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1 **Mobilization of phenolic antioxidants from grape, apple and pitahaya residues via solid**
2 **state fungal fermentation and carbohydrase treatment**
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22 ABSTRACT

23

24 This paper reports two strategies to mobilize phenolic antioxidants from lyophilized and oven-
25 dried black grape (*Vitis vinifera x* (*Vitis labrusca x Vitis riparia*)) pomace, and apple (*Malus*
26 *domestica* cv. Jonagold) and yellow pitahaya (*Hylocereus megalanthus*) peel, core, peduncle and
27 seed mixtures: a solid-state *Rhizomucor miehei* NRRL 5282 fermentation-based process and a
28 carbohydrate-cleaving enzyme treatment using *R. miehei* NRRL 5282 cellulase and *Aspergillus*
29 *niger* pectinase cocktails. Both methods proved to be suitable to increase the extractable phenolic
30 content and improve the antioxidant properties of the phenolics as determined by 1,1-diphenyl-2-
31 picrylhydrazyl radical inhibition or ferric reducing antioxidant power analyses. During solid-state
32 fermentation, maximal phenolic yields obtained in lyophilized grape, apple and pitahaya residues
33 were 1956 ± 31, 477 ± 37 and 495 ± 27 mg gallic acid equivalents (GAE)/100 g dry matter (DM),
34 respectively, while they were 1385 ± 71, 362 ± 27 and 615 ± 26 mg GAE/100 g DM in oven-
35 dried samples, respectively. The major individual phenolics produced enzymatically from the
36 substrates were identified by HPLC as gallic acid, 4-hydroxybenzoic acid, vanillic acid, (+)-
37 catechin and (–)-epicatechin, with yields ranging from 0.58 ± 0.06 to 215.81 ± 17.17 mg/100 g
38 DM depending on the substrate and the pretreatment. The obtained phenolic-enriched extracts
39 could potentially be applicable as sources of natural antioxidants.

40

41 Keywords: Fruit byproducts treatment; Phenolic compounds; *Rhizomucor miehei*; Cellulase;
42 Pectinase

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45 **1. Introduction**

46 Increasing interest can be observed towards the utilization of fruit byproducts for
47 production of biologically active phenolics with antioxidative benefits. This can be conducted via
48 solid-state fermentation (SSF) with filamentous fungi as fermenting organisms (Dey,
49 Chakraborty, Jain, Sharma, & Kuhad, 2016), or by direct enzymatic treatment of the substrate
50 (Oszmiański, Wojdyło, & Kolniak, 2011; Xu et al., 2014). Most plant derived phenolic
51 antioxidants occur in conjugated forms with sugar residues, which reduce their bioavailability
52 (Pandey & Rizvi, 2009). Carbohydrate-cleaving enzyme preparations, i.e. cellulases and
53 pectinases, can hydrolyze these glycosides releasing the phenolic aglycones (McCue & Shetty,
54 2003; Mandalari et al., 2006). Consequently, fermentation with filamentous fungi with high
55 carbohydrase activity may facilitate this process and enhance the bioavailability of the
56 antioxidant phenolics. In this context, certain members of the fungal group Mucoromycotina (a
57 representative group of the former class Zygomycetes) can be excellent producers of such
58 enzymes (Papp et al., 2016). Among them, *Rhizomucor miehei* NRRL 5282 has been well-
59 characterized from its cellulase production by our group (Takó et al., 2010, 2015).

60 Grapes are rich in polyphenols, including flavonoids, stilbenes and proanthocyanins
61 which found primarily in the skin and seeds (Ali, Maltese, Choi, & Verpoorte, 2010).
62 Consequently, about 70% of these phenolics remain within the grape pomace after fruit
63 processing (Ratnasooriya & Rupasinghe, 2012). Apple peel, seed and core residues, which
64 constitute about 25-35% of the fresh fruit, are the primary waste products of juice and sauce
65 production. They contain substantial amounts of phenolics (Kalinowska, Bielawska,
66 Lewandowska-Siwkiewicz, Priebe, & Lewandowski, 2014), and are frequently used as substrates
67 to produce aroma compounds (Madrera, Bedriñana, & Valles, 2015) and natural antioxidants
68 (Ajila et al., 2012). Varieties of pitahaya, also known as dragon fruit, contain large amounts of

69 bioactive phytochemicals (Kim et al., 2011). The peel represents about 33% of the fruit weight,
70 and its disposal causes major problems in the pitahaya juice processing industries (Le Bellec &
71 Vaillant, 2011). Since the abovementioned fruit residues have high nutrient content, they could
72 support the growth of filamentous fungi in solid conditions.

73 In the present study, free antioxidative phenolic content of oven-dried and lyophilized
74 Othello black grape, Jonagold apple and Yellow pitahaya residues was evaluated and compared
75 after SSF with the high-yield cellulase producer *R. miehei* NRRL 5282 isolate. Because β-
76 glucosidase of this fungus can release phenolic aglycones from their glycosidic bond (Krisch,
77 Bencsik, Papp, Vágvölgyi, & Takó, 2012), changes in the enzymatic activity have also been
78 monitored during the fermentation. Additionally, phenolics mobilization was also investigated by
79 direct enzymatic treatment of the residues using a cellulolytic cocktail produced from *R. miehei*
80 NRRL 5282 on wheat bran. In this case, commercial *Aspergillus niger* pectinase was also added
81 to the reaction mixtures to improve the extraction efficiency of the phenolic compounds, and
82 decrease the tissue rigidity in the residues.

83 **2. Materials and methods**

84 *2.1. Substrate preparation and fermentation conditions*

85 Othello black grape (*Vitis vinifera x* (*Vitis labrusca x Vitis riparia*)) and Jonagold apple
86 (*Malus domestica* cv. Jonagold) were purchased at local market in Szeged, Hungary. Yellow
87 pitahaya (*Hylocereus megalanthus*) was purchased at local market in Guayaquil, Ecuador. The
88 fruits were processed at the Institute of Food Engineering, University of Szeged. In case of the
89 grape samples, the skin, stem and seeds were obtained after juice pressing. The apple and
90 pitahaya fruits were peeled, and the flesh was separated from the other parts of the fruit. A
91 mixture of peels, cores, peduncles and seeds was subjected to substrate pretreatment. These by-

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92 products constitute the bulk of the pomace remained after juice and sauce production, therefore,
93 the substrates used were close to those generated at industrial level. To pretreat the substrates, the
94 residues were dried immediately at 65 °C for 18 h, or frozen at -20 °C for 12 h and subsequently
95 lyophilized for 24 h. Then, both types of samples were grinded in a commercial mill into particles
96 of 3 mm mean diameter. The chopped samples were stored in dark at room temperature and used
97 for subsequent fermentation and enzyme treatment experiments.

98 For SSF, 2 g of chopped sample, 0.5 g of soy flour as a nitrogen source and 5 mL distilled
99 water were mixed in 100-mL Erlenmeyer flasks and autoclaved at 121 °C for 25 min. The flasks
100 were then inoculated with 5×10^6 spores of *R. miehei* NRRL 5282 and incubated at 37 °C for 18
101 days. Six parallel fermentation tests were carried out, three for distilled water extraction and three
102 for ethanol:distilled water 50:50 extraction. During incubation, six flasks were taken on every
103 second day for extraction and subsequent analytical measurements.

104 *2.2. Extraction processes*

105 The fungus-byproduct ferments were extracted with 30 mL of distilled water or a solution
106 of ethanol:distilled water 50:50 macerating them with a spatula and incubated at 4 °C for 24
107 hours. After the extraction, the samples were filtered through gauze and centrifuged at 16,200× *g*
108 for 20 min at 4 °C. The supernatant was stored at -20 °C until analytical measurements. To avoid
109 the inhibition effect of ethanol (Takó et al., 2010), aqueous extracts were used to measure the β-
110 glucosidase activity in the samples, while crude extracts made with ethanol:water 50:50 solution
111 were utilized for total phenolic content (TPC), antioxidant activity and HPLC measurements.

112 *2.3. Enzymatic treatment*

113 Cellulolytic cocktail produced under wheat bran-based SSF by *R. miehei* NRRL 5282
114 (Takó et al., 2010) and *A. niger* pectinase (Sigma-Aldrich, Germany) were used to investigate the

115 enzyme catalyzed enrichment of free phenolics. The cellulase cocktail diluted in 50 mM acetate
116 buffer (pH 5.0) had filter paper degrading (FPase), endoglucanase (EG), cellobiohydrolase
117 (CBH) and β-glucosidase (BGL) activities of 45, 122, 21 and 1036 enzyme-activity units (U),
118 respectively, measured according to the descriptions of Takó et al. (2015). The pectinase powder
119 had 1.1 U/mg polygalacturonase activity, as declared by the manufacturer. For treatment 1
120 (designated as S1), 600 mg of oven-dried or lyophilized fruit residue was mixed with 6 ml of
121 cellulase cocktail (75, 203, 35 and 1727 U/g dry matter FPase, EG, CBH and BGL activities,
122 respectively). In the case of treatment 2 (designated as S2), this reaction mixture was
123 supplemented with 0.6 mg of pectinase (1.1 U/g dry matter). Enzyme-free (designated as C1),
124 and 0.6 mg pectinase supplemented (designated as C2) acetate buffer (50 mM, pH 5.0) / fruit
125 residue mixtures were used as controls. The reaction mixtures were incubated at 50 °C for 5 h
126 under constant stirring (200 rpm) and subsequently centrifuged at 16,200× *g* for 10 min. The
127 resulted clear supernatant was used for TPC, antioxidant activity and HPLC measurements. The
128 reaction conditions (pH 5.0, 50 °C and 5-h incubation time) were selected according to the
129 studies of Krisch et al. (2012) and Xu et al. (2014). All enzymatic treatment tests were performed
130 in three independent experiments.

131 *2.4. β-Glucosidase activity assay*

132 β-Glucosidase activity was determined using a reaction mixture containing 20 µL of 7
133 mM *p*-nitrophenyl β-D-glucopyranoside (Sigma-Aldrich, Germany), 160 µL of 100 mM sodium
134 acetate buffer (pH 5.0) and 20 µL of diluted extract. After 30-min incubation at 50 °C, the
135 reaction was stopped by addition of 50 µL of 100 mM sodium carbonate, and the *p*-nitrophenol
136 release was measured at 405 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech,
137 Germany). The standard curve was established using a stock solution of 10 mM *p*-nitrophenol in

138 the concentration range of 1-10 mM in sodium acetate buffer (100 mM, pH 5.0). One unit of β-
139 glucosidase activity was defined as the amount of enzyme that releases 1 μM *p*-nitrophenol per
140 minute under the conditions of the assay.

141 *2.5. Determination of total phenolics*

142 TPC was measured using a reaction mixture containing 20 μL of ethanol (96%), 100 μL
143 of distilled water, 10 μL of 50% Folin-Ciocalteu's reagent (Sigma-Aldrich, Germany) and 20 μL
144 of extract diluted in ethanol (96%). The mixture was left at room temperature for 5 min, then, the
145 reaction was initiated by addition of 20 μL of sodium carbonate (5%), and maintained in dark
146 place for 60 min. After incubation, absorbance was measured at 725 nm (SPECTROstar Nano,
147 BMG Labtech, Germany). The standard curve was established using gallic acid in the
148 concentration range of 0-100 mg/mL. The content of total phenolics was expressed as mg of
149 gallic acid equivalents (GAE) in 100 grams dry matter (DM).

150 *2.6. Determination of free radical scavenging activity*

151 The free radical scavenging activity of the extracts was determined using 1,1-diphenyl-2-
152 picrylhydrazyl (DPPH, Sigma-Aldrich, Germany) solution in 100 μM concentration prepared
153 freshly before the measurement. The mixture was shaken vigorously until complete dissolution
154 and left in dark at room temperature until use. The reaction mixture contained 150 μL of 100 μM
155 DPPH and 25 μL of extract diluted in 50% ethanol solution. The control sample contained 150
156 μL of 100 μM DPPH and 25 μL of 50% ethanol. Absorption was measured at 517 nm
157 (SPECTROstar Nano, BMG Labtech, Germany), and the results were plotted as percent (%)
158 DPPH radical scavenging capacity against the control: (control absorbance - extract absorbance) /
159 (control absorbance) x 100.

160 *2.7. Ferric reducing antioxidant power assay*

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161 The reagent solution prepared for ferric reducing antioxidant power (FRAP) analyses
162 contained 80 mL of 300 mM acetate buffer (pH 3.6), 8 mL of 10 mM 2,4,6-Tri(2-pyridyl)-s-
163 triazine (TPTZ; Sigma-Aldrich, Germany) diluted in 40 mM hydrochloric acid, 8 mL of 20 mM
164 iron(III) chloride and 4.8 mL of distilled water. The power of phenolic extracts on ferric reducing
165 was determined by mixing of 200 μL FRAP reagent and 6 μL extract diluted in 50% ethanol. The
166 reaction mixture was incubated at 37 °C for 30 min and then absorbance was measured at 593 nm
167 using a SPECTROstar Nano reader (BMG Labtech, Germany). The standard curve was
168 established using 1 mM iron(II) sulfate solution in the concentration range of 0.1-1.0 mM. The
169 FRAP of the crude extracts was expressed as μM Fe(II)/g DM.

170 *2.8. HPLC analysis of phenolics*

171 Phenolic compounds were separated on Prodigy ODS 3 C18 (250 x 4.6 mm, 5 μ;
172 Phenomenex, USA) column using the mixture of solvent A (H_2O:acetic acid, 97:3) and solvent B
173 (MeCN:acetic acid, 97:3) as mobile phase at a flow rate of 1 mL/min; detection was carried out at
174 280 nm with a SPD-20AVP UV-VIS detector (Shimadzu, Germany). The following gradient
175 program was used during the separation: 0 min 8% B, 20 min 25% B, 25 min 25% B, 40 min
176 60% B, 45 min 60% B, 46 min 8% B, and 60 min 8% B. The oven temperature and injection
177 volume were 40 °C and 3 μL, respectively. The detector response was linear for all examined
178 standard compounds (gallic, vanillic, syringic, *p*-coumaric, 4-hydroxybenzoic and cinnamic
179 acids, and (+)-catechin, (–)-epicatechin, polydatin, resveratrol and quercetin; Sigma-Aldrich,
180 Germany) with correlation coefficients greater than 0.995. Data recording and evaluation were
181 carried out using ClassVP v5.1 chromatographic software.

182 *2.9. Statistical analyses*

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183 Assays were performed in triplicates and data were expressed as an average of the
184 replicates ± standard deviation. The significant differences between the independent variables
185 were analyzed by the two-way analysis of variance (ANOVA) test followed by the Tukey's
186 Multiple Comparison Test using the program GraphPad Prism version 6.00 (GraphPad Software
187 Inc., San Diego, USA). Pretreatment (oven-drying and lyophilization), and other two parameters
188 namely the fruit residue and the type of the enzyme treatment (cellulase, pectinase and
189 cellulase/pectinase) were considered as independent variables in the SSF and enzymatic treatment
190 experiments, respectively. The fruit residue and the type of the enzyme treatment (cellulase and
191 cellulase/pectinase) were considered as factors in the individual phenolic compound analyses. A
192 P value of < 0.05 was considered as statistically significant. The relationship between β-
193 glucosidase activity, TPC and antioxidant potential (FRAP assay) in the SSF experiments was
194 investigated by applying the Pearson's correlation test.

195 **3. Results and discussion**

196 *3.1. Solid-state fermentation on oven-dried and lyophilized fruit residues*

197 *3.1.1. Black grape substrates*

198 Negative associations were found between β-glucosidase activity and reducing power
199 (FRAP) for both oven-dried (DBG) (r = -0.477) and lyophilized black grape (LBG) (r = -0.111)
200 pomaces (Fig. 1A). β-Glucosidase activity showed a slow increase from the 7th day of the
201 fermentation and reached maximal yields at the 15th day. The slow increase in the activity may
202 due to the high sugar content of the medium. These compounds, for example cellobiose and
203 glucose, can bind to the active site of the enzyme inhibiting its activity if they are present in
204 excess. Additionally, interaction of polyphenols and free aglycones with glucosidases may also

205 reduce the enzyme activity (Cairns & Esen, 2010; Ximenes, Kim, Mosier, Dien, & Ladisch,
206 2010; Yu & Ahmedna, 2013).

207 The high phenolic content observed at the beginning of the fermentation (Fig. 1A) is
208 related to the flavanol, stilbene and proanthocyanin content of the crude pomace (Ali et al.,
209 2010). Polyphenol concentration in DBG samples decreased from 1236 ± 74 to 875 ± 50 mg
210 GAE/100 g DM, while it reached 1956 ± 31 mg GAE/100 g DM TPC at the 7th day on the LBG
211 residue (Fig. 1A). The decrease in TPC may be due to degradation and/or enzymatic
212 polymerization of the released phenolics by the fermenting fungus. Phenolic compound
213 degradation and polymerization have been documented for other filamentous fungi as well
214 (Mendonça, E., Martins, A., & Anselmo, A. M. 2004; Santos & Linardi, 2004; Correia, McCue,
215 Magalhães, Macêdo, & Shetty, 2004).

216 In red grape pomaces, the main phenolic compounds are gallic acid, catechin and
217 epicatechin (Makris, Boskou, & Andrikopoulos, 2007). HPLC analysis detected an increase in
218 the concentration of these components when LBG pomace was used as a fermenting medium.
219 The highest gallic acid, (+)-catechin and (–)-epicatechin yields, i.e. 0.79 ± 0.12, 3.49 ± 0.52 and
220 1.37 ± 0.08 mg/100 g DM, respectively, were determined at the 7th day of fermentation. In case
221 of the DBG samples, only the gallic acid content improved reaching 1.27 ± 0.03 mg/100 g DM at
222 the 7th day of incubation.

223 Antioxidant potential in the fermented samples varied depending on the incubation time.
224 FRAP for the DBG and LBG samples was the highest at the 3rd (146 ± 9 and 225 ± 11 μM
225 Fe(II)/g DM, respectively) and 7th days (150 ± 10 and 224 ± 11 μM Fe(II)/g DM, respectively).
226 The ability of polyphenolics to inhibit DPPH was 53 ± 1 and 51 ± 1% for DBG and LBG,
227 respectively, in the 7th-day extracts (Fig. 1A). Fermentation on LBG resulted in higher free
228 phenolic yield than that on DBG ($p < 0.05$), which is directly related to the increased FRAP.

229 *3.1.2. Apple substrates*

230 β-Glucosidase activity of the oven-dried (DA) and the lyophilized (LA) apple substrates
231 showed a considerable increase from the 7th to the 10th day of bioconversion (Fig. 1B). TPC of
232 DA is slightly decreased during the fermentation, but the yield on LA increased continuously
233 reaching 477 ± 37 mg GAE/100 g DM TPC at the 5th day (Fig. 1B). Similarly, Ajila et al. (2012)
234 described an increase in the free phenolic content after bioconversion of apple samples by
235 *Phanerochaete chrysosporium*. β-Glucosidase activity reached its maximum by the 6th to 8th
236 day, which coincided with the liberation of phenolic compounds. In the current study, however,
237 TPC increased slowly and reached a maximum on the 5th fermentation day before the β-
238 glucosidase activity increased.

239 Hydroxybenzoic acids are one of the major polyphenolic compounds of apple residues
240 (Watkins & Liu, 2011). Interestingly, among hydroxybenzoic acids analyzed by HPLC, only the
241 gallic acid content of LA samples demonstrated an increase during the fermentation. The highest
242 gallic acid concentration was 0.79 ± 0.06 mg/100 g DM at the 5th sampling day. However, free
243 gallic acid may be formed not only from its glucosidic derivatives but by hydrolysis of the gallic
244 acid esters of flavan-3-ol (Rentzsch, Wilkens, & Winterhalter, 2009).

245 FRAP was the highest at the 13th and 10th days for DA and LA, respectively, and
246 subsequently reduced during later course of fermentation (Fig. 1B). In this context, the phenolic
247 compound stability and antioxidant activity might be affected by a number of factors including
248 enzymatic degradation (Santos & Linardi, 2004), oxidation of phenolics and storage conditions of
249 the extracts (Heras-Ramírez et al., 2012; Shah, Rahman, Shamsuddin, & Adzahan, 2015). It is
250 remarkable that the FRAP increased in parallel to the β-glucosidase activity in both fermentation
251 systems. Furthermore, the DPPH radical scavenging activity showed a slight increase up to the

252 5th day of fermentation (Fig. 1B). In the case of LA-based fermentation, this may be related to
253 the TPC increment during the first phase of fermentation.

254 *3.1.3. Yellow pitahaya residues*

255 Despite the large antioxidant compound content of the fruit, fermentative production of
256 bioactive phenolics from pitahaya residues has not been studied so far. In case of the lyophilized
257 pitahaya (LP) sample, β-glucosidase activity found to be correlating with TPC ($r = 0.866$) and
258 FRAP ($r = 0.41$) suggesting that the *R. miehei* β-glucosidase has an important role in the
259 liberation of phenolics with reducing power (Fig. 1C). A similar trend has been reported during
260 the bioconversion of cranberry and pineapple pomaces by *Lentinus edodes* and *Rhizopus*
261 *oligosporus*, respectively (Zheng & Shetty, 2000; Correia et al., 2004). β-Glucosidase activity on
262 LP was maximal at the 10th day, while it was the highest at the 13th day in case of the oven-dried
263 pitahaya (DP) residue. TPC of DP substrate exhibited a decrease up to the 13th fermentation day,
264 then, a moderate increase could be observed, potentially due to the β-glucosidase action (Fig.
265 1C). In contrast, TPC of LP did not decrease during the fermentation, moreover, it increased in
266 parallel to the β-glucosidase activity.

267 Gallic acid, 4-hydroxybenzoic acid, (+)-catechin and (–)-epicatechin content of DP
268 samples increased during the fermentation (Table 1). The quantity of gallic acid and (–)-
269 epicatechin increased considerably and hydroxycinnamate *p*-coumaric acid was also detected
270 after 15 days. Hydroxycinnamates were previously identified in large quantities in red and white
271 pitahaya varieties (Mahattanatawee et al., 2006). The initial concentration of gallic acid in LP
272 samples was 2.46 ± 0.11 mg/100 g DM. After fermenting for 15 days, it increased to 10.38 ± 0.34
273 mg/100 g DM. The content of 4-hydroxybenzoic acid, vanillic acid, syringic acid, (+)-catechin,
274 quercetin and polydatin decreased, while the content of *p*-coumaric acid, cinnamic acid, (–)-

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275 epicatechin and resveratrol did not change after fermentation (data not shown). Anyway, there is
276 little information in the literature regarding to the phenolic content of pitahaya samples.

277 *3.1.4. Effect of substrate pretreatment on free phenolics enrichment*

278 Proper selection of the sample preparation technique is important to achieve high phenolic
279 yield during the fermentation. It influences not only the growth of fermenting fungus but affects
280 the stability of the fruit byproducts and phenolics (Tseng & Zhao, 2012). Comparison of the best
281 data indicated the highest β-glucosidase activity on the pitahaya substrates ($p < 0.05$). The final
282 activity yields were LP > DP > LA/DBG > DA > LBG (Fig. 2A). Except for grape residues, β-
283 glucosidase activities were higher when lyophilized substrates were used ($p < 0.05$). But
284 fermentation on LBG samples resulted higher maximal TPC (Fig. 2B) and FRAP (Fig. 2C)
285 compared to the other supports tested ($p < 0.05$). In the case of apple and pitahaya samples, there
286 were no significant differences in the FRAP between the two pretreatments (Fig. 2C). But the
287 best DPPH scavenging activity obtained during LP sample fermentation was superior than that
288 can be measured on DP ($p < 0.05$) (Fig. 2D). In general, bioactive compounds in fruit byproducts
289 were sensitive to heat and may undergo chemical degradation, isomerization or polymerization
290 when subjected to elevated temperature (Yu & Ahmedna, 2013). Freeze-drying at low
291 temperature and vacuum conditions is a more gently process preventing the bioactive
292 compounds, especially the polyphenolics, from thermal degradation (Michalczyk, Macura, &
293 Matuszak, 2009).

294 It is important to mention that the highest TPC for DA, and TPC, FRAP and DPPH
295 scavenging activity for DP substrates presented on Fig. 2 were obtained at the initial phase of
296 fermentation. Then, a decrease was observed in these values (Fig. 1). This may be caused by the
297 action of phenolic degrading and/or stress induced polymerizing enzymes of the fermenting

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298 fungus, similar to that observed for black grape substrates (see 3.1.1.) and reported in other
299 studies (Vattem, Lin, Labbe, & Shetty, 2004; Dulf, Vodnar, & Socaciu, 2016). However, the
300 recovery of some small phenolics of DP sample was increased during the fermentation (Table 1),
301 which may be explained by the hydrolysis of total phenolic compounds (Buenrostro-Figueroa et
302 al., 2017).

303 By comparing the fruit samples, black grape pomace exhibited the highest TPC and FRAP
304 ($p < 0.05$), followed by data obtained for pitahaya and apple samples (Fig. 2B and C). DPPH
305 scavenging activities of fermented black grape substrates was also superior to that detected for
306 the other supports tested ($p < 0.05$) (Fig. 2C). Interestingly, SSF of the LBG sample exhibited the
307 lowest β-glucosidase production and the highest TPC and FRAP among the fruit residues and
308 pretreatments tested ($p < 0.05$). In our previous experiments the *R. miehei* was not able to grow
309 on black grape seed extracts presumably due to the antifungal effect of the substrate (Kotogán,
310 Papp, Vágvölgyi, & Takó, 2013). In the current study, we hypothesized that the antioxidant and
311 antifungal power of polyphenolics found in the LBG pomace partly inhibited the β-glucosidase
312 production and/or activity during the fermentation.

313 *3.2. Enzymatic treatment of oven-dried and lyophilized fruit residues*

314 *3.2.1. Evolution of total phenolics and antioxidant activity*

315 TPC and FRAP of LBG pomace showed significant ($p < 0.05$) increase after cellulase and
316 cellulase/pectinase treatments (Figs. 3A and B). For DBG samples, cellulase treatment also
317 improved the TPC and FRAP compared to the enzyme-free control. However, interestingly, this
318 was not observed when the reaction mixture was supplemented with pectinase (Figs. 3A and B).
319 Regarding to DPPH radical scavenging activity (Fig. 3C), only pectinase addition resulted a
320 slight increase in the inhibition. Nevertheless, the *R. miehei* cellulase/*A. niger* pectinase

321 combined treatment was effective to liberate free phenolics and increase the antioxidant activities
322 of the LBG samples. In a previous study testing tannase treatment of grape pomace, the TPC
323 increased up to 6 times, and it was observed that the pectinase additive could release gallic acid
324 from galloylated catechins and thus, increase the antioxidant activity of the extracts (Chamorro,
325 Viveros, Alvarez, Vega, & Brenes, 2012).
326 For apple residues, both cellulase and cellulase/pectinase treatments positively affected
327 the TPC. After 5-h incubation only in cellulolytic surrounding, the overall TPC showed about 1.7
328 and 1.3 times increase ($p < 0.05$) in DA and LA samples, respectively, to that detected for
329 untreated samples (Fig. 4A). Furthermore, statistically significant ($p < 0.05$) increase in the
330 phenolics yield was also observed when cellulase/pectinase catalysts were used in combination.
331 This could be attributed to the cell wall depolymerization effect of pectinase, which is responsible
332 to the release of soluble dry mass, including phenolic substances, from the residues (Will,
333 Bauckhage, & Dietrich, 2000). In most papers, the optimal pectinase concentration for phenolics
334 liberation is different, but it strongly depends on the type of the commercial enzyme used for the
335 reaction. The Pectinex AFP L-4, Pectinex Yield Mash and Pectinex XXL pectinases, for
336 example, proved to be strong biocatalysts for the liberation of phenolics from apple pomaces,
337 while the Pectinex Ultra SPL did not affect the free phenolics yield (Oszmiański et al., 2011).
338 The TPC of LA substrates was slightly higher in both enzymatic treated and untreated systems
339 than that detected for the corresponding DA samples. The antioxidant activity followed this trend
340 (Figs. 4B and C); however, DPPH radical scavenging activity calculated for cellulase/pectinase
341 treated DA residues was slightly superior compared to the other samples.
342 TPC and FRAP of the DP and the LP samples exhibited an increase after a 5-h incubation
343 with the cellulase/pectinase cocktail (Figs. 5A and B). As the pectin content of the pitahaya
344 residues is relatively low compared to those of grapes and apple (Mahattanatawee et al., 2006), it

345 was expected that the pectinase treatment does not influence significantly its extractable phenolic
346 content. However, the added pectinase resulted in about 1.4- and 1.2-fold increase in the TPC
347 recovery and FRAP of the DP sample, respectively, compared to the enzyme-free control ($p <$
348 0.05) (Fig. 5A and B). Furthermore, the cellulase/pectinase combined treatment affected
349 positively the DPPH radical scavenging activity of the DP substrate (Fig. 5C). To our knowledge,
350 no characterization of the TPC and antioxidant activity of pitahaya residues after cellulolytic and
351 pectinolytic enzyme treatment has been reported so far.

352 *3.2.2. Liquid chromatography analysis of individual phenolic compounds*

353 Chromatographic data indicate that total yield of the tested individual phenolics increased
354 after enzymatic treatments (Tables 2 and 3), which agrees with the results obtained for TPC
355 determination. However, it is worth to note that the Folin-Ciocalteu's reagent used for the TPC
356 assay reacts not only with phenolic compounds but also with amino acids, sugars and other
357 reducing substances present in the extracts (Rapisarda et al., 1999). It can cause differences
358 between the degree of phenolic content changes determined by spectrophotometric and
359 chromatographic methods.

360 With regards to the pectinase supplemented treatments, chromatographic measurements
361 showed a decrease in the amount of some individual phenolics. This was most obvious for the
362 data of the DBG extracts (Table 2). Similarly, TPC in spectrophotometric assays displayed a
363 decrease after cellulase/pectinase addition to the DBG samples (see Fig. 3A). A probable reason
364 for this phenomenon can be the transformation of the phenolics into volatile compounds during
365 incubation (Sandri, Fontana, Barfknecht, & da Silveira, 2011; Shah et al., 2015).

366 During the cellulase treatment, (+)-catechin and (–)-epicatechin were found in the largest
367 concentration in grape pomaces (Tables 2 and 3). Compared to the control treatments, their

368 quantities in the DBG and LBG samples had increased about 6 and 26 times, respectively ($p <$
369 0.05). The contents of these flavan-3-ols also increased when apple residues were incubated with
370 the cellulase cocktail. Furthermore, chromatography detected an about 10 times increase ($p <$
371 0.05) in the (–)-epicatechin content of LA after addition of pectinase (Table 3). This could ascribe
372 to the decomposition of apple pectin fiber, supporting the liberation of epicatechin from its
373 bounded form with insoluble fiber and pectin. Anyway, the bioavailability of the entrapped apple
374 epicatechin is limited (Hollands et al., 2013), which could be enhanced by the *R. miehei*
375 cellulase/*A. niger* pectinase combined treatment. However, a decrease in the flavan-3-ol content
376 was detected in some samples after pectinase addition, which may be due to both oxidation and
377 several polymerization reactions (Ye, Yue, & Yuan, 2014).

378 The content of hydroxybenzoates (gallic, 4-hydroxybenzoic, vanillic and syringic acids),
379 and hydroxycinnamates (*p*-coumaric and cinnamic acids) increased by different degrees. The
380 LBG residues contained the highest level of these phenolics and, except for cinnamic acid, their
381 amounts increased by about 10 to 20 times ($p < 0.05$) after the enzymatic treatments (Table 3).
382 The same trend was reported during the treatment of grape pomace using Novoferm 106
383 pectinolytic and Cellubrix ®L cellulolytic enzyme mixtures (Maier, Göppert, Kammerer,
384 Schieber, & Carle, 2008). The DP samples showed the highest initial gallic acid concentration
385 among the fruit residues studied. Moreover, its content increased to 1.7 times ($p < 0.05$) after
386 cellulase/pectinase treatment (Table 2).

387 The detected stilbenoids during enzymatic treatment were resveratrol and polydatin, the
388 glycoside form of resveratrol. Except for DP samples, the initial content of resveratrol in residues
389 was below the detection limit (Tables 2 and 3). This low level may be due to the poor
390 extractability of resveratrol from the residues in the applied aqueous environment. But the
391 resveratrol yield increased in all lyophilized and the DA samples after treatment with cellulase.

392 Polydatin, which also displays a broad variety of beneficial effects on human health (Ravagnan et
393 al., 2013), was found in the largest concentration in the DP samples. Compared to the initial
394 content, it showed an about 1.6 times increase ($p < 0.05$) during enzyme treatments (Table 2).
395 The amount of polydatin in DP residues was higher than that of the resveratrol. Similar difference
396 between the concentration of resveratrol and its glycosidic form has been reported for grape
397 samples by Romero-Pérez, Ibern-Gómez, Lamuela-Raventós, & De la Torre-Boronat (1999).
398 Although many fruit products and processing residues have extensively been investigated in the
399 literature in terms of their resveratrol and resveratrol glycoside content, as we know, there is no
400 data about these phenolics in pitahaya samples.

401 Quercetin was observed after cellulase treatment of both DBG and LBG pomace extracts
402 (Tables 2 and 3), and after cellulase/pectinase treatment of LBG and LA samples (Table 3). In
403 line with our previous study testing sour cherry pomace (Krisch et al., 2012), it could be a result
404 of the hydrolysis of the quercetin aglycone from quercetin glycosides by the *R. miehei* cellulase.
405 No quercetin was detected in the pitahaya samples irrespectively of the enzymatic treatments.

406 **4. Conclusions**

407 In the present work, we have studied the influence of solid-state *R. miehei* fermentation
408 and enzymatic treatments with *R. miehei* cellulase and *A. niger* pectinase cocktails on the
409 mobilization of antioxidative phenolic aglycones from black grape, apple and pitahaya residues.
410 Our investigations demonstrated positive associations between the β-glucosidase activity and the
411 TPC and FRAP during the fungal growth on lyophilized pitahaya substrate. In general, both
412 chromatographic and spectrophotometric data confirmed increased TPC after cellulase treatment,
413 coinciding with an improved FRAP. Except for oven-dried black grape samples, application of
414 combined cellulase and pectinase treatment also enhanced the TPC compared to the enzyme-free

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415 sample. The concentration of individual phenolics changed by different degrees after the
416 enzymatic treatments depending on the substrate and the pretreatment: (+)-catechin and (–)-
417 epicatechin were the major small compounds in the black grape and apple residues while the
418 content of gallic and vanillic acids increased significantly in case of the oven-dried pitahaya
419 samples.

420 In conclusion, both SSF and enzymatic treatments had a positive effect on the release of
421 free phenolics from the residues involved. However, although we have not yet investigated such
422 enzyme activities, phenolic degrading and/or stress induced polymerizing enzymes of the
423 fermenting fungus may cause free phenolic loss during the fermentation, which underlines the
424 better applicability of direct enzymatic treatment. Nonetheless, our investigations provide useful
425 data on the application of *R. miehei* and its cellulase to generate free phenolics from fruit wastes.
426 The phenolic compound-enriched extracts then can be used as a source of antioxidant natural
427 products.

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434

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578 **Figure captions**

579

580 **Fig. 1.** β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP)
581 and free radical scavenging activity (DPPH) of oven-dried and lyophilized black grape (A), apple
582 (B) and yellow pitahaya (C) byproducts during solid-state fermentation by *R. miehei* NRRL 5282.
583 Results are presented as averages from three replicates; error bars represent standard deviation.

584

585 **Fig. 2.** Comparison between pretreatment methods and fruit residue types according to the best β-
586 glucosidase activity (A), total phenolic content (B) and antioxidant activity (C and D) data
587 achieved on oven-dried and lyophilized pomaces during solid-state *R. miehei* NRRL 5282
588 bioconversion. Results are presented as averages from three replicates. The different letters above
589 the columns show significant differences according to two-way ANOVA followed by Tukey's
590 Multiple Comparison Test ($p < 0.05$; independent variables: pretreatment and the fruit residue).

591

592 **Fig. 3.** Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (GS1) and *A. niger* pectinase
593 supplemented (GS2) treatments on the TPC (A), and FRAP (B) and DPPH scavenging (C)
594 activity of oven-dried and lyophilized black grape pomaces. Enzyme-free (GC1), and only
595 pectinase contained (GC2) mixtures were used as controls. Results are presented as averages
596 from three replicates. The different letters above the columns indicate significant differences
597 according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$;
598 independent variables: pretreatment and the type of the enzyme treatment).

599

600 **Fig. 4.** Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (AS1) and *A. niger* pectinase
601 supplemented (AS2) treatments on the TPC (A), and FRAP (B) and DPPH scavenging (C)

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602 activity of oven-dried and lyophilized apple samples. Enzyme-free (AC1), and only pectinase
603 contained (AC2) mixtures were used as controls. Results are presented as averages from three
604 replicates. The different letters above the columns indicate significant differences according to
605 two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent
606 variables: pretreatment and the type of the enzyme treatment).

607

608 **Fig. 5.** Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (PS1) and *A. niger* pectinase
609 supplemented (PS2) treatments on the TPC (A), and FRAP (B) and DPPH scavenging (C)
610 activity of oven-dried and lyophilized yellow pitahaya residues. Enzyme-free (PC1), and only
611 pectinase contained (PC2) mixtures were used as controls. Results are presented as averages from
612 three replicates. The different letters above the columns indicate significant differences according
613 to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent
614 variables: pretreatment and the type of the enzyme treatment).

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616 **Table 1**

617 Liberation of phenolic substances (mg/100 g DM) from oven-dried pitahaya residues fermented
618 with *R. miehei* NRRL 5282. Concentration of vanillic acid, syringic acid, cinnamic acid,
619 quercetin, polydatin and resveratrol compounds were below the detection limit (<DL). The
620 measurements were performed from the ethanol:water 50:50 solutions.

621

Fermentation time (day)	Gallic acid	4-Hydroxybenzoic acid	*p*-Coumaric acid	(+)-Catechin	(–)-Epicatechin
0	<DL*	<DL	<DL	<DL	<DL
3	1.24 ± 0.09	<DL	<DL	<DL	1.88 ± 0.30
7	7.88 ± 1.00	2.06 ± 0.38	<DL	4.49 ± 0.19	14.32 ± 0.49
15	37.93 ± 2.19	3.33 ± 0.31	0.77 ± 0.01	6.77 ± 0.29	25.49 ± 1.21

622
623 * Detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively,
624 and 0.49 mg/L for the other phenolics tested.

625 Values are averages from measurements performed in triplicates ± standard deviation.

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Table 2

Concentration of phenolic acids and other phenolic compounds (mg/100 g DM) detected in oven-dried black grape, apple and yellow pitahaya extracts pre- and post enzymatic treatment. The measurements were performed from the supernatants of the reaction mixtures.

Compounds	Fruit residues								
	Black grape			Apple			Pitahaya		
	Control[*]	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase
Gallic acid	2.28 ± 0.32[a]	6.21 ± 0.46[b]	3.61 ± 0.26[ab]	2.17 ± 0.24[a]	<DL	0.58 ± 0.06[a]	19.68 ± 1.31[c]	27.53 ± 2.02[d]	34.16 ± 2.95[e]
4-Hydroxybenzoic acid	<DL[**]	6.31 ± 0.52[a]	2.88 ± 0.38[b]	<DL	1.99 ± 0.17[b]	1.78 ± 0.28[b]	5.44 ± 0.52[a]	5.04 ± 0.64[a]	5.93 ± 0.84[a]
Vanillic acid	2.11 ± 0.21[a]	12.61 ± 0.93[b]	11.27 ± 0.39[b]	<DL	3.56 ± 0.34[a]	3.18 ± 0.31[a]	5.16 ± 0.35[a]	11.99 ± 1.13[b]	15.86 ± 1.35[c]
Syringic acid	2.27 ± 0.31[a]	7.97 ± 0.80[b]	14.58 ± 0.81[c]	<DL	2.09 ± 0.21[a]	2.29 ± 0.33[a]	5.94 ± 0.66[b]	6.19 ± 0.93[b]	7.69 ± 0.77[b]
p-Coumaric acid	<DL	2.56 ± 0.16[a]	10.35 ± 0.35[b]	<DL	<DL	1.78 ± 0.37[a]	2.61 ± 0.28[a]	3.21 ± 0.60[ac]	4.41 ± 0.84[c]
Cinnamic acid	<DL	<DL	<DL	<DL	<DL	<DL	1.59 ± 0.18[a]	1.66 ± 0.37[a]	1.73 ± 0.35[a]
(+)-Catechin	3.59 ± 0.42[a]	22.14 ± 1.42[b]	7.67 ± 0.52[c]	<DL	8.93 ± 0.55[c]	6.22 ± 0.57[c]	22.86 ± 2.08[b]	18.03 ± 0.78[d]	3.74 ± 0.51[a]
(–)-Epicatechin	3.44 ± 0.38[a]	21.73 ± 0.91[b]	7.82 ± 0.42[c]	<DL	2.59 ± 0.26[a]	3.35 ± 0.41[a]	14.02 ± 0.93[d]	6.95 ± 0.84[c]	10.87 ± 1.21[e]
Quercetin	<DL	6.11 ± 0.32	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Polydatin	<DL	3.97 ± 0.34[a]	<DL	<DL	<DL	<DL	22.56 ± 1.17[b]	28.56 ± 1.11[c]	36.56 ± 3.15[d]
Resveratrol	<DL	<DL	<DL	<DL	1.99 ± 0.24[a]	<DL	2.46 ± 0.44[a]	2.82 ± 0.62[a]	<DL
Total	13.69	89.61	58.18	2.17	21.15	19.18	102.32	111.98	120.95

[*] without enzymatic treatment; [**] <DL, below detection limit; detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively, and 0.49 mg/L for the other phenolics tested.

Values are averages of measurements performed in triplicates ± standard deviation; mean values within a row with different letters are significantly differ according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent variables: fruit residue and type of the enzyme treatment).

Table 3

Concentration of phenolic acids and other phenolic compounds (mg/100 g DM) detected in lyophilized black grape, apple and yellow pitahaya extracts pre- and post enzymatic treatment. The measurements were performed from the supernatants of the reaction mixtures.

Compounds	Fruit residues								
	Black grape			Apple			Pitahaya		
	Control[*]	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase
Gallic acid	3.05 ± 0.59^{a}	43.77 ± 4.10^{b}	37.23 ± 3.10^{b}	<DL	5.11 ± 0.74^{a}	3.91 ± 0.86^{a}	2.24 ± 0.35^{a}	2.61 ± 0.34^{a}	2.56 ± 0.24^{a}
4-Hydroxybenzoic acid	4.21 ± 0.66^{a}	68.23 ± 4.27^{b}	67.45 ± 6.11^{b}	2.35 ± 0.41^{a}	8.32 ± 0.98^{c}	7.30 ± 0.92^{c}	1.53 ± 0.21^{a}	1.67 ± 0.38^{a}	1.61 ± 0.25^{a}
Vanillic acid	2.75 ± 0.37^{a}	60.26 ± 6.11^{b}	60.27 ± 6.64^{b}	1.52 ± 0.35^{a}	14.64 ± 1.42^{c}	12.49 ± 0.57^{c}	<DL	2.87 ± 0.34^{a}	2.84 ± 0.36^{a}
Syringic acid	2.45 ± 0.48^{a}	33.06 ± 2.68^{b}	35.46 ± 3.47^{b}	0.76 ± 0.15^{a}	3.23 ± 0.44^{a}	3.92 ± 0.49^{a}	2.08 ± 0.18^{a}	2.29 ± 0.24^{a}	2.22 ± 0.16^{a}
p-Coumaric acid	1.83 ± 0.36^{a}	10.35 ± 1.16^{b}	15.36 ± 2.11^{c}	0.89 ± 0.09^{a}	0.95 ± 0.11^{a}	2.97 ± 0.67^{a}	<DL	1.80 ± 0.19^{a}	1.81 ± 0.18^{a}
Cinnamic acid	<DL[**]	1.51 ± 0.36^{a}	1.49 ± 0.35^{a}	<DL	<DL	<DL	<DL	<DL	<DL
(+)-Catechin	7.48 ± 0.89^{a}	209.83 ± 22.31^{b}	200.51 ± 20.33^{b}	<DL	33.23 ± 3.62^{c}	2.81 ± 0.55^{a}	<DL	<DL	<DL
(–)-Epicatechin	8.75 ± 0.82^{a}	215.81 ± 17.17^{b}	188.13 ± 19.93^{b}	6.71 ± 0.84^{a}	10.05 ± 1.22^{a}	99.92 ± 9.68^{c}	2.79 ± 0.34^{a}	2.83 ± 0.28^{a}	2.77 ± 0.34^{a}
Quercetin	<DL	8.54 ± 0.81^{a}	7.61 ± 0.71^{a}	<DL	<DL	6.49 ± 0.75^{a}	<DL	<DL	<DL
Polydatin	2.63 ± 0.43^{a}	<DL	<DL	<DL	<DL	<DL	3.35 ± 0.41^{a}	3.51 ± 0.37^{a}	3.49 ± 0.36^{a}
Resveratrol	<DL	4.28 ± 0.61^{a}	4.67 ± 0.53^{a}	<DL	0.51 ± 0.02^{b}	<DL	<DL	2.17 ± 0.19^{c}	2.12 ± 0.17^{c}
Total	33.15	655.64	618.18	12.23	76.04	139.81	11.99	19.75	19.42

[*] without enzymatic treatment; [**] <DL, below detection limit; detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively, and 0.49 mg/L for the other phenolics tested.
Values are averages of measurements performed in triplicates ± standard deviation; mean values within a row with different letters are significantly differ according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent variables: fruit residue and type of the enzyme treatment).

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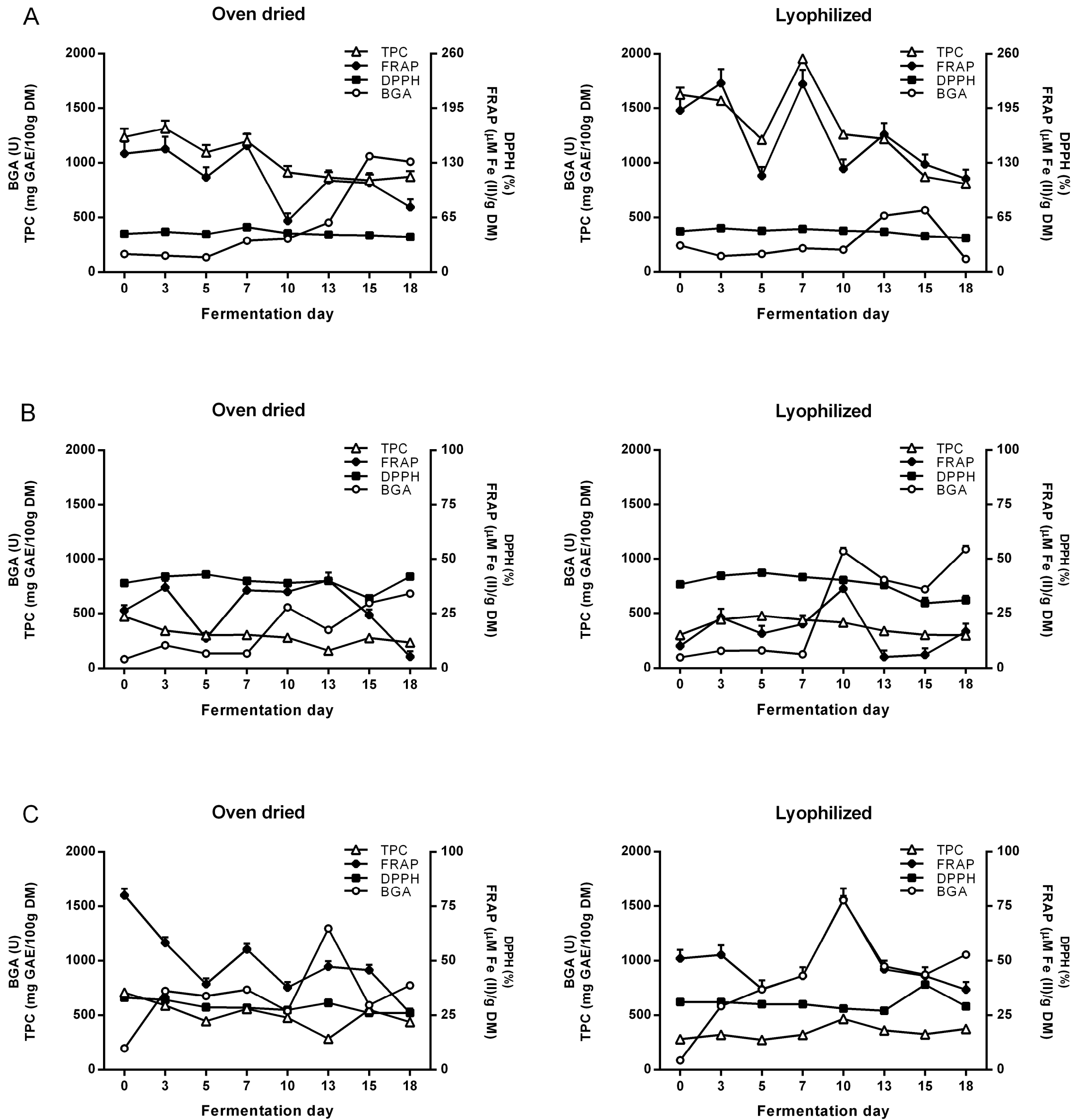
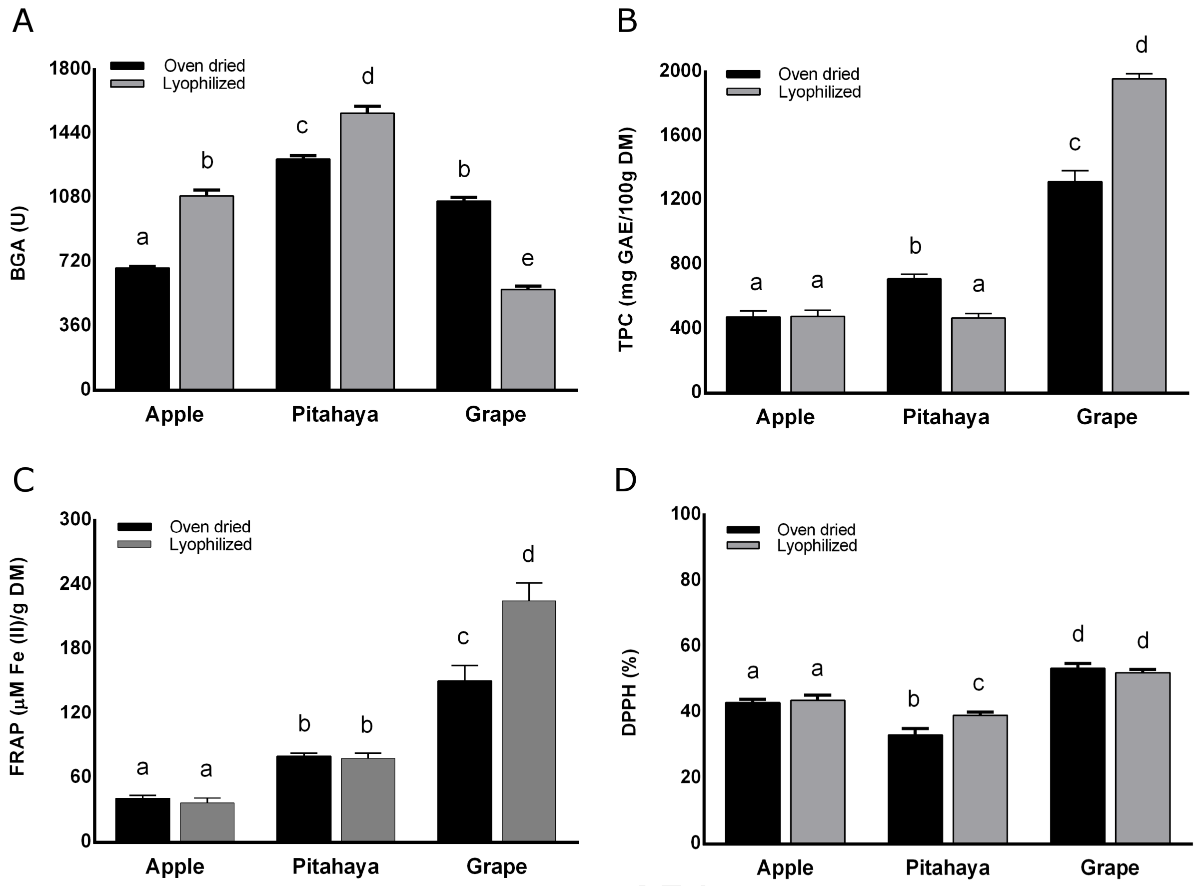


A
Oven dried
Lyophilized
TPC
FRAP
DPPH
BGA
BGA (U)
TPC (mg GAE/100g DM)
DPPH (%)
FRAP (µM Fe (II)/g DM)
Fermentation day
B
Oven dried
Lyophilized
C
Oven dried
Lyophilized



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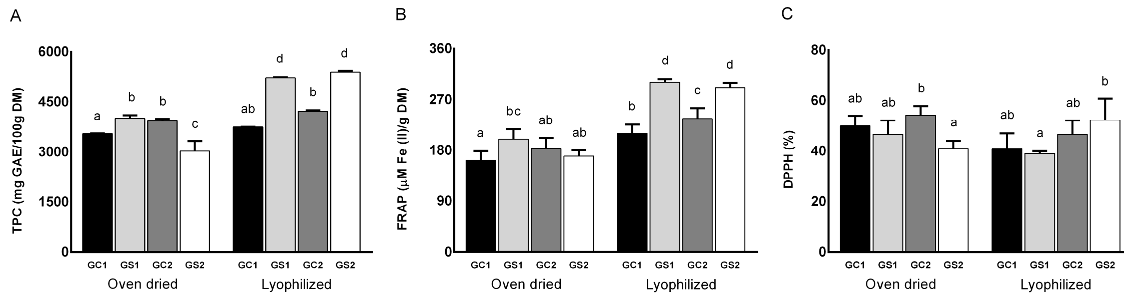

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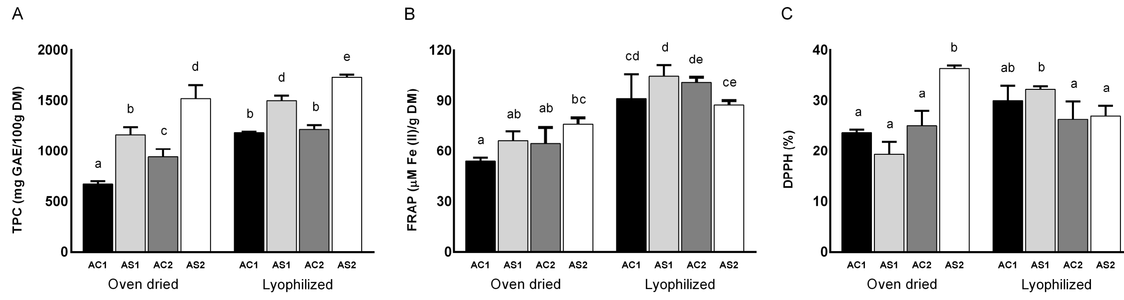

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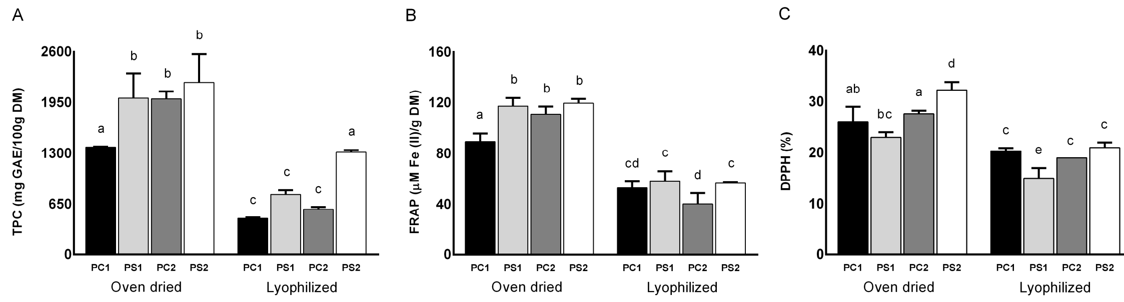

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Highlights

- Liberation of phenolics from lyophilized and oven-dried fruit residues was studied.
- *Rhizomucor miehei* fermentation and carbohydrase treatments were conducted.
- Both strategies improved the total phenolic content (TPC) and antioxidant capacity.
- The effect of substrate pretreatment process on TPC and activity was examined.
- The major phenolic products were hydroxybenzoic acids and flavan-3-ols in extracts.

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