B. licheniformis* and *B. amyloliquefaciens* hydrolysed rutin but no activity
355 toward *para*-nitrophenol- β -D-glucoside was detected.. Supernatant contains several
356 glycosidases that could have been involved in rutin degradation. Thus, an α -L-rhamnosidase
357 could have first released isoquercetin from rutin and quercetin would have been further
358 released by a β -D-glucosidase. Complementary studies, such as purification and
359 characterisation of the supernatant enzymes must be performed to confirm that rutin
360 degradation is carried out by a diglycoside-specific glycosidase. This assumption is consistent
361 with the presence of a strain specific gene encoding a predicted Glycosyl Hydrolase 78 (GH
362 78) (<https://www.ncbi.nlm.nih.gov/protein/ATI75006.1/>, website visited the 2021, June 4th) in

363 the sequenced genome of *B. licheniformis* ATCC 9789. However, to our knowledge, this is
364 the first time that rutin deglycosylation is carried out by a *B. licheniformis* strain.

365

366

367 Monoterpenoids

368 Monoterpenoids are the compounds responsible for the characteristic floral aroma, and
369 displaying the highest OAVs (Odor Active Value) in Muscat grape (Wu et al., 2019). In our
370 study, the most released volatile compound is neric acid, reaching 378 µg/kg of grape
371 pomace. It is not the first time this compound is shown to be the predominant terpene in grape
372 (Dziadas & Jeleń, 2016) but it is usually found in lower concentrations in both Muscat and
373 non-Muscat grapes (Hernandez-Orte et al., 2008, 2009; Jesús Ibarz et al., 2006). While there
374 is not much information about neric acid glycosides in Muscat grape, our results tend to
375 indicate that this aglycon is mainly linked to a rutinosidic moiety. Geraniol and nerol, two
376 monoterpenoids released in our study, display one among the highest OAVs in Muscat grape
377 (Lukić et al., 2017). It is known that rutinose is the main glycosidic moiety of these two
378 volatile compounds in Muscat grape (Flamini et al., 2014; Williams et al., 1982) and their
379 release are of great importance when considering valorisation of grape pomace. No released
380 linalool nor α -Terpineol were observed for both strains, probably because tertiary alcohols are
381 less susceptible to β -glycosidase hydrolysis than other terpenoids due to steric hindrance with
382 the enzyme (Gil et al., 2005).

383

384 Benzenoids

385 - Both 2-phenylethanol and benzyl alcohol were release by *B. licheniformis* supernatant. It
386 has been shown that these volatile compounds are found more likely in rutinosidic glycosides
387 than in monoglucosides in Muscat grape (Williams et al., 1983). A recent study, which
388 characterised glycosidic moieties of Muscat, showed that neither benzyl alcohol nor 2-
389 phenylethanol could have been identified linked to a monoglucoside (Caffrey et al., 2020).
390 These compounds are rarely released in high content by exogenous application of enzymes in
391 grape. Indeed, most of studies focused on application of enzymes from yeasts (Tufariello et
392 al., 2021). Yeasts showed β -D-glucosidic activities, releasing high content of monoterpenoids
393 but very low or no amount of benzenoids (de Ovalle et al., 2021; Gil et al., 2005). When 2-
394 phenylethanol was released, it was usually in wine model system and it was related to the
395 fermentation metabolism of yeast rather than to the glycosidic activity (Lukić et al., 2017;
396 Trinh et al., 2010). It might be possible that the usually low releases of both 2-phenylethanol
397 and benzyl alcohol by yeast glucosidases is related to the low contents of β -D-glucosidic
398 precursors of these two compounds. The only opposite example is the release of as much as
399 81 $\mu\text{g}/\text{kg}$ of benzyl alcohol and 696 $\mu\text{g}/\text{kg}$ of 2-phenylethanol from glycosidic precursors in
400 Muscat grape by the yeast *Kloeckera apiculata* (Mendes Ferreira et al., 2001). Thus, the
401 application of both yeast enzymes and enzymes produced by this *B. licheniformis* strain could
402 lead to the release of both monoterpenoids and benzenoids if they work synergistically.

403

404 **Conclusion**

405 *B. licheniformis* is known for its cellulolytic and proteolytic activities (Baruzzi et al., 2006;
406 Tamang et al., 2016; Vlasenko et al., 2010) but for the first time, a strain *B. licheniformis*
407 showed a rutin deglycosylation activity. This activity was linked to the revelation of grape
408 aroma potential. It can be a tool for further grape pomace valorisation, through varietal

409 aromatic production or through flavonoids deglycosylation. The strain showed high activity
410 toward benzenoic compounds, which might be mostly linked to rutinose moiety in grape.
411 Application of such enzymes in grape can target specifically rutinosidic moieties and can
412 avoid off-flavours production led by fungal enzymes side activities. Future work to purify the
413 enzyme(s) involved and to characterise the glycosidic fraction in grape must be considered to
414 ensure direct link between the activity of this strain and the actual rutinosidic-glycoside
415 potential of grape.

416 **Abbreviations used**

417 CMC, carboxymethylcellulose; *B.*, *Bacillus*; BCIC, 5-bromo,4-chloro,3-indolyl- β -D-
418 cellobioside; *p*NP, *para*-nitrophenyl; DMSO, dimethylsulfoxide; LAB, lactic acid bacteria;
419 BSA, bovin serum albumin; OAV, odor active value

420

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427

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Figure 1: Means of *p*NPCase activity (nkat/mL, *n*=3) for each *Bacillus* isolate. Error bars represent SDs. Black bars represent *B. subtilis* isolates, white bars represent *B. thuringiensis* isolates, dot bars represent *B. licheniformis* isolates and hatched bar represents *B. amyloliquefaciens* isolate. Different letters above bars indicate significant differences between isolates from the same species at *P*<0.05 (HSD, Turkey's test). No statistical differences were observed for any of *B. thuringiensis* and *B. licheniformis* groups of isolates (*P*=0.234 and *P*=0.193 respectively).

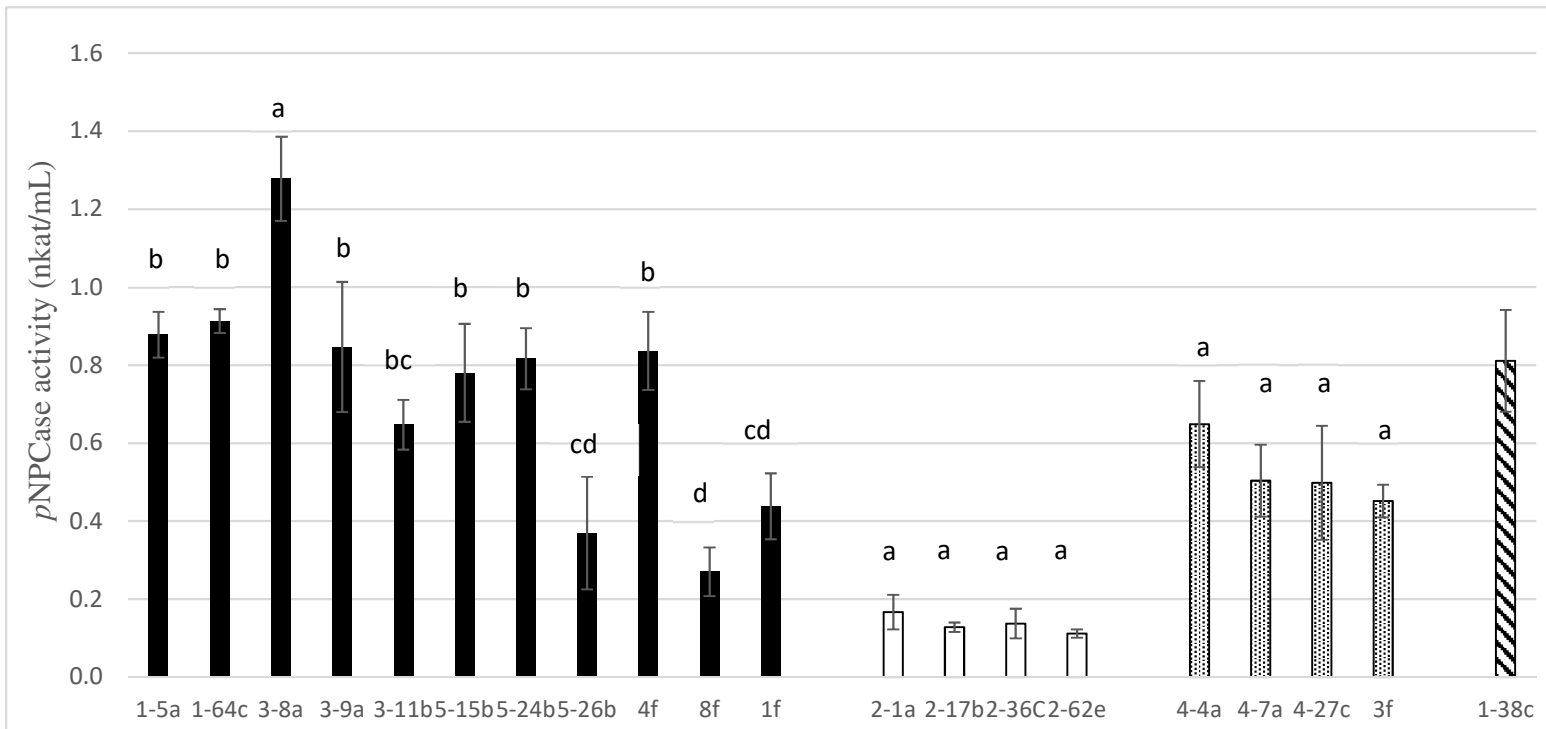


Figure 2: Means of main volatile compounds released ($\mu\text{g}/\text{kg}$, $n=3$) after hydrolysis of an extract of glycosidic grape pomace by *Bacillus* strain supernatants. Error bars represent SDs. black bars represent strain *B. amyloliquefaciens* 1-38c and dots bars represent strain *B. licheniformis* 4-4a. Stars represent statistical difference between the strains for each compound (t-test ; $P<0.05$)

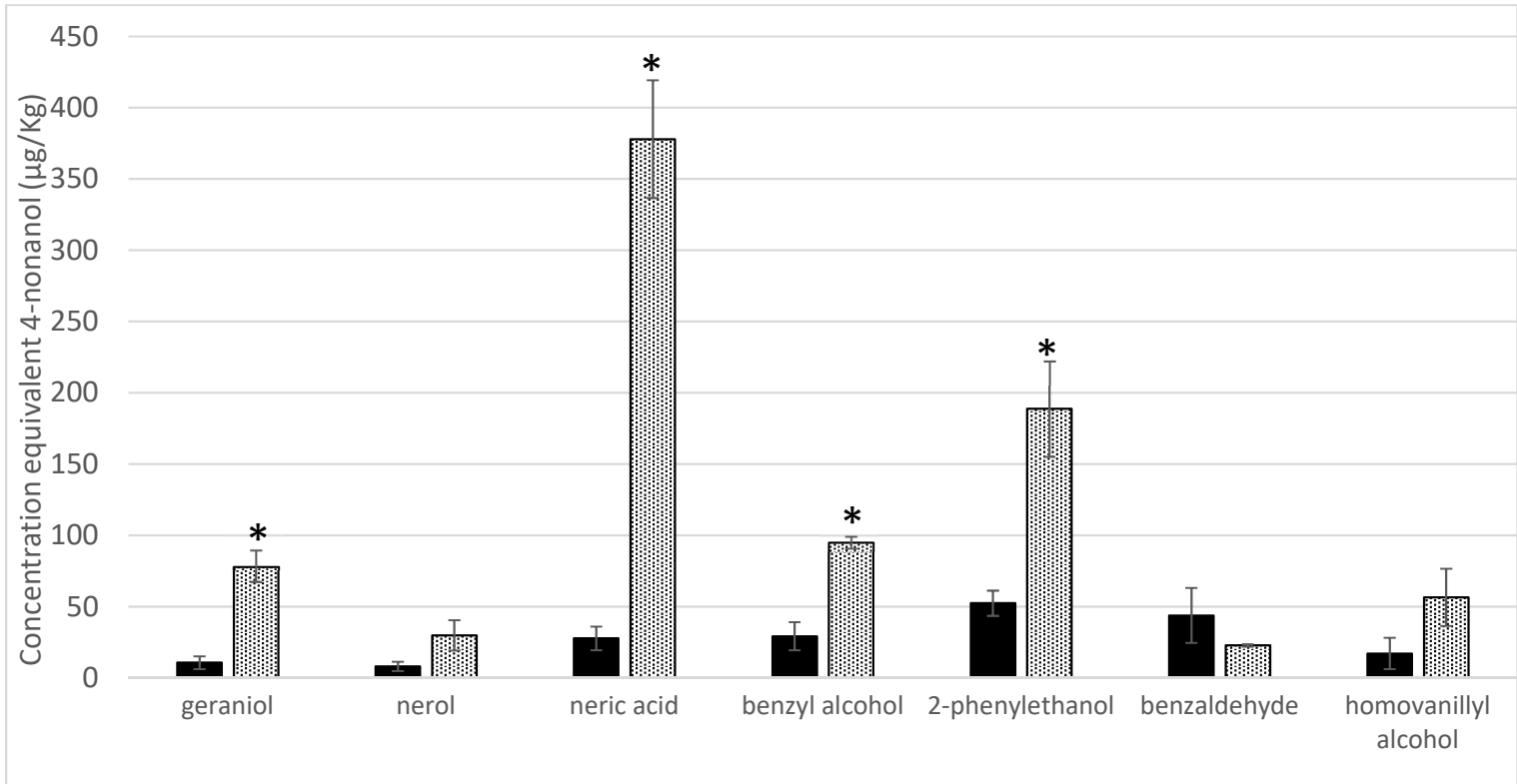


Table 1: Linear retention index and CAS number of each identified compound

compound class	volatile compound	LRI ^a		CAS number
		sample	reference	
monoterpenoids	β -linalool	1098	1100	78-70-6
	geraniol	1248	1268	106-24-1
	trans linalool oxide	1069	1086	34995-77-2
	nerol	1222	1220	106-25-2
	2,7-Octadiene-1,6-diol, 2,6-dimethyl-	1338	1325	64142-78-5
	α -terpineol	1192	1207	98-55-5
	epoxylinalool	1168	1163	14049-11-7
	neric acid	1349	1347	4613-38-1
	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	1186	1197	13741-21-4
	1,7-Octadiene-3,6-diol, 2,6-dimethyl-	1269	1277	51276-33-6
	α -citral	1264	1174	5392-40-5
	β -citral	1235	1204	106-26-3
	thymol	1289	1292	89-83-8
	7-Octene-2,6-diol, 2,6-dimethyl-	1226	1189	29210-77-3
	nerol oxide	1150	1153	1786-08-9
benzenoids	benzyl alcohol	1032	1029	100-51-6
	benzaldehyde	959	921	100-52-7
	2-phenylethanol	1109	1060	60-12-8
	phenyl acetaldehyde	1040	1051	122-78-1
	vanillin	1390	1435	121-33-5
	methyl vanillate	1508	1525	3943-74-6
	homovanillyl alcohol	1521	1542	2380-78-1
norisoprenoids	3-hydroxy- β -damascone	1604	1646	102488-09-5
	4-Hydroxy- β -ionone	1666	1647	27185-79-1
	Dihydro-3-oxo- β -ionol	1709	1646	116296-75-4
aliphatic alcohol	1-octanol	1070	1069	111-87-5
	4-nonanol (standard)	1089	1078	5932-79-6

^a LRI, Linear retention index on Elite-5MS. LRI reference values according to NIST database.

Table 2. Specific and supernatant activities ($n=3$) towards para-nitrophenol- α -L-rhamnoside and rutin for selected strains.

Supernatant / enzyme	Specific activity (nkat/mg)		Supernatant activity (nkat/mL)	
	<i>p</i> NPR	Rutin ($\cdot 10^{-3}$)	<i>p</i> NPR	Rutin ($\cdot 10^{-3}$)
<i>B. amyloliquefaciens</i> 1-38C	34 \pm 2.0	0.62 \pm 0.13	2.2 \pm 0.4	0.077 \pm 0.009
<i>B. licheniformis</i> 4-4a	12 \pm 3.2	0.41 \pm 0.61	1.8 \pm 0.6	0.12 \pm 0.021
<i>B. subtilis</i> 3-8a	34 \pm 1.5	ND	0.3 \pm 0.1	ND
<i>B. thuringiensis</i> 2-1a	ND	ND	ND	ND
AR 2000	17 \pm 1.0	0.23 \pm 0.015	39 \pm 2.4	1.06 \pm 0.09

AR2000 is used as control (4.5 g/L). "ND" = Not Detected. Detection limits: for *p*NPR: 0.10 nkat/mL; for rutin: 0.065 $\cdot 10^{-3}$ nkat/mL

Table 3. Released volatile compounds ($n=3$) after hydrolysis of an extract of glycosidic grape juice by *Bacillus* strain supernatants or AR2000 (4.5 g/L).

Compound class	<i>B. amyloliquefaciens</i> 1-38c	<i>B. thuringiensis</i> 2-1a	<i>B. subtilis</i> 3-8a	<i>B. licheniformis</i> 4-4a	AR2000
monoterpenoids	73 ± 7.6	64 ± 12	67 ± 16	62 ± 5.0	2832 ± 273
benzenoids	63 ± 22	12 ± 4	21 ± 7.3	114 ± 25	1139 ± 78
<i>of which</i>					
2-phenylethanol	21 ± 5.9	ND	8.3 ± 2.1	80 ± 17	165 ± 22
Phenyl acetaldehyde	13 ± 4.1	ND	ND	11 ± 2.2	ND
norisoprenoids	ND	ND	ND	ND	130 ± 31
TOTAL	136 ± 30	76 ± 16	88 ± 24	176 ± 30	4101 ± 382

Results are shown in µg/kg of grape juice. “ND” = Not Detected ; Detection limits: 1.0 µg/kg of grape juice

Table 4. Released volatile compounds ($n=3$) after hydrolysis of an extract of glycosidic grape pomace by *Bacillus* strain supernatants or AR2000 (4.5 g/L).

Compound class	<i>B. amyloliquefaciens</i> 1-38c	<i>B. licheniformis</i> 4-4a	AR2000 (4.5 g/L)
monoterpenoids	67 ± 29	506 ± 97	2746 ± 322
benzenoids	168 ± 54	400 ± 77	1415 ± 147
norisoprenoids	ND	ND	260 ± 38
TOTAL	235 ± 83	906 ± 164	4421 ± 507

Results are shown in $\mu\text{g}/\text{kg}$ of grape pomace. “ND”=Not Detected ; Detection limits: 1.0 $\mu\text{g}/\text{kg}$ of grape pomace