Effect of olive mill waste (OMW) supplementation to Oyster mushrooms substrates on the cultivation parameters and fruiting bodies quality

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Abbreviations: DPPH: 2,2-diphenyl-1-picrylhydrazyl, MnP: manganese-dependent peroxidase, MiP: manganese-independent peroxidise, OMW: olive mill waste, WS: wheat straw

ABSTRACT

Seven Oyster mushroom strains were cultivated in wheat straw (WS) bags supplemented with 0 up to 90% olive mill waste (OMW), a solid residue obtained from a two-phases olive oil production system. All mushroom strains could grow but high OMW concentrations resulted in a significant yield, biological efficiency and productivity decrease, retarding of pinning and flushing and loss of fruiting bodies quality. However, most of the mushroom strains showed no significant differences on cultivation parameters and fruiting bodies quality (except for colour) between control (WS) substrates or substrates supplemented up to 50% OMW (w/w). Addition of 50% OMW to WS substrates turned mushrooms lighter and less reddish. This colour shift was not due to enzymatic oxidations or inhibition since laccase and peroxidase activities were not differing from control. Total phenolic content and antioxidant activity were also similar and no phenolic compounds from OMW were detected in the fruiting bodies. Some of the analysed *Pleurotus* strains were better suited to grow on OMW supplemented substrates than others.

43

44 ## 1. Introduction

45 The traditional olive oil production process (a three-phase continuous centrifugation
46 system) generates two types of wastes, an olive press-cake (OPC) and a dark liquid
47 effluent called olive oil wastewater or vegetation water (OMWW). Environmental
48 regulations encouraged producers to change their production systems because of the
49 large amount of highly polluting OMWW generated. These residues were directly
50 discharged in soils and receiving waters producing a strong negative impact on the
51 environment. Nowadays, modern olive oil factories (using a two-phase olive oil
52 production) discard a fibrous lignocellulosic paste with high moisture content called
53 olive mill waste (OMW), which includes a very high content of organic load and
54 harmful phenolic compounds too but in a lower volume than the traditional wastes.

55 To preserve the ecology of olive oil producers countries, degradation of these wastes,
56 particularly OMWW, have been intensively studied using thermal (combustion and
57 pyrolysis) and physico-chemical (e.g. precipitation/flocculation, ultrafiltration and
58 reverse osmosis, adsorption, chemical oxidation processes and ion exchange) processes,
59 agronomic applications (e.g. land spreading), animal-breeding methods (e.g. direct
60 utilisation as animal feed or following protein enrichment) and biodegradations in
61 bioreactors (by solid state fermentation using aerobic or anaerobic microorganisms)
62 (McNamara et al., 2008; Morillo et al., 2009). One of the most promising processes was
63 to decontaminate the OMWW using edible fungi or fungal enzymes. White-rot fungi
64 were able to secrete specific ligninolytic enzymes (polyphenol oxidases, peroxidases
65 etc.) causing significant phenolic removal (Fountoulakis et al., 2002; Aggelis et al.,
66 2003; Olivieri et al., 2006). Moreover, if edible mushrooms were utilized, a double
67 target could be achieved, on the one hand their enzymes were able to degrade OMWW

68 toxicity and on the other hand, they were able to produce sensory appreciated fruiting
69 bodies (Zervakis et al., 1996). Apparently, mushroom species such as *Pleurotus* strains
70 (Oyster mushrooms), *Agaricus bisporus* (common Buttom mushroom) or *Lentinula*
71 *edodes* (Shiitake mushrooms) were able to colonize commonly used solid substrates
72 supplemented with OMWW (up to certain concentration) and produce fruiting bodies
73 with commercially acceptable yields (Kalmis and Sargin 2004; Kalmis et al., 2008;
74 Altieri et al. 2009; Lakhtar et al., 2010). Despite the large number of studies describing
75 the capacity of the *Pleurotus* varieties (*P. sajor-caju, P. cornucopiae, P. ostreatus, P.*
76 *pulmonarius, P. eryngii* etc.) to grow and detoxify OMWW only a few studies describe
77 the possibility of cultivating *Pleurotus* on the waste obtained from the modern olive oil
78 production process (OMW) although at the present, the major olive oil producing
79 companies from the Mediterranean countries are shifting their systems from the three-
80 to the two-phases process. Most of the latter studies were focused on the
81 decontamination / degradation of the phytotoxic phenolic compounds by the mushroom
82 enzymes (Saavedra et al., 2006; Sampedro et al., 2007) or on the effect of these
83 compounds on the bacterial flora endemic from the *Pleurotus* cultivation substrate
84 (Soler-Rivas et al., 2006). However, the effect of the OMW supplementation on the
85 *Pleurotus* growth and quality of produced fruiting bodies was not evaluated. On the
86 other hand, the previous studies describing the OMW effect used only one or two
87 *Pleurotus* species and results might largely differ depending on the selected strain since
88 each individual variety has its own specific substrate requirements and capabilities.

89 If edible mushrooms can be cultivated on olive mill wastes, it is wise to take into
90 consideration the fact that OMWs have toxic compounds harmful for human
91 consumption (Aggelis et al., 2003; McNamara et al., 2008) and mushrooms grown on
92 these substrates might absorb them and become toxic too. On the other hand, some of

the phenolic compounds present in the OMWs have been described as beneficial for human health (such as phenols with antioxidant activity) (Visioli et al., 1999; Mulinacci et al., 2001). Thus, absoption of these other compounds might have interesting influence on the fruiting bodies quality and nutritional value.

The most important quality parameters defined by the consumer in mushrooms are colour, texture and odour (Diamantopoulou and Philippoussis 2001; Caglarirmak, 2007). Good quality *Pleurotus* fruit bodies have to be off-white to brown, with tender but not soft texture, and have to posses the characteristic ´*Pleurotus* odour´ due to the presence of particular volatile compounds (Caglarirmak, 2007). Mushroom cap colour quality is also connected to the presence of oxidative enzymes. During mushrooms development, oxidative enzymes and its substrates are separated by membrane boundary layers within the cells. As a result of handling, natural ageing or infections, enzymes and substrates might intermix and enzymes become activated. Thus, enzymes such as peroxidases and laccases catalyze oxidative reactions yielding brown melanins and similar complex pigments contributing to quality loss.

The aim of the present work was to study the cultivation parameters and fruiting bodies quality of five *P. ostreatus* and two *P. pulmonarius* strains cultivated on wheat straw supplemented with several OMW concentrations (the waste obtained from two-phase olive oil production system).

2. Material and methods

2.1 Biological material

Mushroom strains used in this study were the commercial strains 2191, 2204, 2171 of *Pleurotus ostreatus* and *Pleurotus pulmonarius* 2204, obtained from the mother culture

117 collection of Mycelia co. (Gent, Belgium). *Pleurotus ostreatus,* strains K15 and
118 *Pleurotus pulmonarius* P17, were supplied by the spawn company Fungisem S.A.
119 (Autol, Spain). *Pleurotus ostreatus,* strain 1111 was kindly donated by INETI (Intituto
120 Nacional de Engenharia Tecnologia e Inovaçao) (Lisboa, Portugal). Ordinary
121 commercial Oyster mushrooms fruiting bodies were purchased at the local supermarket
122 as standard mushrooms to compare the quality parameters evaluated. Olive mill waste
123 (OMW) was obtained from a two-phase continuous olive oil mill plant extraction
124 (Cooperativa de Olivicultores de Fatima, Fatima, Portugal) and stored at –25 ^{o}C as it
125 was acquired. Wheat straw used for mushroom cultivation was purchased at Hoge Born
126 (Wageningen, The Netherlands). Wheat grains used to prepare spawn were supplied by
127 Koopmans Meel BV (Leeuwarden, The Netherlands).

128

129 *2.2 Culture media and spawn preparation*

130 Subcultures of the mother mycelia from the commercial strains were grown on Petri
131 plates containing 10 g L^{-1} of malt extract (Difco), 5 g L^{-1} of mycological peptone
132 (Difco) and 15 g L^{-1} of agar (nº 3 Oxoid) (MMP medium). Mycelia incubation took
133 place at 25 ^{o}C. Spawn substrate was prepared by mixing rye grains with tap water in a
134 ratio (2 : 1) (w/v) and sterilized in an autoclave 30 min. Sterilized spawn (20 g) was
135 added to the top of colonised Petri plates (after 7 incubation days) to allow further
136 mycelial colonisation of the grains. Plates were afterwards incubated at 25 ^{o}C during 7
137 days. Afterwards, colonized grains were extracted from the plate and used to inoculate
138 substrate bags.

139

140 *2.3. Lab scale production of fruiting bodies*

141 Specific substrate mixtures were prepared as follows: wheat straw was chopped (2 – 5
142 cm) and left overnight soaking up tap hot water. The excess of water was drained on a
143 sieve during 20 min. Afterwards, the soaked straw was mixed and homogenized with
144 0% (control), 25%, 50%, 60%, 70%, 80% and 90% of OMW (expressed in dry weights
145 of both straw and OMW). 100% OMW was also tested as substrate. Homogenized
146 substrates (300 g) were placed in plastic bags (15 x 25 x 3 cm) and sterilized in
147 autoclave during 30 minutes.

148 The sterilized substrates were inoculated with 10% (w/w) of fully colonized spawn
149 from the selected strains and left incubated in dark at 25°C during 15 days. When
150 substrates were fully colonized temperature was changed to 16 °C and 85% r.h., in the
151 presence of light, in a day/night cycle and approx. 550 ppm CO_2 concentration to induce
152 fructification. Two holes (approx. 2 cm diam.) were made in one side of each bag.
153 After few days (depending on substrate and strain) pinheads were eye visible.
154 Mushrooms obtained from the first and second flush were harvested and used to
155 perform the below described experiments. Substrate bags were prepared in triplicate for
156 each *Pleurotus* strain and for each type of substrate.

157

158 *2.4. Evaluation of the cultivation parameters*

159 Several cultivation parameters were evaluated during *Pleurotus* strains cultivation on
160 control substrates (containing only wheat straw, WS) and on substrates including OMW
161 supplementation. Moisture content and pH of the substrates were measured using
162 standard methods (pH meter, drying procedure). The incubation time required before
163 pinning (pinhead appearance) and for harvesting of the first and second flushes was
164 recorded. Yield (expressed as grams of fresh mushrooms harvested at maturity per gram

165 of substrate, w/w), biological efficiency (BE%, defined as the percentage ratio of the
166 fresh weight of harvested mushroom per gram of substrate (dw)) and productivity (BE%
167 per day of mushroom production) were monitored and defined as described by Zervakis
168 et al. (1996) and Curvetto et al. (2002). Other parameters taken into consideration were:
169 average weight of individual mushroom strain (determined as the quotient between the
170 total weight of individual strain harvested by their total number), and average
171 dimensions (defined as the quotient between the fresh mushroom area of individual
172 strain harvested by their total number) were evaluated according to Philippoussis et al.
173 (2001) immediately after harvesting. Results were obtained from three replicates for
174 each *Pleurotus* strain and for each type of tested substrate.

175

176 *2.5. Fruiting bodies texture*

177 Cap texture was monitored with a Texture Analyser (Stable Micro Systems,
178 Godalming, UK). Measurements were performed immediately after harvesting and
179 removing the stem. The cap was compressed in a distance of 1.5 cm from the edge, with
180 a probe SMS P/0.25S at 1 mm s^{-1} and 0.98 N force load into the fresh mushroom,
181 according to the method of Diamantopoulou et al. (2001). Results were expressed as the
182 force (N) needed to compress the cap and produce the first fracture in the mushrooms.
183 Results were the average of ten measurements for each mushroom strain and each
184 substrate.

185

186 *2.6. Fruiting bodies cap colour*

187 Cap colour was measured with a Minolta Chromometer CR-10 following the method
188 of Soler-Rivas et al. (1997). Measurements were the average of six mushrooms per

189 strain and per type of substrate. The cap colour was evaluated immediately after
190 harvested. The standardized $L^*a^*b^*$ scale system was used to define the colour.

191

192 *2.7. Oxidative enzyme activities*

193 Mushroom powders (166.7 mg mL^{-1}) were mixed with citric-phosphate buffer (0.1 M,
194 pH 5) for laccase activity or succinic-lactic acid buffer (0.1 M, pH 4.5) for peroxidase
195 activity, shaken on a vortex for 2 min, centrifuged (5 min, 14 000 rpm) and maintained
196 on ice. Supernatants were used as enzyme source.

197 Laccase (Lac) activity was measured using ABTS (2,2'-azino-bis(3-ethylbenz-
198 thiazoline-6-sulfonic acid) as substrate and following the method of Gutierrez et al.
199 (1994). Peroxidases activities (total peroxidases, manganese-dependent peroxidase and
200 Mn-independent peroxidases) were measured following the method of Mata and Savoie
201 (1998) using as substrate 3-Methyl-2-benzothiazolinone hydrazone hydrochloride
202 (MBTH, Fluka) which interact with 3-dimethylaminobenzoic acid (DMAB, Aldricht) in
203 the presence of the enzyme, and with/without H_2O_2 , manganese (Mn) or EDTA.

204 All enzymatic activities were monitored using a Perkin Elmer UV/vis Spectrometer
205 Lambda 2S, by measuring absorbance of the oxidation products. The molar extinction
206 coefficients of the oxidation products from ABTS and from MBTH/DMAB were
207 respectively ε_{436} = 29 300 and ε_{590} = 32 900 M^{-1} cm^{-1}.

208 Enzymatic activities were defined as the amount of enzyme that transforms 1 mol of
209 substrate $second^{-1}$ (katal) per gram of dry weight of mushroom.

210

211 *2.8. Phenolic compounds from mushrooms and OMW*

212 Mushroom fruiting bodies cultivated and harvested as previously described where
213 frozen and lyophilized (Soler-Rivas et al., 2006). Dried samples were ground on a
214 mortar with liquid nitrogen and stored at –25 ^{o}C for further use.

215 The total phenol concentration of mushroom powders (20 mg) was determined by the
216 Folin–Ciocalteu method according to the procedure of Mau et al. (2002). Absorbance at
217 750 nm was measured in a Perkin-Elkmer UV/Vis Spectrometer Lambda 2S. Gallic acid
218 was used as standard for quantification.

219 The phenolic compounds characteristic of olive mill waste were evaluated on the
220 prepared mushroom powders using the HPLC equipment (Waters) and methods
221 developed and described in Soler-Rivas et al. (2006). Briefly, samples were injected
222 onto a reversed phase HPLC column (Novapack C18 150 x 3.9 mm, Waters) and eluted
223 with a flow rate of 1 ml min^{-1} and a mobile phase containing: (A) acetic acid/water
224 (2.5%) and (B) acetonitrile following a gradient: from 0 to 10 min, 0 % B, from 10 to
225 40, 10% B, from 40 to 70, 40 % B, up to 72, 100 % B. Peaks were monitorized using a
226 diode array detector (Waters), identified on the basis of their retention times and spectra
227 compared to standards and quantified at 280 nm.

228

229 *2.9. DPPH scavenging capacity*

230 Dried mushroom powders (25 mg), obtained as above described, were mixed with 1
231 mL methanol, shaked during 5 min in a Vortex and centrifuged for 5 min at 14 000 rpm.
232 The antioxidant activity of the obtained supernatants (100µl) was determined using a
233 1mM $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) as radical solution according to Mau et al.
234 (2002). Absorbance at 515 nm was measured after 30 minutes incubation.

235

236 # 3. Results

237 *3.1. Effect of OMW addition on cultivation parameters*

238 Control substrates containing only wheat straw showed a moisture content of 84.7%
239 and a pH of 7.35 while fresh OMW showed 65.7% moisture and 4.9 pH. Thus, addition
240 of OMW to the substrate induced a slight reduction of the water content (79.8, 72.9, 72,
241 72.4, 71.6 and 65% with respectively 25, 50, 60, 70, 80 and 90% supplementation) and
242 a pH decrease (5.63, 5.34, 5.15, 5.20, 5.23 and 5.05 for these respective
243 supplementation levels).

244 Several Oyster mushroom strains were cultivated on wheat straw mixed with several
245 OMW concentrations. On average, addition of this residue up to 50 % (w/w) did not
246 influence the incubation time required for pinheads appearance compared to control
247 mushrooms cultivated on standardized substrate (Table 1). Pinheads from all mushroom
248 strains were visible 17 – 21 days after spawn inoculation except for *Pleurotus ostreatus*
249 1111 which needed, on average, 30 days. Higher OMW supplementations (60 – 80%)
250 provoked a generalized delay on pinning of approx. 4 - 7 days except for *P.*
251 *pulmonarius* 2204 that was able to initiate fructification on 60% OMW at similar
252 incubation time than control. None of the investigated strains was able to grow on 100%
253 OMW and on 90% on average 30.6 days were required for primordia initiation.

254 On average, OMW additions up to 70% did not provoke a delay on the harvesting time
255 for the first flush. All the fruiting bodies were harvested after approx. 6-7 days after
256 pinheads appearance. The first flush of mushroom strains cultivated on substrates
257 including OMW concentrations higher than 50% was harvested later, because they
258 needed a longer interval to initiate fructification. Only OMW additions of 80 and 90%

OMW induced a flushing delay. The second flush was harvested earlier from those substrates containing higher OMW supplementation.

The mushroom yield obtained from the different substrates was also evaluated. Results showed similar profile than when their biological efficiency and their productivity were plotted. Mushroom yields on substrates including up to 50% OMW showed 0.13 g mushroom/g substrate meaning an 85% BE. Higher addition resulted in lower yields ranging from 0.09 up to 0.04 g/g (64 – 28% BE) with 60 up to 90% OMW supplementation. If the cultivation days were taken into consideration, the productivity of the cultivation process was, on average, slightly higher on substrates including 50% OMW than control substrates (Fig. 1). However, when individual mushroom strains were considered, productivity differences between 0, 25 and 50% OMW supplementation were not significant in four of the strains and in two of them the OMW addition was even detrimental (*P. ostreatus* 1111 and *P. pulmonarius* P17). Only for *P. ostreatus* K15 addition of 25 or 50% OMW provoked a remarkable increase on productivity. OMW additions at concentrations higher than 50% reduced the productivity of all the analyzed strains.

3.2. Effect of OMW addition on physical quality parameters

Under the described lab-scale cultivation conditions, *Pleurotus* mycelia grown on wheat straw were able to produce, on average, 20 fruiting bodies (in the two obtained flushes). Similar amount of mushrooms were harvested from substrate bags containing up to 50% OMW. Higher OMW supplementation resulted in lower amount of fruiting bodies (10 – 4) but of higher size. Mushrooms grown on 90% OMW showed a cap average size of 6.5 cm^2 while those grown on substrates with OMW concentrations

283 lower than 60% reached 3 cm^2. Thus, the total weight of harvested mushroom remained
284 almost constant (6 – 8 g) since the amount of fruiting bodies was compensated by
285 higher size. Only *P. ostreatus* K15 showed 1.3 and 3 folds higher fruiting bodies weight
286 when it was grown respectively on 25 and 50% OMW addition compared to control
287 substrate.

288 Fruiting bodies texture was also negatively influenced by OMW addition to the
289 substrate provoking a softening of the mushrooms (Fig. 2). The firmness values
290 recorded for all the analyzed *Pleurotus* strains were decreasing with increasing of OMW
291 concentration in the substrate. However, for a few strains texture was not significantly
292 affected by addition of 25 or 50% OMW.

293 The cap colour of the produced fruiting bodies (measured by the CIE *Lab* system)
294 changed significantly and correlated to the OMW concentration added to the substrate
295 (Fig. 3). This colour shifting was observed in all analyzed *Pleurotus* strains. The
296 lightness (*L* parameter) changed, on average, from 60 up to 85 indicating that
297 mushroom strains were getting lighter with increasing OMW concentrations in the
298 substrate (Fig. 3a). The *a* parameter was also highly influenced since all mushroom
299 strains shifted from red to green concomitantly with the increase of OMW addition (Fig.
300 3b). Changes on the *b* parameter with increasing OMW concentrations were strain
301 dependent. Some of them such as *P. ostreatus* K-15 and 1111 turned bluer whereas
302 other strains such as *P. pulmonarius* 2204 and *P. ostreatus* 2171 turned more yellow.
303 Changes were larger when OMW was supplemented at concentrations higher than 50 or
304 60%. The rest of strains showed only slight changes on *b* parameter towards yellow
305 (Figure 3c). Thus, when the total colour change was calculated as ΔE compared to
306 control mushrooms (Figure 3d) a linearly correlated colour shifting was observed with
307 the amount of OMW added to the substrate. Values were ranging from 5 to 20 (on

308 average) from 25 to 90% OMW. Total colour changes were particularly high in both *P.*
309 *pulmonarius* strains.

310

311 *3.3. Effect of OMW addition on chemical quality parameters*

312 *Pleurotus* fruiting bodies showed both laccase and peroxidase activities. Laccase
313 activity in all the studied strains ranged from approx. 1.5 to 3 nkat/g mushroom (dw)
314 independently of the evaluated strain and OMW concentration in the substrate. Only the
315 *P. pulmonarius* strains cultivated on substrates containing 80% OMW showed a
316 significantly higher laccase activity than the others (Fig. 4a). The seven mushroom
317 strains showed lower laccase than peroxidase activity being the latter influenced by the
318 OMW concentration added to the substrate (Fig. 4b). No significant differences were
319 observed between peroxidase activities in control mushrooms or mushrooms cultivated
320 on substrates including 50% OMW. However, higher OMW additions resulted in
321 higher peroxidase activities. Particularly, *P. ostreatus* strains showed an increase in
322 their Mn-dependent peroxidase levels of approx. 2 to 4 folds the control or 50% OMW
323 samples but no significant differences were found between *P. ostreatus* strains
324 cultivated on 60, 70 or 80%. However, the values seemed to increase with OMW
325 concentration in *P. pulmonarius* strains and in these strains, the increase was mainly due
326 to increases on Mn-independent peroxidases (Table 2).

327 The total phenolic content and antioxidant activity of the seven mushroom strains did
328 not significantly change with addition of 0, 50, 60, 70 or 80% OMW. Total phenol
329 values were very similar for all the studied strains ranging from 0.13 to 0.22 mg/ml
330 gallic acid equivalents. None of the phenolic compounds identified and detected in
331 OMW (Soler-Rivas et al., 2006) was found on the fruiting bodies. Methanol extracts

332 obtained from *Pleurotus* fruiting bodies (independently of the substrate mixture used for
333 cultivation) were able to scavenge 45 to 70% of the DPPH radical under the assay
334 conditions, only *P. pulmonarius* P17 showed a DPPH scavenging capacity of approx.
335 80%.

336

337 ## 4. Discussion

338 OMW addition to wheat straw substrates provoked a slight reduction of the water
339 content and a pH decrease. Moisture content was still in the optimal range for
340 cultivation (above 70%) common for this and other agro-residues based substrates
341 (Hernandez et al., 2003). The use of an alkaline medium was usually suggested to
342 reduce competing microorganisms and possible contaminating deuteromycetes (Stölzer
343 and Grabbe, 1991). However, no $CaCO_3$ or similar compounds were added during the
344 substrate preparation because few years later, Hernandez et al. (2003) demonstrated that
345 fungal or bacterial contaminants could be avoided by keeping the relative humidity of
346 the fruiting room not higher than 85%. Moreover, *Pleurotus* mushrooms were able to
347 colonize other substrates with similar pHs ranges (7.5 to 5.6) (Yildiz et al., 2002).

348 When other *Pleurotus* varieties were cultivated on mixtures containing OMWW
349 (Kalmis and Sargin, 2004) a significant delay on the cropping periods was observed
350 with increasing OMWW concentrations. Similarly, OMW supplementation in
351 concentrations higher than 50% is not encouraged since they induced retarding of
352 pinning and flushing and reduction on their yield, biological efficiency and productivity.
353 However, if the OMW was applied at 50% or lower concentration, their biological
354 efficiency was in the range of other agro-wastes tested as substrates to cultivate Oyster
355 mushrooms such as mixtures of switch grass or cottonseed hulls (Royse et al., 2004),

356 hazelnut, tilia, European aspen leaves, paper waste, sawdust etc. (Yildiz et al., 2002) or
357 viticulture wastes, paddy straw or sesame straw (Kurt & Buyukalaca, 2010). Therefore,
358 the *Pleurotus* strains selected in this study could use this lignocellulosic waste including
359 up to 50% OMW as substrate to grow and to produce fruiting bodies. One particular
360 strain, *Pleurotus ostreatus* K-15, seemed to grow better than in a standardized
361 commercial substrate such as wheat straw. Thus, the following point to study was the
362 quality of those fruiting bodies produced from OMW. Two particular characteristics
363 were considered as a ´quality mushrooms´: i) that they showed a physical appearance
364 similar to those commercially available and ii) that they did not absorb or modify their
365 level of phenolic compounds due to OMW addition.

366 According to the results, OMW additions up to 50% did not significantly influence the
367 fruiting bodies number, size or texture if compared with control samples cultivated on
368 wheat straw substrates including no OMW or commercial strains purchased at a local
369 supermarket; only cap colour was affected. Oyster mushrooms cultivated on substrates
370 including 50% OMW turned less red and greener and they became lighter. This
371 discoloration might not be considered as quality depreciation since the *Pleurotus* strains
372 commercially available at the supermarkets showed a wide range of colours from white
373 to dark brown and they are all easily accepted by consumers. However, the colour
374 change indicates that the chemical composition of these fruiting bodies has changed.
375 Melanins and melanin-complexes are the responsible compounds for the mushroom cap
376 colour. In *Pleurotus* mushrooms, these brown pigments are generated by the oxidative
377 reactions catalyzed by enzymes such as peroxidases and laccases on phenols or phenols-
378 like compounds. Thus, in this case, the colour shift might be due to certain compounds
379 absorbed directly from the OMW able to inhibit the oxidative enzymes since turning
380 whiter and less red might indicate lower melanin production. However, no inhibitors of

381 oxidative enzymes have been previously described on olive oil or derivative products.
382 On the contrary, polyphenol oxidases from edible mushrooms were able to effectively
383 degrade olive-related phenolic compounds such as hydroxytyrosol, tyrosol,
384 methylcatechol, etc (Canfora et al. 2008; Espin et al. 2001). So if these were absorbed
385 by the fruiting bodies, a darkening of the caps should have been expected.

386 The total phenolic content, antioxidant activity and laccase and peroxidase activities
387 from mushrooms cultivated on substrates including 50% OMW were similar to those
388 with no OMW addition suggesting that neither oxidative enzyme inhibition nor
389 significant absorption of phenol-related-compounds occurred. Moreover, tyrosol,
390 hydroxytyrosol 4-methylcatechol, caffeic acid and other more complex molecules
391 described in OMW (Soler-Rivas et al., 2006) were not detected on any of the fruiting
392 bodies analyzed (including those cultivated on substrates with very high OMW
393 concentrations).

394 A discolouration of the cultivation substrate or media was also observed when
395 *Pleurotus* mycelia were grown on OMW and OMWW-containing substrates (Soler-
396 Rivas et al., 2006) or OMWW-containing liquid cultures (Kissi et al. 2001;
397 Fountoulakis et al. 2002). The colour reduction was suggested to be due to the
398 degradation of lignocellulosic fibres containing aromatic phenolic compounds by the
399 mushroom oxidative enzymes secreted to the cultivation media. In those cases, colour
400 change correlated with total phenol degradation and with the increase on oxidative
401 enzymes activities, therefore the discolouration mechanism occurring inside the
402 mushroom fruiting bodies should be different. Moreover, mushroom fruiting bodies do
403 not have the metabolic pathways necessary to absorb such complex phenolic
404 compounds or lignin-like molecules. They can only secrete the oxidative enzymes and
405 degrade these extracellularly.

406 Thus, it could be concluded that Oyster mushrooms can be cultivated on wheat straw
407 substrates supplemented up to 50% OMW with no significant changes in their
408 cultivation parameters or quality of the produced fruiting bodies, as only a colour
409 change was detected. The reason for this colour change remains unclear and needs
410 further investigation although it can be suggested that apparently it is not a direct effect
411 of the phenolic compounds from the OMW. Some specific *Pleurotus* strains such as i.e.
412 *Pleurotus ostreatus* K-15 are better suited to grow on this substrate than others.

413

414 ## Acknoledgements

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523 **Table 1:** Cultivation time (days) required for pinhead formation (days after inoculation
524 until appearance of visible primordia) and for the harvesting of first and second flushes
525 of seven *Pleurotus* strains cultivated in several substrates including mixture with
526 different OMW supplementations.

		OMW (%)						
	Strain	**0**	**25**	**50**	**60**	**70**	**80**	**90**
Pinheads	**Average**	**21**	**21.0**	**21.1**	**25.6**	**27.3**	**27.6**	**30.6**
	PO 2191	21	21	21	29	35	38	40
	PO 2204	17.5	17.5	17.5	25	22.5	24	25.7
	PO -K15	21	21	21	29	29	36.5	41
	PO 1111	31.5	30.7	29	29	29	35	39
	PO 2171	21	21	21	25	27	34	36
	PP 2204	17.5	17.5	17.5	17.5	22	25.5	32.5
	PP -P17	17.5	18.5	21	25	26.5	0	0
1st Flush	**Average**	**28.2**	**27.4**	**27.2**	**32.2**	**32.6**	**38.3**	**43.1**
	PO 2191	28.5	26.5	25.8	36	35	39.8	45
	PO 2204	24.5	24.5	24.5	30	28.5	36.3	43.5
	PO -K15	27.5	27.5	28	34.5	34.5	48.5	50
	PO 1111	40	36.5	33.8	33.8	37.5	37	42.8
	PO 2171	26.5	26.5	26.5	33	34.2	36.8	41.5
	PP 2204	24.5	24.5	25.5	26.7	26.7	31.5	36
	PP -P17	26	26	26	31.5	32	0	0
2nd Flush	**Average**	**47.4**	**46.7**	**44.5**	**48.5**	**48.4**	**51.2**	**53.8**
	PO 2191	50	45	40	51	52.5	0	0
	PO 2204	43	45	40	43.5	42.5	47	0
	PO -K15	50	47	43.5	47.5	50	57.5	0
	PO 1111	54	55	57.5	53.5	54	57.5	57.5
	PO 2171	45	45	43.5	50	50	51	0
	PP 2204	43	42.5	40	42.5	40	43	50
	PP -P17	47	47.5	47	51.5	50	0	0
Pin to 1st Fl.	**Average**	**7.2**	**6.4**	**6.0**	**6.6**	**5.3**	**10.7**	**12.5**
1st F to 2nd F	**Average**	**19.2**	**19.3**	**17.3**	**16.3**	**15.8**	**12.9**	**10.6**

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528

529 **Table 2:** Mn-dependent (MnP) and Mn-independent (MiP) peroxidase activities (nkat g^{-1}) of seven *Pleurotus* strains cultivated in several substrates including mixture with different OMW supplementations.

532

	PO 2191		PO 2204		PO -K15		PO 1111		PO 2171		PP 2204		PP -P17	
OMW	**MnP**	**MiP**	**MnP**	**MiP**	**MnP**	**MiP**	**MnP**	**MiP**	**MnP**	**MiP**	**MnP**	**MiP**	**MnP**	**MiP**
0%	1.63	0.87	0.57	3.28	0.63	3.07	0.15	3.35	1.12	1.18	1.04	0.86	1.17	1.83
50%	1.59	0.86	0.69	2.31	0.46	1.29	0.10	3.10	1.10	1.00	1.32	2.28	1.22	2.58
60%	4.39	0.61	2.74	3.36	4.24	2.36	2.15	2.95	4.02	3.48	1.01	3.19	1.56	3.34
70%	4.79	0.71	1.16	3.14	3.76	1.54	3.02	3.58	4.22	3.18	1.46	4.24	1.64	6.71
80%	3.81	0.59	2.12	3.78	2.88	3.62	2.73	3.57	2.82	2.88	1.02	4.78	1.37	7.93

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534

535 **Fig. 1:** Productivity (biological efficiency per cultivation days) of seven *Pleurotus*
536 strains cultivated on wheat straw supplemented with different OMW concentrations.

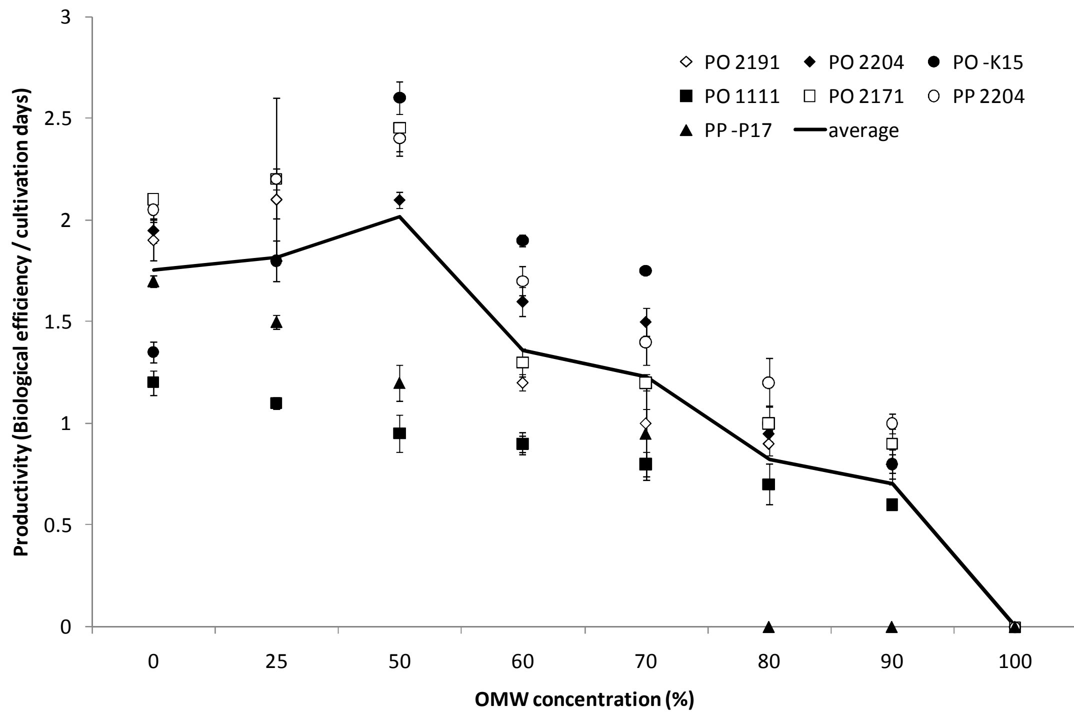
537 **Fig. 2:** Firmness values (N) measured in the seven *Pleurotus* strains grown on
538 substrates with increasing OMW concentrations and their average compared with the
539 average of commercial fruiting bodies.

540 **Fig. 3:** Cap colour measured in the seven *Pleurotus* strains grown on substrates with
541 increasing OMW concentrations and their average compared with the colour average of
542 commercial fruiting bodies. Values obtained for the a) *L*, b) *a* and c) *b* parameters and
543 d) total colour change defined as ΔE.

544 **Fig. 4:** a) Laccase and b) total peroxidase activities in several *Pleurotus* strains
545 cultivated in substrates with different OMW concentrations.

546

547 Figure 1:



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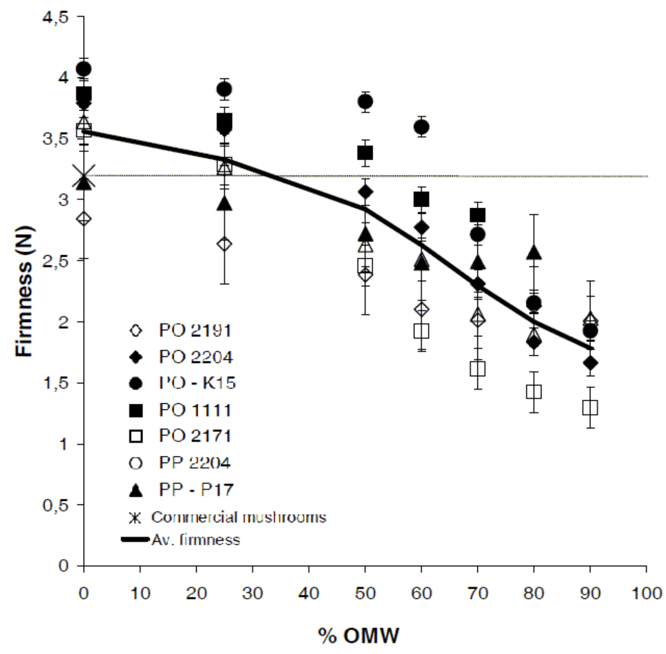
550 Figure 2:



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553 Figure 3:

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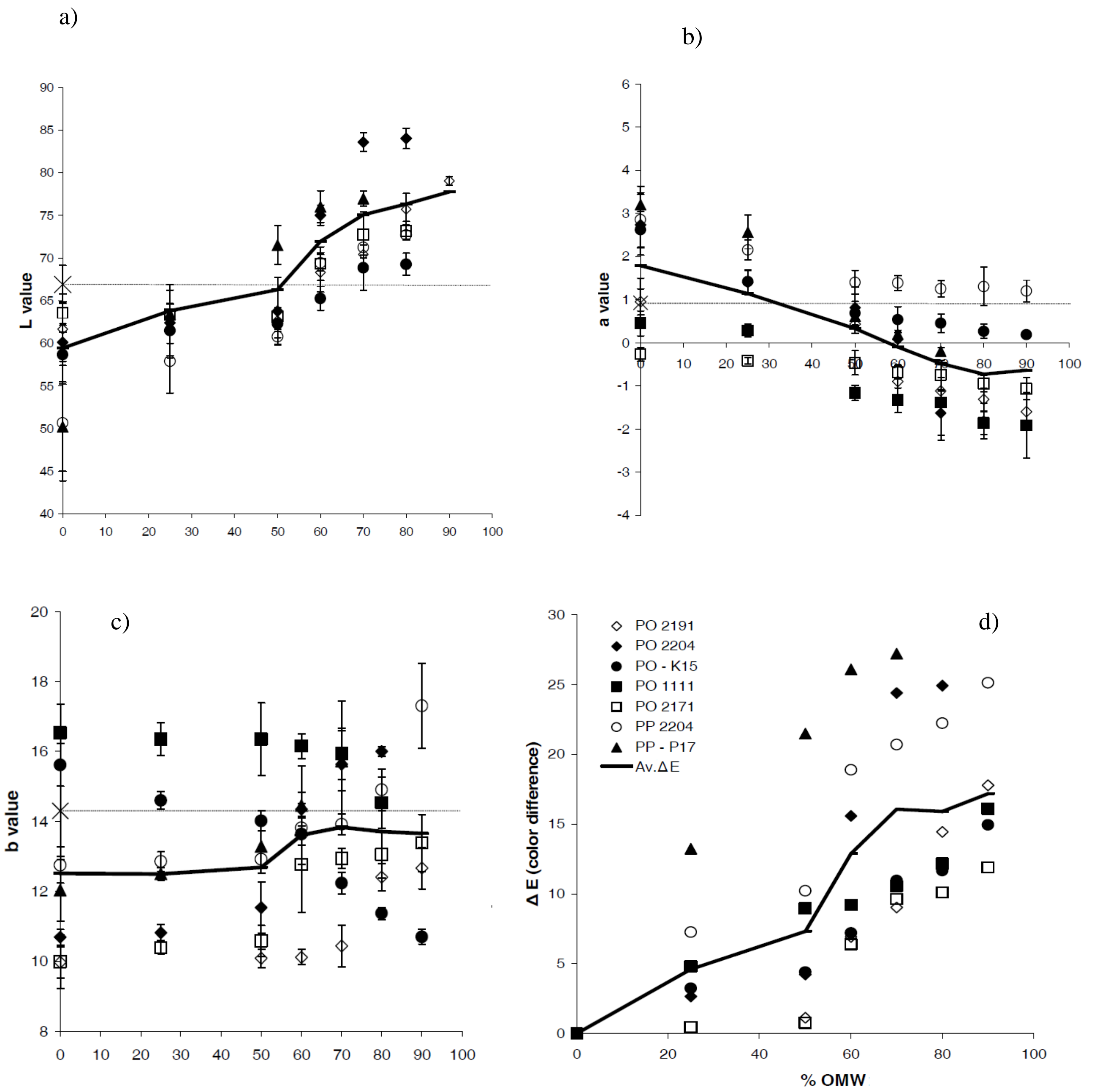

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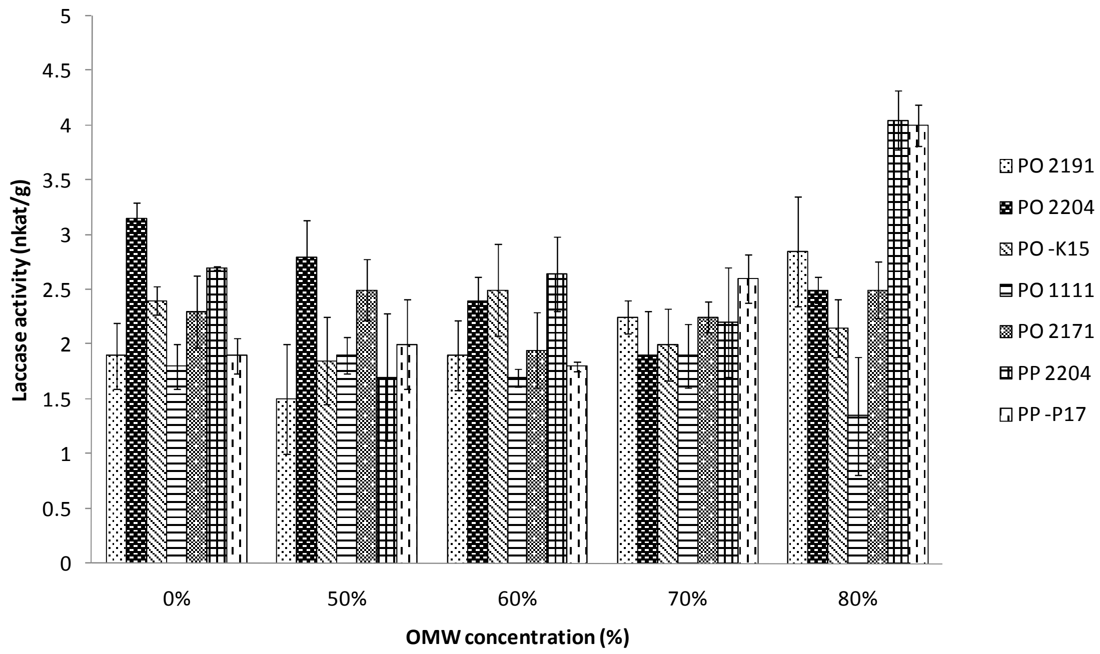
557 Figure 4:

558 a)



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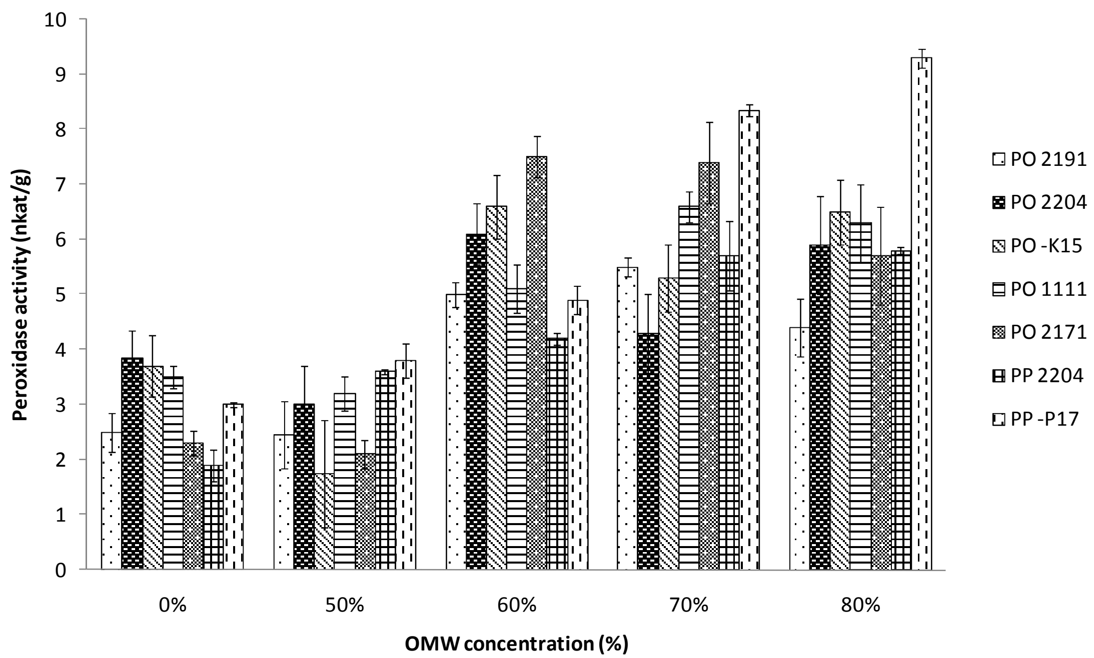
560 b)



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