3
4   **Enhancing laccase production by white-rot fungus *Funalia floccosa LPSC 232* in co-culture with**
5   ***Penicillium commune* GHAIE86**
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Abstract

To obtain enzymatic preparations with higher laccase activity levels from *Funalia floccosa* LPSC 232, available for use in several applications, co-cultures with six filamentous microfungi were tested. A laccase non-producing soil fungus, identified as *Penicillium commune* GHAIE86, showed an outstanding ability to increase laccase activity (3-fold as compared to that for monoculture) when inoculated in 6-d-old *F. floccosa* cultures. Maximum laccase production with the *F. floccosa* and *P. commune* co-culture reached 60 U/mL, or twice that induced by chemical treatments alone. Our study demonstrated that co-culture with soil fungi might be a promising method for improving laccase production in *F. floccosa*. Although the enhancement of laccase activity was a function of *P. commune* inoculation time, two laccase isoenzymes produced by *F. floccosa* remained unchanged when strains were co-cultured. These data are compatible with the potential of *F. floccosa* in agricultural applications in soil, whose enzyme machinery could be activated by soil fungi such as *P. commune*.

Keywords: laccase activity enhancement; fungal interaction; co-culture; white-rot fungus; soil fungus; isoenzymes

Introduction

Funalia floccosa LPSC 232 (formerly known as *Coriolopsis rigida*) is a model white rot fungus which produces extracellular laccases (E.C. 1.10.3.2), with promising enzymes for industrial applications such as the paper, textile and food industries as well as for detoxification treatments and biosensor development (Saparrat et al. 2014). This fungus is therefore an environmentally friendly candidate to produce large amounts of these enzymes. In addition, this fungus has potential for promoting the growth and development of several plants of economic importance such as blueberries, tomato and eucalyptus (Almonacid et al. 2015; Arriagada et al. 2012, 2014) in co-inoculation with mycorrhizal fungi. It could also be used in the degradation of polycyclic aromatic hydrocarbons (Gómez et al. 2006), the decolouration of industrial anthraquinone dyes (Sánchez-López et al. 2008), the transformation and detoxification of the phenolic content of olive mill waste (Sampedro et al. 2004) and the bioremediation of soil contaminated by crude oil (Colombo et al. 1996).

Culture systems reported to produce laccases from *F. floccosa* include inoculation in liquid media and on lignocellulosic-rich solid matrices (Alcántara et al., 2007). To our knowledge, enzyme levels obtained using these procedures are low and need to be supplemented with high-cost chemical inductors as compounds related to lignin and its derivatives, phenolic and other aromatic compounds, copper ions and industrial waste water (Saparrat et al. 2004, 2014). However, chemical inducers are expensive and, in some cases, toxic and ineffective, with their possible practical applications being limited due to their availability or high cost (Saparrat et al. 2010, 2014). Currently, the search for economical and safe methods for the production of laccase is therefore one of the most interesting areas of enzyme research (Saparrat et al. 2014). In this regard, its production on a large scale is hampered by several technical constraints, including inoculum formulations and their mode of application (Baldrian 2008). One of the

73 new sustainable strategies to obtain laccases from white rot fungi is co-cultivation with other fungi (Ma et
74 al. 2015). The combination of ligninolytic fungi have dramatic dynamic effects on the production of
75 lignocellulose-active enzyme, which may lead to divergent degradative processes of dead wood and forest
76 litter (Mail et al. 2017), antibiotic (Gao et al. 2018) and decolorization of industrial dye (Kumari et al.
77 2016). Other studies have shown that co-cultivation of white-rot fungi, such as *Lentinula edodes*,
78 *Pleurotus ostreatus*, *Trametes versicolor* and *Phanerochaete chrysosporium*, with filamentous fungi, such
79 as *Trichoderma* spp. and *Paecilomyces carneus*, increases laccase production (Chan-Cupul et al. 2014;
80 Flores et al. 2009, 2010; Mata et al. 2005; Savoie et al. 2001). Although the mechanisms involved in this
81 laccase induction have not been fully elucidated, they relate to their role in biological interactions or
82 stress defence (Crowe and Olsson 2001). The production of laccase by white-rot fungi in co-culture with
83 filamentous fungi other than *Trichoderma* sp. has been poorly documented and, to date, there are no
84 studies of *F. floccosa* laccase production in co-culture. This study was therefore undertaken in order to: a)
85 evaluate the production of laccase by the white-rot fungus *F. floccosa* in co-culture with six micro fungi
86 strains in order to make high levels of this activity available for use in various applications; b) determine
87 whether, by establishing co-cultures, the inoculation time of the microfungi affects the laccase activity of
88 *F. flocossa*; and c) to characterize the natural isoenzymes secreted in the interaction.
89
90 **Material and methods**
91
92 *Fungal strains*
93
94 *Funalia floccosa* LPSC 232 (Spegazzini Institute Culture Collection) isolated from decaying wood
95 collected from a subtropical rain forest in Argentina (Ibañez 1998), *Penicillium* GHAIE86 isolated from
96 coffee plantation soil in Veracruz, México, *Penicillium chrysogenum* EEZ 10, *Penicillium*
97 *brevicompactum* EEZ32 and *Fusarium graminearum* BAFC 122 isolated from soil from Castañar de Ibor,
98 Granada (Spain) and Buenos Aires (Argentina), respectively, *Mucor racemosus* EEZ113 isolated from the
99 dry residue of olive oil industry and *Paecilomyces farinosus* BAFC F8846 isolated from *Funneliformis*
100 *mosseae* sporocarps were obtained from the collection of the Estación Experimental del Zaidín (EEZ),
101 CSIC (Granada, Spain) and the culture collection of the Faculty of Exact and Natural Sciences, University
102 of Buenos Aires (BAFC) (Argentina). All strains were maintained at 4 ºC on malt extract agar plates.
103
104 *Co-cultures of F. floccosa and microfungi for laccase activity evaluation*
105
106 All fungi were grown separately at 28 ºC in 250 mL Erlenmeyer flasks containing 70 mL of a glucose-
107 yeast medium on a rotary shaker at 80 rpm (Evans and Niven 1951). The flasks were inoculated with 2
108 cm^2 agar plugs covered by 7-d-old mycelia. After 7 d, the mycelia were collected and homogenized with
109 distilled water (1:1 v/v, mycelia:water). One millilitre of the suspension was used as inoculums (mycelia,
110 500 mg).
111 Laccase production by *F. floccosa* grown in monoculture and in co-cultures, with microfungi
112 inoculated at the same time was studied in 250 mL Erlenmeyer flasks containing 70 mL of medium and

113 incubated on static cultures for 21 d as described by Guillén et al. (1992). Each experiment was

114 performed in triplicate.

115   Laccase activity was assayed by the oxidation of 5 mmol/L 2,6-dimethoxyphenol (DMP) to

116 coerulignone in 100 mmol/L sodium acetate buffer (pH 5.0) (Muñoz et al. 1997).

117

118 *Morpho-physiological characteristics of isolate Penicillium GHAIE86*

119

120 Morphological identification was carried out according to the method described by Pitt (2000). The

121 isolate was inoculated as 3-point cultures in Czapek yeast extract agar (CYA, 25 ºC and 37 ºC), malt

122 extract agar (MEA, 25 ºC) and 25 % glycerol nitrate agar (G25N, 25 ºC) and incubated for 7 d in

123 darkness.

124   Texture, pigmentation and colony diameter as well as sporulation were recorded in each medium.

125 The structures differentiated, such as conidiophores, phialides and conidia, in these cultures were

126 examined using an Olympus microscope Model CX41 UC-MAD3.

127

128 *Molecular identification of Penicillium GHAIE86*

129 Genomic DNA from the fungus was isolated from 7-d glucose-yeast medium mycelium using a Genomix

130 DNA extraction kit (Talent, Italy) according to the manufacturer's instructions. The eluted DNA was

131 stored at -20 ºC and used as a template for PCR amplifications. The ITS region was amplified by PCR

132 using the primers and protocol described elsewhere (White et al. 1990). The PCR-amplified fragment was

133 purified using the UltraCleanTM PCR Clean-Up TM kit (MoBio Laboratories Inc., USA) according to

134 the manufacturer's instructions and was sent for direct sequencing to STABVIDA®. Automated

135 sequencing of both strands was performed using the BigDye Terminator Kit from Applied Biosystems

136 and the 96-capillary 3730xL DNA Analyzer from Applied Biosystems. The sequence was corrected using

137 Chromas version 1.43 (Griffith University, Brisbane, Australia) and was analyzed and edited using

138 Bioedit Sequence Alignment Editor version 7.0.9.0 (Hall 1999). The sequence was deposited in the

139 GenBank database under accession number KY174328.

140

141 *Optimization of co-culture F. floccose-P.commune for laccase production*

142

143 Laccase production by static *F. floccosa* cultures grown in monoculture and in co-culture with *P.*

144 *commune* inoculated at the same time or after 3 and 6 d of *F. floccosa* inoculation and its characterization

145 was carried out in 250 mL Erlenmeyer flasks containing 70 mL of medium as described previously. The

146 inoculum of both fungi was performed as indicated previously. All the cultures were maintained for 21 d

147 at 28 ºC under static conditions. Three replicate cultures of each treatment were tested. Samples were

148 taken after 12, 18 and 21 d from the cultures and the supernatants, separated from mycelia by

149 centrifugation at 8,000 *g* for 10 min, were analyzed for laccase activity.

150

151

152

153   *Laccase purification*

154

155   A crude enzyme preparation with laccase activity was obtained both from 21-d-old cultures of *F. floccosa*

156   grown alone and in co-culture with *P. commune* added after 6 d of incubation. The culture liquid was

157   separated from mycelia by centrifugation at 20,000 *g* for 20 min, dialyzed against 10 mmol/L sodium

158   acetate (pH 5.0) through a 12-14 kD a cut off membrane filtration (Spectrum) and was then concentrated

159   by ultrafiltration (Pall Filtron, 3-kDa cut off membrane).

160   1 mL samples of the crude enzyme preparation were applied to a Superdex 200 column

161   (Amersham Pharmacia Biotech HR 16/60) equilibrated with a 50 mmol/L phosphate buffer (pH 7.1)

162   containing 150 mmol/L NaCl at a flow rate of 0.4 mL/min. A laccase peak was pooled, concentrated

163   (Filtron Microsep, 3-kDa cutoff) and dialyzed against 10 mmol/L sodium acetate (pH 5.0) by using a PD-

164   10 desalting column (Amersham Biosciences). Then, 1 mL samples were applied to a Mono-Q anion-

165   exchange column (Pharmacia HR 5/50) equilibrated with the same buffer. The laccase isoenzymes were

166   eluted with a linear NaCl gradient from 0 to 250 mmol/L for 50 min and from 250 mmol/L to 1 mol/L for

167   7.5 min at a flow rate of 0.4 mL/min. Fractions of 2 mL were collected and the laccase peaks were

168   pooled, concentrated and stored with 10 % (w/v) glycerol at -4 ºC.

169   Proteins were determined according to the Bradford method, using bovine albumin as standard and

170   the BioRad kit assay (Bradford 1976).

171

172   *Isoelectric focusing (IEF), zymogram and molecular mass of laccases*

173

174   A preliminary characterization of laccase activity from a crude preparation of 21-d-old static *F. floccosa*

175   cultures grown in monoculture and in co-culture with *P. commune* inoculated after 6d of incubation was

176   carried out using IEF and by estimating molecular mass.

177   The isoelectric point of the laccases was determined by zymograms on 5 % polyacrylamide gels

178   with a thickness of 1 mm and a pH range from 3 to 10 (Amersham Pharmacia). The sample (20 µL)

179   contained 5-10 mU of laccase activity. The anode and cathode solutions were 1 mol/L phosphoric acid

180   and 1 mol/L sodium hydroxide, respectively. The pH gradient was measured on the gel by means of a

181   contact electrode. Protein bands with laccase activity were detected by using 5 mmol/L DMP in 200

182   mmol/L sodium acetate buffer (pH 5.0) after the gels were washed for 10 min with the same buffer (Díaz

183   et al. 2010).

184   The molecular mass of isoenzymes was estimated by size-exclusion chromatography. This

185   chromatography was carried out on a Superdex 200 column as described above. The column was

186   calibrated with blue dextran (2,000 kDa), albumin (67 kDa), ovoalbumin (43 kDa), chymotrypsinogen A

187   (25 kDa) and ribonuclease A (13.7 kDa).

188

189   *Statistical analysis*

190

191 The differences in laccase production among the treatments were assessed using one-way ANOVA with
192 Tukey's honest significance difference (HSD) post-hoc test. Normal distribution and heteroscedasticity of
193 data were tested by the Shapiro–Wilk and Breusch–Pagan tests, respectively.
194
195 **Results and discussion**
196
197 *Screening of microfungi with ability to enhance laccase production by F. floccose*
198
199 We analyzed the levels of extracellular laccase activity from 21-d-old static *F. floccosa* cultures grown in
200 monoculture and in co-culture with each of the six microfungi. We found that *F. floccosa* grown in static
201 cultures produced a similar level of laccase activity to that reported previously for this fungus (Saparrat
202 et al. 2002) (Fig. 1).
203   Although any of these tested fungal strains produced extracellular laccase activity when grown
204 axenically on the glucose-yeast medium under static conditions (data not shown), most of them were
205 found to increase the laccase activity of *F. floccosa* (Fig.1). However, while the co-cultures with *M.
206 racemosus* and *P. farinosus* showed similar levels of laccase activity compared to those from the *F.
207 floccose* monoculture, the highest induction of laccase activity was obtained in the co-cultures of *F.
208 floccosa* with *Penicillium* GHAIE86.
209
210 *Morpho-physiological characteristics and molecular identification of Penicillium GHAIE86*
211
212 The coloration and characteristics of the colonies developed on the different media used as well as the
213 microscopic morphology of the differentiated conidial system were typical representatives of *P. commune*
214 according to Pitt's description (Pitt 2000) included in subgenus *Penicillium*, section *Penicillium*.
215   The nucleotidic ITS sequence of isolate *GHAIE86* showed 100 % homology with the ITS
216 sequences of *P. commune* and one sequence from *P. camemberti*. However, following morphological
217 identification, our strain was classified as *P. commune*.
218   For a long time *P. commune* was considered as a food-borne fungus, frequently associated with
219 cheese and cottonseed meal spoilage (Samson et al. 1996; Wagner et al. 1980). However, in the last decade,
220 different reports have shown that this species proliferates in very distant and varied environments. It has
221 been isolated from soil samples collected in China (Liu et al. 2013), Canada (Out et al. 2016) and Kingdom
222 of Saudi Arabia (Mohamed et al. 2016). There are also studies that have used *P. commune* strains isolated
223 from: rhizospheric soil samples collected in India (Jain et al. 2013), marine sediments from southern China
224 Sea (Gao et al. 2011; Shang et al. 2012), and leaves and berries of *Vitis vinifera* from Portugal (Oliveira et
225 al. 2017). Taking into account this information it is feasible to assume that *P. commune* is a cosmopolitan
226 species with a high saprophytic ability to colonize different types of substrates.
227
228 *Laccase produced by F. floccosa LPSC 232 and P. commune GHAIE86 cocultures established with
229 simultaneous and delayed inoculation*
230

231   We also tested laccase activity in combined static *F. floccosa* and *P. commune* cultures at different

232   inoculation times. Fig. 2 shows that laccase activity from *F. floccosa* simultaneously inoculated with *P.*

233   *commune* was lower than that from *F. floccosa* monoculture. In soil fungus samples inoculated after 3 d

234   of *F. floccosa* incubation, we observed an increase in laccase production after 12 d of *F. floccosa-P.*

235   *commune* co-culture. The highest production of laccase activity in co-cultures of both fungi was obtained

236   when fungus *P. commune* was added to a 6-d *F. floccosa* culture (Fig. 2), which was three times higher

237   than that of the *F. floccosa* control. Our results suggest that the production of laccase by *F. floccosa* in

238   interaction with *P. commune* is dependent on a subsequently longer inoculation time as reported by Chan-

239   Cupul et al. (2014) for the *P. carneus-Trametes maxima* couple.

240   Several findings demonstrate that fungal laccases can be involved in physiological processes such

241   as lignin degradation, detoxification reactions and also as a virulence factor in interactions with other

242   organisms (Crowe and Olsson 2001). This latter role in biological interactions is considered to be species-

243   specific and also related to nutritional conditions (Chan-Cupul et al. 2014; Flores et al. 2009). Another

244   possible hypothesis is that laccases are released by basidiomycetes as part of a defensive response to

245   mycelial invasion, such as sequestering of nutrients already occupied by competitive/antagonistic

246   organisms (Baldrian 2004). It should be noted that the presence of *P. commune* did not affect *F. floccosa*

247   growth rates, since, in co-cultures, growth of this latter fungus was identical to that estimated for

248   monocultures, as shown by the typical colour of the *F. floccose* mycelium and the presence of clamp

249   connections (Supplementary Fig. 1).

250   The induction of laccase activity in *F. floccosa* by phenols from agro-industrial residues or heavy

251   metals such as Cu^{2+} has been previously reported (Saparrat et al. 2002; 2010). All these supplements

252   have several limitations because they can be highly toxic and adversely affect the laccase-producing

253   fungus as well as by their wastes that can pollute the environment. Therefore, our study shows data about

254   the use of an environmentally safe strategy to improve laccase (sustainable) production in *F. floccosa*

255   using biological techniques such as the inoculation with *P. commune*. Although different authors have

256   proposed the use of biological induction to increase laccase activity, as seen in the co-culture of

257   *Hypholoma fasciculare* with *Bacillus subtilis* (Griffith et al. 1994), *P. ostreatus* (Velázquez-Cedeño et al.

258   2004) and *Trametes sp.* AH28-2 (Zhang et al. 2006) with *Trichoderma*, *Trametes maxima* with *P.*

259   *carneus* (Chan-Cupul et al. 2014) and *Rhizostonia solani* with *Pseudomonas fluorescent* (Crowe and

260   Olsson 2001), laccase activity levels obtained in these studies were very low. For this reason, our main

261   concern in this study was to find a fungus capable of stimulating the *F. floccosa* laccase synthesis to a

262   significant degree. Previous studies indicated the induction of different enzymes like endoglucanase and

263   β-glucosidase in co-cultures of *Aspergillus niger* and *Fusarium oxysporum* (Hernández et al., 2018).

264   However, these studies also observed that when more than two strains were cultured, the relationships of

265   competition were established and a decrease of the amount of enzymes and the extracellular protein

266   isoforms produced. For this reason, the selection of the appropriate fungus and also the conditions of the

267   co-culture are the important factors to be considered. Our results demonstrate the potential of *P. commune*

268   for producing high levels of laccase activity in *F. floccosa*. This is the first study to demonstrate the

269   capacity of *P. commune* to enhance laccase production in white-rot fungi under co-culture conditions,

270   although further studies need to be carried out to elucidate the physiological mechanisms involved in

271	these responses. Several findings show the degradative effect of *F. floccosa* on numerous aromatic
272	compounds and complex matrices involving its extracellular laccase activity (Saparrat et al. 2010, 2014).
273	Colombo et al. (1996) have reported the ability of this fungus to degrade the aliphatic and aromatic
274	fractions of a crude oil from an artificially contaminated soil that was also colonized by *P. chrysogenum*,
275	which showed a low degradative capacity. Therefore, our study suggests the potential role of soil fungi in
276	laccase induction, such those belonging to the genus *Penicillium,* which might activate the degradation of
277	xenobiotics by *F. floccosa* in polluted soils. Saparrat et al. (2010) have reported the existence of at least
278	three laccase genes in the genome of this fungus, which showed a differential regulation at the
279	transcriptional level, although just two laccase isoenzymes encoded by the *lcc1* gene have been the only
280	ligninolytic enzymatic components found. One explanation for this increased laccase production could be
281	the synergistic relationship between *F. floccosa* and *P. commune.* The capacity of *P. commune* to produce
282	secondary metabolites under competitive conditions is probably related to the induction of laccase activity
283	in *F. floccosa.* In fact, a defence mechanism in *F. floccosa* to counteract the deleterious effect of
284	amphotericin B via the induction of laccase has been proposed, which leads to the highest levels of
285	extracellular laccase activity, even higher than copper (Saparrat et al. 2010). Further studies are necessary
286	to determine whether the secondary metabolites from *P. commune* such as amphotericin B are the agents
287	responsible for laccase induction in *F. floccosa*. Concomitantly, Svahn et al. Svahn et al. (2015) have
288	recently reported the production of amphotericin B by a *Penicillium nalgiovense* isolate from soil. The
289	remarkable ability of *Penicillium* species to produce several bioactive molecules as well as the high
290	diversity and environmental distribution of this fungal genus (more than 200 species are currently listed in
291	the Index Fungorum database) are well known. However, their potential use in co-culture systems, as
292	compared to those used with other microfungi belonging to *Trichoderma* spp has received little attention.
293	With regard to inoculation time, our results show that asynchronous inoculation of microfungi in
294	co-cultures with *F. floccosa* has a significant effect on laccase activity, as has been reported previously by
295	Dwivedi et al. (2011).The interspecific fungal interactions are quite complex and their physiological
296	mechanisms are little understood (Boddy 2000; Iakovlev and Stenlid 2000). Combative mechanisms in
297	fungal competition are closely related to the initial conditions of the resource to be colonized. The lowest
298	laccase activity levels (below those from *F. floccosa* monocultures) recorded under simultaneous
299	inoculation conditions may be due to the rapid development of the microfungi. These microfungi
300	produced large amounts of spores which germinated quickly and formed masses of mycelium
301	(characterized by a lack of clamp connections), taking advantage of the nutrients and inhibiting the
302	combative mechanisms of *F. floccosa*. Interspecific fungal interactions, besides playing a key role in
303	community structures and ecological processes, are a promising strategy not only to induce enzymes but
304	also to provide a high diversity of molecules such as pheromones and secondary metabolites with
305	important biotechnological applications (Bertrand et al. 2013).
306	
307	*Purification and characterization of laccase isoenzymes produced in co-cultures F. floccosa LPSC 232*
308	*and P. commune GHAIE86*
309	

310 To determine the possible induction of new *F. floccosa* laccase isoenzymes under co-culture conditions,

311 we purified the laccases produced by *F. floccosa* when grown in monoculture and those produced in co-

312 cultures with *P. commune*. In order to design the purification process, we carried out a preliminary

313 characterization of the extracellular proteins with laccase activity from a crude preparation obtained from

314 21-d-old-culture liquid supernatants of *F. floccosa* grown in monoculture and in co-culture with *P.*

315 *commune* inoculated after 6 d of *F. floccose* inoculation due to its higher laccase activity levels.

316 Isoelectric focusing of crude enzyme preparations obtained from an extracellular supernatant of *F.*

317 *floccosa* monocultures and from the co-culture of *F. floccosa* and *P. commune* showed two bands with a

318 pI of 3.4 and 3.6 in all cases (Fig. 3). These pI values are in line with those reported in the literature, as

319 the pI values for fungal laccases, ranging from 2.6 to 6.9, are generally in the acidic range (Baldrian 2006;

320 Saparrat et al. 2008).

321 The purification process was carried out using two chromatographic separations involving

322 exclusion molecular chromatography followed by ion-exchange chromatography. The conditions for *F.*

323 *floccosa* laccases purification were similar for all the treatments. During the first stage of chromatography

324 (Superdex 200), a major protein peak containing laccase activity was obtained (Fig. 4A and 5A). The

325 chromatographic profile of the *F. floccosa* laccase from co-cultures with *P. commune* was identical to the

326 profile of the monoculture grown for this study and to that previously described by Díaz et al. (2010).

327 Using size-exclusion chromatography, we estimated the molecular mass of both laccase produced by *F.*

328 *floccosa* and that from co-cultures *F. floccosa-P.commune* to be 60 kDa. Previous studies carried out with

329 *F. floccosa* laccase indicated a molecular mass of 66 kDa (Díaz et al. 2010; Saparrat et al. 2002). Several

330 fungal laccases show a molecular mass of 60 and 80 kDa (Guo et al. 2008).

331 In the second chromatographic step involving a high-resolution ion-exchange column (Mono-Q), it

332 was necessary to resolve two laccase activity peaks (LacI and LacII). These isoenzymes detected in co-

333 cultures of *F. floccosa* and *P. commune* have elution times (48 and 50 min, respectively) and NaCl

334 concentrations (14 and 16 %, respectively) similar to those for the *F. floccosa* monocultures analyzed in

335 this study and previously described by Díaz et al. (2010) (Fig 4B and 5B). Different systems and culture

336 conditions, such as growth in co-cultures with other organisms, are known to induce the expression of

337 new laccase isoenzymes (Baldrian 2004). One of the main objectives of this study was therefore to

338 identify and compare the enzymes produced by *F. floccosa* in mono or co-culture with a second organism

339 such as *P. commune*. However, our findings provided by size-exclusion and ion-exchange

340 chromatography, show laccase isoenzymes (LacI and LacII) produced by *F. floccosa*, either in

341 monoculture or in co-culture with *P. commune* (Fig. 5A and 5B) to be identical.

342 At the end of the purification process, LacI and LacII were purified 2.9- and 1.7-fold in

343 monoculture conditions, and 2.9- and 2.1-fold in co-culture conditions (Tables 1), respectively. Although

344 the yield from purification was similar in both types of *F. floccosa* culture, higher specific activity was

345 detected in laccase isoenzymes produced in co-cultures than in those produced in *F. floccosa*

346 monocultures. This is probably due to the different culture conditions or to the different catalytic

347 properties among laccases from the same organism, as has been described for *Pycnoporussanguineus*

348 (Dantán-González et al. 2008) and *Panus tigrinus* (Quaratino et al. 2008).

349

350   **Conclusions**

351

352   The interaction between *F. floccosa* LPSC 232 and *P. commune* GHAIE86 enhances laccase production.

353   The inoculation time of *P. commune* on *F. floccosa* static cultures plays an important role in laccase

354   enhancement. The increase in laccases produced by *F. floccosa* in a co-culture system could be an

355   attractive alternative to those required in a monoculture system using chemical inductors.

356

366

367   **Author contributions**

368   R.D. and G.H. contributed equally to this study.

369

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523 **Fig. 1.** Extracellular laccase activity of static *F. floccosa* LPSC 232 cultures grown in

524 monoculture (F.flo) and dual co-culture with *P. commune* GHAIE 86 (P.co), *P.*

525 *chrysogenum* EEZ 10 (P.ch), *P. brevicompactum* EEZ 32 (P.br), *M. racemosus* EEZ 113

526 (M.ra), *F. graminearum* BAFC 122 (F.gra) and *P. farinosus* BAFC F8846 (P.fa) after 21

527 d of incubation. Values are means of three replicates. Error bars correspond to standard

528 deviation. Bars with the same letter are not significantly different (Tukey test, $P < 0.01$).

529

530 **Fig. 2.** Time course of laccase activity in the extracellular fluid of *F. floccosa* LPSC 232

531 static cultures grown in monoculture (monoculture;▲) and in dual co-culture with *P.*

532 *commune* GHAIE 86 inoculated at the same time (■) or after 3 (●) and 6 d (**x**) of *F.*

533 *floccosa* inoculation. The results shown are mean values of 3 replicate flasks.

534

535 **Fig. 3.** Isoelectric focusing of laccase activity in the extracellular fluid of *F. floccosa*

536 LPSC 232 cultures grown in monoculture (monoculture; lane 1) and in dual co-culture

537 with *P. commune* GRAIE 86 inoculated after 6 d of *F. floccosa* inoculation (lane 2) at pH

538 3.0–8.0. The gel was stained with 5 mmol/L DMP in 200 mmol/L sodium acetate buffer

539 (pH 5.0).

540

541 **Fig. 4.** Purification of laccases from 21-d-old static *F. floccosa* LPSC 232 cultures by

542 chromatography on Superdex 200 (A) and Mono-Q (B) columns. Absorbance at 280 nm

543 (solid line), NaCl gradient (dashed line) and laccase activity (■) are indicated.

544

545 **Fig. 5.** Purification of laccases from 21-d-old static *F. floccosa* LPSC 232 cultures grown

546 in dual co-culture with *P. commune* GRAIE 86 inoculated after 6 d of *F. floccosa*

547 inoculation by chromatography on Superdex 200 (A) and Mono-Q (B) columns.

548   Absorbance at 280 nm (solid line), NaCl gradient (dashed line) and laccase activity (■)

549   are indicated.

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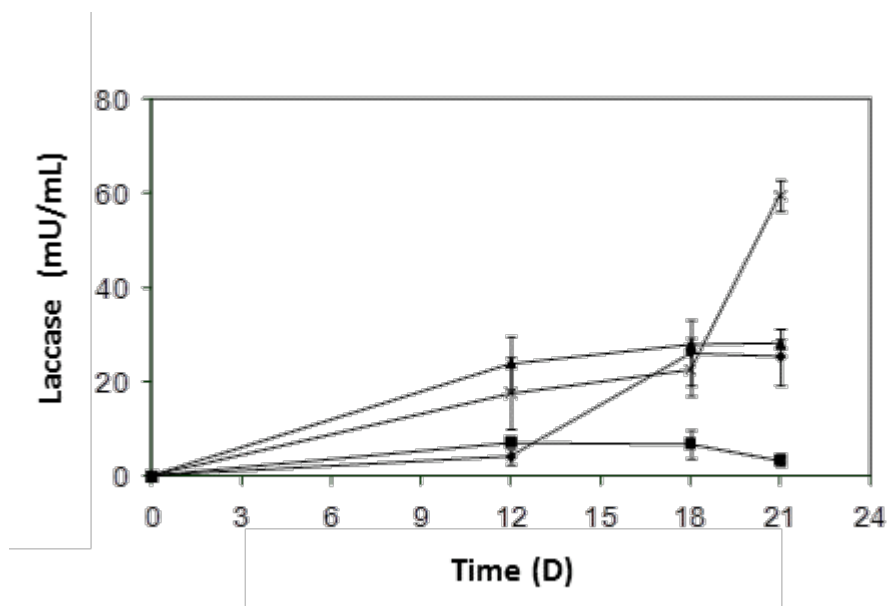551   Fig. 1

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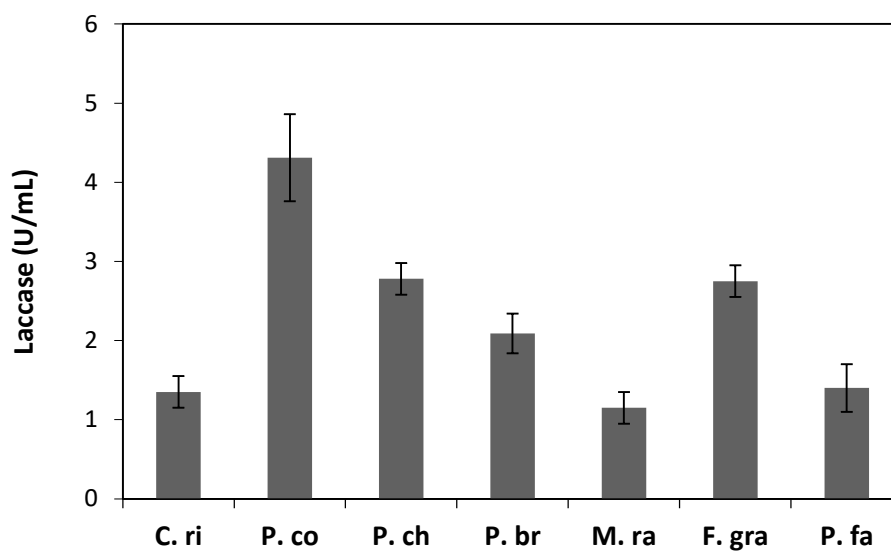
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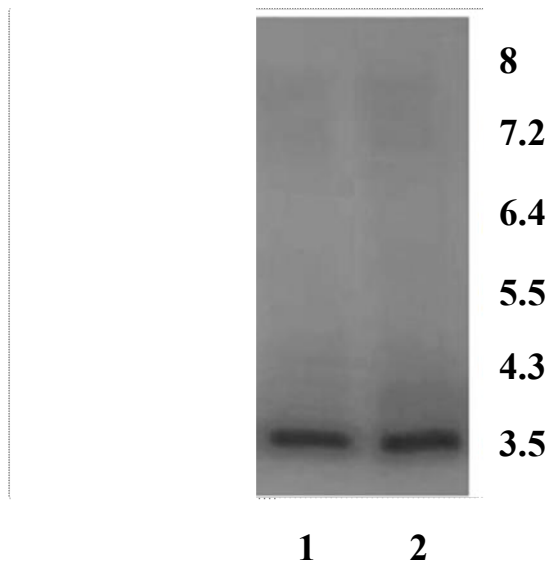
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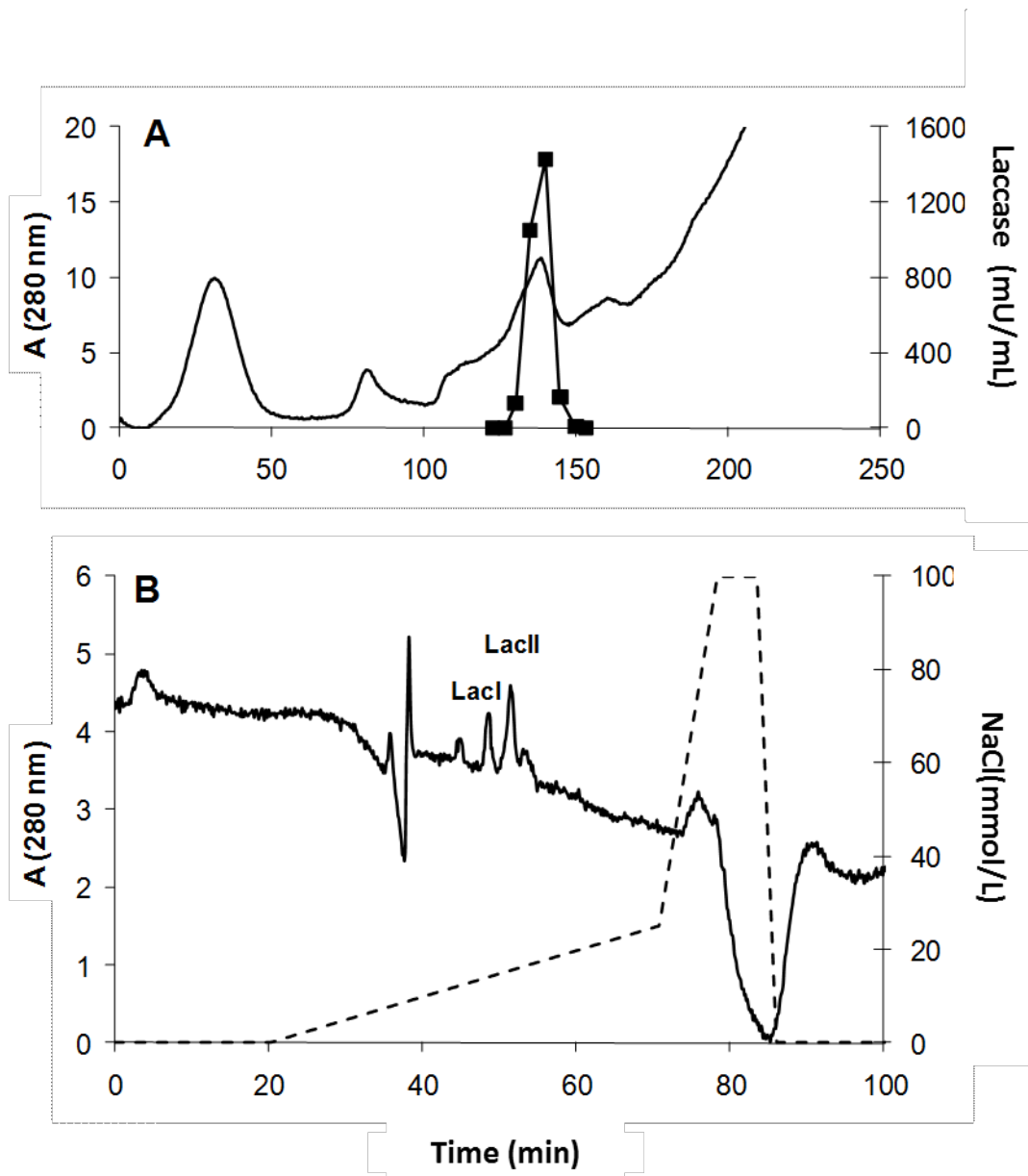566

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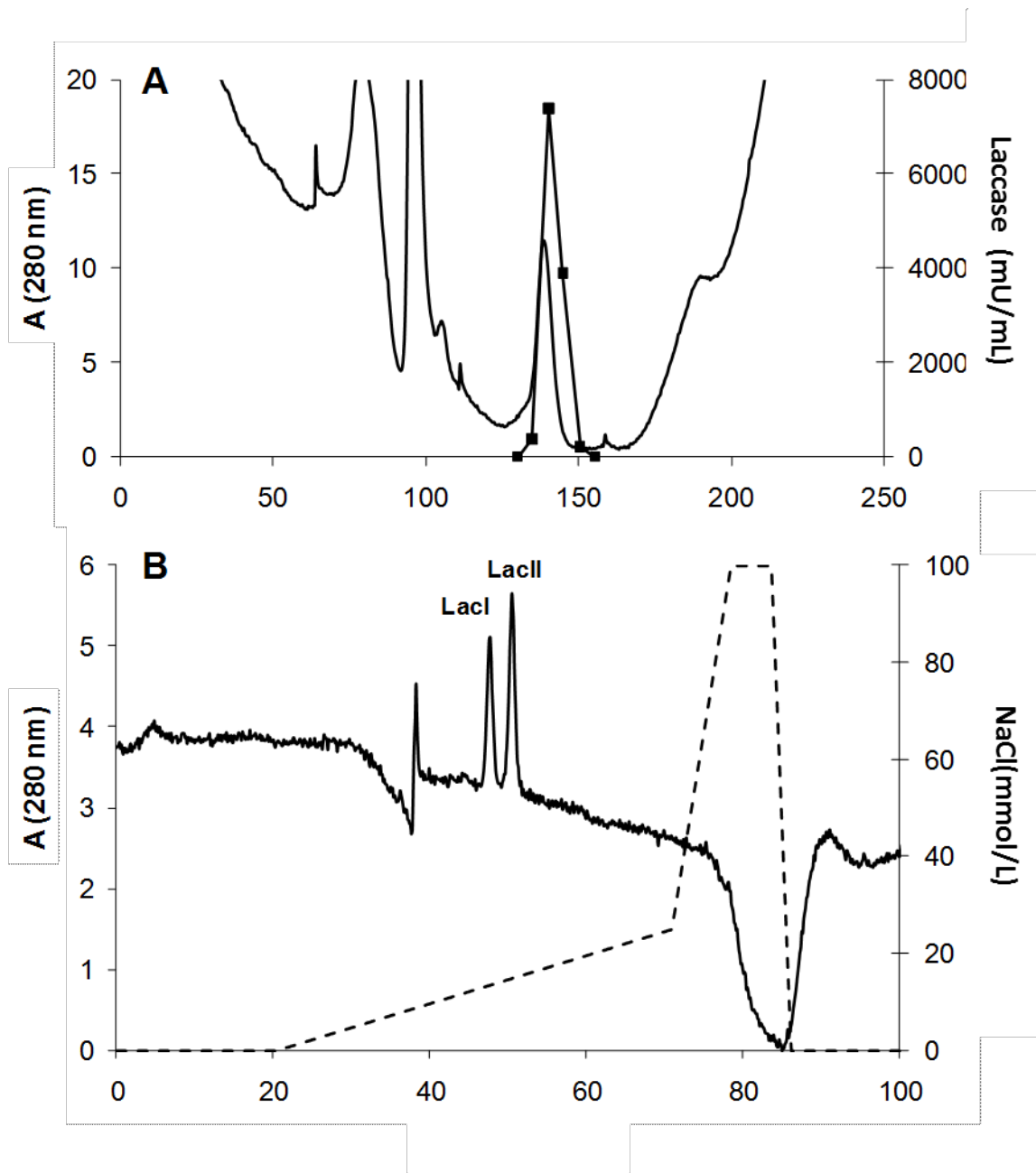570   Fig. 4

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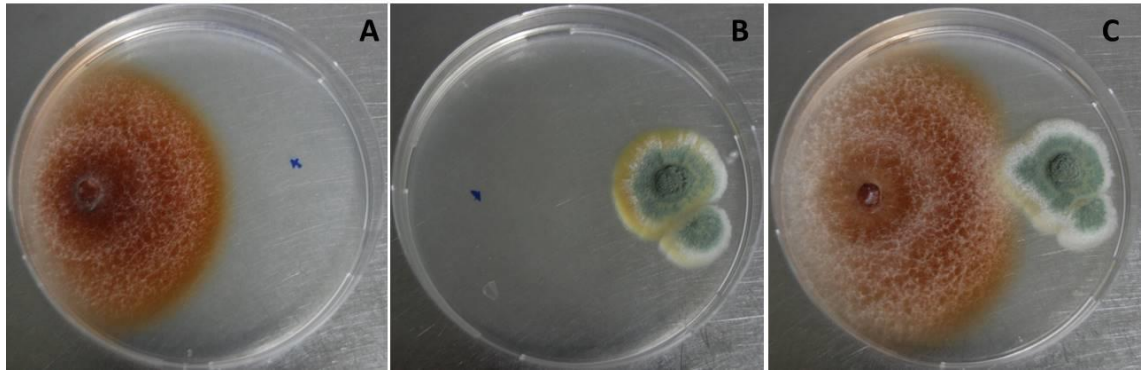577   Fig. 5

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583   **Table 1.** Purification of extracellular laccase isoenzymes from 21-d-old static *F. floccosa* LPSC 232 cultures grown in monoculture (F.flo) and in

584   dual co-culture with *P. commune* GRAIE86 (F.flo+P.co) added (supplemented) after 6 d of incubation. Data are the means of 3 different

585   preparations.

Purification step

	Protein (mg/l)		Activity (U/l)		Specific activity (U/mg)		Yield (%)		Purification factor (fold)	
	F.flo	F.flo+P.co	F.flo	F.flo+P.co	F.flo	F.flo+P.co	F.flo	F.flo+P.co	F.flo	F.flo+P.co
Culture liquid	114.2	100.8	13335.8	24434.1	116.7	242.5	100.0	100.0	1.0	1.0
Superdex200	9.5	9.5	1923.0	1922.9	202.4	202.4	14.4	7.9	1.7	0.8
Mono-Q (LacI)	2.4	2.1	802.0	1500.1	340.5	715.2	6.0	6.1	2.9	2.9
Mono-Q (LacII)	5.1	3.6	1009.0	1855.9	199.1	509.9	7.6	7.6	1.7	2.1
Mono-Q (LacI+LacII)	7.4	5.7	1811.0	3356.0	540.0	1225.1	14.0	13.7	5.0	5.0

586

587   **Supplementary Fig. S1.** *F. floccosa* LPSC 232 and *P. commune* GHAIE 86 growing in

588   monoculture on solid culture medium (A and B, respectively) and co-culture of *F.*

589   *floccosa* LPSC 232 with *P. commune* GHAIE 86 (C).

590

591

592