Simultaneous valorization and detoxification of the hemicellulose rich liquor from the organosolv fractionation

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Abstract

Fractionation of lignocellulosic biomass with solvents (organosolv process) generates a hemicellulose-rich liquor with a high content of phenolics which is particularly toxic. This work addresses the utilization of this stream as a potential carbon source for the production of ligninolytic enzymes (LE). Among six basidiomycetes species, *Irpex lacteus* and *Ganoderma lucidum* presented the highest activities of manganese peroxidase (646±122 U L^{-1}) and laccase (1,497±161 U L^{-1}), respectively, growing on a medium composed mainly of cellulose fibers, lignin and hemicellulose. The influence of each lignocellulosic fraction on the LE production mechanisms was studied in more detail. The high concentration of phenolic compounds in the hemicellulose-rich stream acted as inducer of LE production, with levels even greater than those of xylose. Acute toxicity tests on *Vibrio fischeri* revealed a substantial reduction of the toxicity after the fungal treatment (by ca. 12–21 times). The proposed valorization and detoxification of this currently non-exploited and abundant by-product stream is a promising strategy to enhance the industrial feasibility of the organosolv fractionation process.

Keywords: manganese peroxidase, laccase, lignocellulose, white-rot fungi, organosolv fractionation

1. Introduction

White-rot fungi are the only group of microorganisms currently known that degrade all basic wood polymers, i.e., cellulose, hemicellulose and lignin into low-molecular weight compounds that can be assimilated for growth (Camarero et al., 2014). This is achieved by their ability to produce several hydrolytic enzymes (cellulases and hemicellulases) and their unique network of oxidative (ligninolytic) enzymes. The study of the extracellular ligninolytic enzyme (LE) system of *Phanerochaete chrysosporium* demonstrated that lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and laccase (EC 1.10.3.2) are the primary enzymes associated with the degradation of lignin (Kuwahara et al., 1984).

The LE system plays a fundamental role in the bioconversion of lignocellulose. Furthermore, LE can also be applied for other purposes, such as the production of second-generation biofuels, organic synthesis (antibiotics, polymers, building blocks), cosmetics (skin-lightening agents), nanobiotechnology (biofuel cells and biosensors for biomedical applications), bioremediation, biopulping and biobleaching in paper industry as well as the food and textile industry (Alcalde, 2015; Yadav and Yadav, 2015).

However, to meet market demands, the large-scale production of these enzymes at low cost is mandatory. Although significant efforts have been devoted to enhance LE production by heterologous protein expression (Alcalde, 2015), the levels of enzymatic production are still rather limited (Eibes et al., 2009) and still have to be obtained from wild strains (Elisashvili and Kachlishvili, 2009).

LE production highly depends on the fungal species, source of lignocellulosic substrate and cultivation method (Elisashvili et al., 2008). The presence of lignocellulose was found to be a prerequisite for LE production by white-rot fungi in submerged cultures (Kapich et al., 2004; Gassara et al., 2012) and the various lignocellulosic fractions have

been reported to influence LE production in a different manner. For example, *P. chrysosporium* was found to secrete laccase in the presence of cellulose, but not glucose (Srinivasan et al., 1995). *T. versicolor* produced higher laccase yields in the presence of natural lignocellulose-containing substrates such as wheat straw or wood, rather than with glucose (Schlosser et al., 1997). Toxic aromatic compounds and lignin also have a remarkable influence on LE production with *T. versicolor* or *Phlebia radiata* (Rogalski et al., 1991a; Rogalski et al. 1991b). Understanding the physiological mechanisms regulating enzyme synthesis by white rot fungi could therefore be useful for improving the efficient production of LE.

The organosolv process, i.e. pulping with ethanol-water, allows the fractionation of the lignocellulosic materials on its main constituents, i.e. cellulose fibers, lignin and hemicellulose fraction (Laure et al., 2014). However, only a few works have studied potential applications of the hemicellulose liquor after lignin recovery (Kautto et al., 2013; Hallberg et al., 2011). The presence of degradation products from the carbohydrates, soluble low molecular weight lignin and other possible inhibitors may limit its conversion to biofuel (Kautto et al., 2013). On the other hand, considering this fraction as waste stream, the large costs associated with wastewater treatment would limit the economic viability of the organosolv biorefinery (Laure et al., 2014).

Recently, the interest on the valorization of agro-industrial residues to produce LE at lower cost has increased (Palma et al., 2016). Several residual streams, such as apple pomace sludge (Gassara et al., 2012), fishery residue, brewery waste, pulp and paper industry sludge (Gassara et al., 2010), sugarcane residue (Maza et al., 2015), olive oil wastewater (Mann et al., 2015), oat husks, waste from paper process industry (Winquist et al., 2008) have been evaluated as carbon source. However, to our knowledge, the

application of the hemicelluloses fraction from organosolv process for ligninolytic enzyme production has not been reported yet.

This study evaluates the production of LEs in submerged fermentations by different fungal strains using the hemicellulose liquor of an organosolv process as carbon source. With the aim of better analyzing the specific requirements of the fungal strains for LE production, the individual or combined addition of the other fractionation products (cellulose fibers and lignin) was evaluated before using the hemicellulose liquor as the only carbon source. Moreover, the acute toxicity of the treated liquor was assessed with the bioluminescent photobacterium *Vibrio fischeri*.

2. Materials and methods

2.1. Lignocellulosic substrates

The lignocellulosic substrates used as carbon source in this study were cellulose fibers (C_6), organosolv lignin (L) and a concentrated C5-sugars liquid fraction (C_5), all provided by the Fraunhofer Center for Chemical-Biotechnological Processes (CBP, Leuna, Germany). The physico-chemical characterisation of these substrates is given in Table 1. The fractionation of beech wood in the organosolv process was performed at the Fraunhofer-CBP. Debarked beech wood chips were pulped with a 1:1 ethanol/water mixture at 170°C for 100 minutes using 0.5% of sulphuric acid (based on dry wood) at a liquor-to-wood ratio of 3.2:1 and the pressure was maintained at 20 bar using nitrogen gas (Laure et al., 2014). The solid fraction obtained after the pulping of the beech wood was washed; disintegrated and dewatered, obtaining the cellulose fibers (C_6). The liquid phase was composed mainly of lignin, C5-sugars from hemicellulose fraction and degradation products. Lignin (L) was precipitated from this stream by the addition of water and filtered for its separation (Schulze et al., 2016). By the recovery of the ethanol

117 from the filtrates of precipitated lignin, a raw hemicellulose fraction was obtained (C_5).
118 This raw hemicellulose stream was subsequently concentrated.

119 *2.2. Microorganisms and inocula preparation*

120 *Irpex lacteus* (Fr. 238 617/93), *Lentinus tigrinus* (PW94-2), *Stereum hirsutum*
121 (PW93-4) and *Phanerochaete chrysosporium* (BKM-F-1767) were obtained from the
122 culture collection of the Department of Chemical Engineering of the University of
123 Santiago de Compostela (Spain). *Bjerkandera* sp. R1 was isolated from a Chilean forest
124 in Temuco and identified as a new anamorph of *Bjerkandera* sp. (Taboada-Puig et al.,
125 2011) *Ganoderma lucidum* was isolated from mushroom spent substrate, kindly provided
126 by Hifas da Terra S.L. (Pontevedra, Spain). All fungal strains were maintained on MEA
127 agar at 4ºC.

128 Mycelia were produced in static cultures for fungal inocula preparation. Five plugs
129 of fungal mycelium from fresh agar plates were inoculated with 200 mL of glucose-
130 peptone medium (Kimura et al., 1990) in Fernsbach flasks and incubated at 30ºC. After
131 7 days, fungal cultures were homogenized in a sterilized blender for 30 s and used as
132 inoculum.

133 *2.3. Culture conditions*

134 Submerged fermentations were performed in 250 mL Erlenmeyer flasks containing 90
135 mL of culture medium and 10 mL of inoculum. The fungal cultures were incubated on a
136 rotary shaker at 150 rpm, initial pH 4.5 and 27ºC. All culture media were prepared with
137 distilled water and contained 1 g L^{-1} peptone (as nitrogen source), 0.5 mM $MnSO_4$ and
138 0.15 mM $CuSO_4$ (as inducers for enzyme production). Six different combinations of
139 cellulose fiber (C_6), organosolv lignin (L), concentrated hemicellulose fraction (C_5),
140 and/or xylose (X) were evaluated as carbon source. The concentration of these
141 components in each culture medium is shown in Table 2. Cellulose and lignin

concentration was calculated considering the humidity and concentration of each of the components in both streams (Table 1). Hemicellulose concentration was expressed in equivalents of TRS, considering that the concentrated hemicellulose fraction had a TRS concentration of 225 ± 5.6 g L^{-1}.

2.4. Analytical methods

An adapted protocol from NREL (National Renewable Energy Laboratory, Golden, USA) was used for the determination of the biomass composition in glucan, xylan and lignin (Sluiter et al., 2007). Acid hydrolysis allows lignin separation from the sugar fraction, and the amount of lignin recovered is determined by weight. The concentrations of xylan and glucan were calculated from the concentration of the corresponding monomeric sugars using a correction of 0.88 and 0.90 for C_5 and C_6 sugars, respectively. The Folin-Ciocalteu's method was used for the quantification of the total phenolic compounds concentration (Singleton and Rossi, 1965).

Total reducing sugars (TRS) were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Glucose, xylose, rhamnose and acetic acid concentrations in the C5-sugars fraction were determined by HPLC (Hewlett Packard chromatograph), equipped with an ION-300 column (López et al., 2011). The concentration of the xylose oligomers was determined by measuring the released xylose after post-hydrolysis catalyzed with sulphuric acid. The liquid sample from the C5-sugar fraction or the fungal culture supernatant was diluted and H_2SO_4 was added to reach a final concentration of 4% (v/v). The final pH of the sample was 0.7. It was autoclaved at 121ºC for 20 min in a sealed flask. Flasks were weighed before and after the thermal treatment to allow correction for any possible evaporation losses. Finally, samples were filtered through 0.45 µm cellulose acetate membranes and analysed by HPLC for glucose, xylose, and arabinose using a 1100 series Hewlett-Packard chromatograph fitted with a refractive

167 index detector operated at 50°C. Other analysis conditions were as follows: Aminex
168 HPX-87H column (BioRad, Hercules, CA); mobile phase, 0.003 mol/L H_2SO_4; flow, 0.6
169 mL/min.

170 *2.5. Enzymatic activity assays*

171 Laccase activity was determined by measuring the oxidation of 5 mM 2,2'-azino-
172 bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate buffer,
173 pH 5 (ε_{436}=29.3 mM^{-1} cm^{-1}) (Taboada-Puig et al., 2011). MnP activity was measured by
174 following the oxidation of 1 mM 2,6-dimethoxyphenol (DMP) at 468 nm in a 50 mM
175 sodium malonate buffer (pH 4.5) containing 1 mM $MnSO_4$ after starting the reaction with
176 0.4 mM H_2O_2 (ε_{468}=49.6 mM^{-1} cm^{-1}) (Taboada-Puig et al., 2011). LiP activity was
177 determined by measuring the oxidation of 2 mM veratryl alcohol to veratraldehyde at 310
178 nm in 50 mM of tartaric acid (pH 3) and 0.4 mM of H_2O_2 (ε_{310}=9.3 mM^{-1} cm^{-1}) (Taboada-
179 Puig et al., 2011). All enzymatic activities were expressed as international units (U),
180 defined as the amount of enzyme that transforms 1 μmol substrate/min or the amount of
181 enzyme that releases 1 μmol product/min at 30ºC.

182 *2.6. Microtox® toxicity assays*

183 Microtox® toxicity assays were performed by using a Microtox® model 500
184 Analyzer. The luminescent marine bacterium *Vibrio fischeri* was the bioassay
185 microorganism used for these experiments. The results were expressed as EC_{50} at 5, 15
186 and 30 min, which corresponds to the volume percentage that causes a reduction in the
187 light output of the Microtox® test organism by 50% in 5, 15 and 30 min of contact,
188 estimated according to the "Basic Test" protocol of the software (Microbics, 1992).

189 *2.7. Data analysis*

190 A statistical analysis was conducted for a correct comparison of results using the
191 software R v.2.12.0 (The R Foundation for Statistical Computing). First, a one-way

192 analysis of variance (ANOVA) was carried out to determine if the results obtained at
193 different conditions were significantly different. Then, if the ANOVA confirms the
194 existence of a significant difference ($p < 0.05$), a post-hoc analysis (Tukey's HSD) was
195 performed for a level of significance (α) of 0.05.
196
197 **3. Results and discussion**
198 *3.1. Fungal screening*
199 Six different white-rot fungi: *I. lacteus*, *Bjerkandera* sp. R1, *S. hirsutum*, *L. tigrinus*, *G.*
200 *lucidum* and *P. chrysosporium*, were screened for ligninolytic enzyme production in a
201 culture medium composed of cellulose fibers, organosolv lignin and hemicellulose
202 fraction (C_6+L+C_5) (Table 2). This medium promoted the growth of all tested fungi in the
203 form of pellets, except *P. chrysosporium*, which did not produce any ligninolytic enzyme.
204 Inhibition of LE production by *P. chrysosporium* was probably caused by a non-identified
205 component from the hemicellulose stream. Components such as total phenolics, acetic
206 acid, Na^+ and SO_4^{2-} were not expected to inhibit LE production (Koutrotsios and Zervakis,
207 2014).
208 Different sugar consumption profiles were observed (Fig. 1A and 1B), but the
209 common trend among all the fungal strains was the unconsumed sugar fraction that
210 remained at the end of the fermentations (approximately 1 g L^{-1} of TRS). *L. tigrinus* and
211 *G. lucidum* were the strains with the fastest sugars consumption rates; in both cases, the
212 TRS concentration was reduced to 1 g L^{-1} at day 4. All fungal cultures presented a lag
213 phase of two days, with the exception of *S. hirsutum,* which had the longest lag phase; it
214 extended up to four days, but thereafter, sugars were depleted in 3d. An increase of TRS
215 at day 5 was detected in the case of *I. lacteus*, probably due to the hydrolysis of the

cellulose or other oligosaccharides contained in the hemicellulose fraction that released some soluble TRS.

Enzyme production varied among individual fungal strains significantly (Fig. 1C and 1D). *I. lacteus* and *Bjerkandera* sp. R1 exclusively produced MnP, while laccase was only detected with *S. hirsutum*, *L. tigrinus* and *G. lucidum*. LiP activity was not detected in any supernatant. None of the strain tested in this study produced high levels of activity of more than one enzyme, despite previous reports about possible simultaneous production of MnP, laccase and/or LiP by *Ganoderma* spp. (de Souza Silva et al., 2005), *I. lacteus* (Fr. 238 617/93, Novotný et al., 2000) and *P. chrysosporium* (ATCC 24275, Gassara et al., 2010).

The maximum activities of MnP (646±122 U L^{-1}) and laccase (1,497±161 U L^{-1}) were obtained with *I. lacteus* and *G. lucidum*, respectively. Different LE production profiles could be observed for these two fungal strains. MnP in *I. lacteus* was firstly detected at day 4 at a very low activity and the production peak was did not occur until day 8. Nevertheless, laccase in *G. lucidum* appeared on day 3 and its activity was maintained for 5 days.

The medium composed of cellulose fibers, lignin and hemicellulose resulted in an appropriate growth substrate. The comparison of the productivity reported here with other published works is not straightforward due to the different methods used to determine enzymatic activity (Elisashvili and Kachlishvili, 2009). However, we can state that the LE production levels are similar or, in some cases, even higher than those previously obtained in submerged fermentation of WRF. Taboada-Puig et al. (2011) achieved a maximum Mn-oxidizing peroxidase activity of 1,400 U L^{-1} in submerged fermentation of *Bjerkandera* sp. R1 using a modified Kirk medium. In cultures with *Dichomitus squalens*, the maximum laccase activity (1,882 U L^{-1}) was obtained after 12 days in a stirred tank

241 reactor, while the maximum MnP activity (449.8 U L^{-1}) occurred after 18 days in a bubble
242 column reactor using an N-limited medium (Babič and Pavko, 2012).
243 *3.2. Effect of carbon source on LE production by I. lacteus and G. lucidum*
244 The carbon source composition of the culture medium was modified by removing the
245 cellulose fibers (C_5+L), the organosolv lignin (C_5+C_6) or both (C_5) to test the effect of
246 these components on the enzyme production by the two most efficient MnP and laccase
247 producers, *I. lacteus* and *G. lucidum*, respectively (Table 2).
248 There was no significant difference among the maximum MnP activities reached
249 with the different media (269±69, 350±30, 263±79 U L^{-1} for C_5, C_5+C_6, C_5+L,
250 respectively, p = 0.585) in *I. lacteus*; however, these values were lower compared with
251 the MnP production with the mixture C_5+C_6+L (646±122 U L^{-1}) (Fig. 2A). Although the
252 MnP activity was lower in the C_5 and C_5+L media, it was maintained stable for longer
253 periods. It is well known that the degradation of cellulose by basidiomycetes involves the
254 action of the array of fungal cellulose-degrading enzymes in combination with a non-
255 enzymatic mechanism based on the Fenton reaction (Baldrian and Valášková, 2008). It
256 has been reported that when cultivated under cellulolytic conditions, i.e. when cellulose
257 is the primary carbon source, *I. lacteus* produces enzymes that act synergistically on
258 cellulose, including hydrolytic enzymes and cellobiose dehydrogenase (CDH), which can
259 provide Fe^{2+}. Together with H_2O_2, Fenton reagent will be formed to produce a hydroxyl
260 radical that can attack cellulose (Hai et al., 2000). It has been previously suggested that a
261 high level of H_2O_2 during secondary metabolism causes loss of peroxidase activity only
262 in a few hours (Taboada-Puig et al., 2011). Regarding the TRS consumption, an increase
263 in TRS was detected only in the media in which the cellulose fraction was present, despite
264 the minor contribution of the C_5+C_6 medium (Fig. 2B). The increase of sugars (TRS)
265 observed during *I. lacteus* fermentation in the medium C_5+C_6+L (Fig. 2B) was confirmed

266 by HPLC analysis (Table 3). Glucose concentration rose from day 5 onwards, reaching a
267 maximal concentration of 1.59±0.21 g L^{-1} on day 7. As mentioned above, the production
268 of CDH by *I. lacteus* under cellulolytic conditions may promote cellulose degradation.
269 The enhanced cellulose degradation in the medium with lignin could be related to the
270 presence of quinones generated during lignin transformation, which could act as an
271 electron acceptor of CDH (Baldrian and Valáŝková, 2008; Henriksson et al., 2000).
272 In the case of *G. lucidum*, hemicellulose fraction as sole carbon source presented the
273 lowest laccase production (218±21 U L^{-1}) and the addition of lignin doubled this value
274 (456±29 U L^{-1}). However, the presence of cellulose boosted laccase production and
275 allowed a stable maintenance of the laccase activity with time. No significant differences
276 were observed in the maximum laccase activity with the media C_5+C_6 and C_5+C_6+L
277 (1,210±274 and 1,328±287 U L^{-1}, respectively, p = 0.945) (Fig. 3A). Faster and similar
278 TRS consumption profiles were detected with the media in which cellulose was present;
279 however, slower consumption rates were observed for the other two media (Fig. 3B).
280 Malarvizhi et al. (2003) observed that *G. lucidum* mycelial growth was faster in media
281 containing xylose or xylan compared to cellulose. Results of TRS consumption (Fig. 3B)
282 suggest the opposite, that growth was promoted in media containing cellulose. However,
283 the direct determination of mycelial biomass concentration by dry weight measurement
284 was not feasible due to the high content of solids different than fungal biomass in the
285 culture broth.
286 It is worth mentioning that the cellulose fibers studied contained a small fraction of
287 lignin (10% w/w), and the possible effect of the presence of this lignin on LE production
288 cannot be neglected, and it cannot be stated that lignin is dispensable for laccase
289 production with *G. lucidum*. In this sense, other authors have reported the favourable

290 effect of lignin (5 g L^{-1}) in the production of laccase by *G. lucidum* (Sharma et al., 2013;
291 Shrestha et al., 2016).

292 HPLC of samples from C_5+C_6+L medium revealed that glucose and xylose were
293 almost completely depleted after 9 days of cultivation with *G. lucidum* (Table 3). In this
294 case, no increase of glucose concentration was detected, although it has been reported
295 that glucan hydrolysis and LE production can take place simultaneously with *G. lucidum*
296 (Manavalan et al., 2012; Tirado-González et al., 2016).

297 Rhamnose was not a preferred monomer, since it was not entirely consumed in both
298 fungal cultures. Xylose oligomers were only partially consumed (0.2 g L^{-1}, <12%) under
299 the conditions evaluated. Manavalan et al. (2012) reported that *G. lucidum* may produce
300 hemicellulose degrading enzymes when cultivated in sugarcane bagasse. Additionally.
301 the secretome analysis of *I. lacteus* during wheat straw pretreatment indicated the
302 secretion of xylanases (Salvachúa et al., 2013). On the contrary, phenolic compounds
303 were significantly reduced at the end of the fermentation by both WRF (Table 3). This is
304 in agreement with previous studies describing white-rot fungi detoxification of
305 wastewater with a high content of phenolic compounds (Asses et al., 2009; Koutrotsios
306 and Zervakis, 2014; Ntougias et al, 2012).

307 In the light of the results obtained, it can be stated that there are no universal
308 conditions equally good for the different fungi, and that the different lignocellulosic
309 fractions exert different effects on the mechanisms for LE production using the same
310 fungus strain. Laccase production by *G. lucidum* was highly influenced by the presence
311 of the cellulose fibers, while high MnP production by *I. lacteus* was favoured by the
312 combination of the cellulose and the lignin in the culture medium. However, while
313 cellulose and lignin obtained from organosolv pulp are the main valuable products of the
314 process, the hemicellulose fraction is considered a secondary stream with less or no direct

315 applications, mainly due to its complex composition and the presence of inhibitory
316 compounds produced by the thermal and chemical decomposition of sugars or other wood
317 components. The valorization of this stream could provide an additional incentive for the
318 biorefinery development.
319
320 *3.3 Hemicellulose fraction from organosolv*
321 The hemicellulose fraction obtained after lignin precipitation and a concentration
322 step is a rich source of monomeric sugars, such as glucose, xylose or rhamnose, and
323 oligomeric sugars, mainly xylose oligomers. However, it also contains a high
324 concentration of acetic acid, released during the xylan hydrolysis, phenolic compounds,
325 and sodium and sulphate ions (Table 1), which are considered to be major inhibitory
326 compounds. In fact, this stream is considered complex and difficult to purify (Bozell et
327 al., 2011). Additionally, Laure et al. (2014) concluded that the costs associated with the
328 treatment of this fraction would be excessive.
329 To further study the influence of this hemicellulose stream, the LE production by *I.*
330 *lacteus* in the C_5 medium and in a synthetic medium, in which xylose was the sole carbon
331 source (X medium), were compared (Fig. 4). A MnP activity of 39±10 U L^{-1} was obtained
332 in the X medium, which is low when compared with the maximum activity reached with
333 the C_5 medium, in which the hemicellulose fraction was used as sole carbon source
334 (301±29 U L^{-1}). On the other hand, when the X medium was supplemented with cellulose
335 fibers and lignin from organosolv (X+C_6+L), MnP activity increased (188±14 U L^{-1})
336 compared with xylose; however, it is still far from the values obtained with the C_5+C_6+L
337 medium (646±122 U L^{-1}). Phenols in the hemicellulose fraction may have an important
338 role promoting the LE production. The supplementation of the culture media with an
339 aromatic compound is one of the most efficient approaches to increase the yield of LE,

and many of the aromatic inducers applied are phenolic compounds, such as, 2,6-dimethylphenol or vanillic acid (Elisashvili and Kachlishvili, 2009). Moreover, it was previously demonstrated that the water-soluble aromatic/phenolic compounds derived from different lignocellulosic substrates increase the production of LE by white-rot fungi (Kapich et al., 2004). Although the phenolic composition of the hemicellulose fraction was not yet determined, it can be considered as a cheap inducer for LE production, since this stream is currently treated as a residue. The contribution of phenolic compounds, the presence of readily available sugars, and the complexity of the hemicellulose matrix represent an advantage for LE production by white-rot fungi.

In order to take better advantage of this waste as ligninolytic enzymes inducer, its concentration was increased from 5 to 10 and 15 g L^{-1} of TRS (Figure 4). The highest concentration of the liquor stream led to the complete inhibition of fungal growth in both species. In this case, the high concentration of phenolics (≈3.25 g L^{-1}) could hamper fungal growth (Buswell et al., 1994). On the other hand, increasing TRS from 5 to 10 g L^{-1} appeared to delay LE production. However, the highest MnP and laccase activities were obtained with 10 g L^{-1}: 501±89 U L^{-1} and 613 ±120 U L^{-1}, respectively.

3.4. Detoxification of hemicellulose-rich liquor

Microtox® tests were performed to evaluate the acute toxicity of the hemicellulose rich stream from the organosolv process diluted to a TRS concentration of 10 g L^{-1}. This stream is extremely toxic to *V. fischeri*, as revealed by the very low concentration required for the 50% inhibition of the luminescence (EC_{50}) (Table 4). To our knowledge, there are no previous reports on the toxicity of hemicellulose-rich liquor from organosolv process. The toxicity of this diluted stream is comparable to that of olive mill wastewaters (EC_{50} ranging from 0.16 to 5.10%, Ntougias et al., 2012; Paixão et al, 1999), which contains high concentrations of recalcitrant compounds such as lignins, tannins, phenolic

365 compounds and long-chain fatty acids that are toxic to microorganisms and plants (Mann
366 et al., 2015).

367 After the fungal treatment and the recovery of the enzyme by ultrafiltration (10
368 kDa), the toxicity of the stream decreased nearly 21 and 12 times for *I. lacteus* and *G.*
369 *lucidum* strains, respectively. The detoxification promoted by *I. lacteus* seemed more
370 effective than that of *G. lucidum*, although the removal of phenolic compounds was very
371 similar in both reactors (≈85%). The analysis of the aromatic composition of both treated
372 liquors could help to clarify the different behaviour observed in the detoxification (Asses
373 et al., 2009). Ntougias et al. (2012) evaluated the detoxification of olive mill wastewater
374 by different WRF, and they observed that *Ganoderma* strains were less efficient than
375 *Pleurotus* sp. in the reduction of toxicity (by ca. 5–8 and 15 times, respectively).
376

377 **4. Conclusions**

378 The different lignocellulosic fractions derived from the organosolv fractionation of beech
379 wood influenced the mechanisms associated with LE production between fungal species.
380 Additionally, the residual liquid stream, containing mainly hemicellulosic sugars and
381 phenolic compounds, was observed to be an excellent LE inducer. The utilization of this
382 residue as the sole carbon source provides an opportunity to produce LE in an economical
383 and simple medium and, importantly, the fungal treatment results in a substantial decrease
384 in the liquor toxicity. Furthermore, the valorization and detoxification of this byproduct
385 stream is a promising strategy that may enhance the industrial feasibility of the organosolv
386 process.
387

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528

Tables

Table 1: Physico-chemical characteristics of the cellulose fibers, organosolv lignin and concentrated hemicellulose fractions obtained from the organosolv process. Mean value ± standard error were calculated using triplicate sets

Parameters	**Cellulose fibers (C_6)**	**Organosolv lignin (L)**	Parameters	**Hemicellulose (C_5)**
pH	3.4	2.6	pH	5.5
Humidity (%, w/w)	69.8 ± 0.4	12.7 ± 0.6	TN (mg L^{-1})	748 ± 55
Ashes (%, w/w)	0.16 ± 0.02	0.14 ± 0.02	Glucose (g L^{-1})	10.8
Glucan (%, w/w dry basis)	73.5 ± 7.4	0	Xylose (g L^{-1})	144.3
Xylan (%, w/w dry basis)	11.3 ± 3.9	2.4 ± 0.09	Rhamnose (g L^{-1})	44.1
			Xylose oligomer (g L^{-1})	75.7
Acid soluble lignin (%, w/w dry basis)	2.1 ± 0.2	2.0 ± 0.1	Acetic acid (g L^{-1})	17.1
			Total phenols (g L^{-1})	40.8 ± 0.8
Total lignin (%, w/w dry basis)	10.4 ± 1.0	92.7 ± 0.9	Na^+ (g L^{-1})	20.7
			SO_4^{2-} (g L^{-1})	18.8

TN: total nitrogen

537

538 **Table 2:** Carbon composition of the different media tested

Medium component (g L^{-1})	**C_5+C_6+L**	**C_5**	**C_5+C_6**	**C_5+L**	**X**	**X+C_6+L**
Cellulose fibers (C_6)	10	--	10	--	--	10
Organosolv lignin (L)	10	--	--	10	--	10
Hemicellulose (C_5)[b]	5	5[c]	5	5	--	--
Xylose (X)	--	--	--	--	5	5

539 [c] Unless otherwise stated

540

Table 3: Concentration of sugars and phenols in the C_5+C_6+L medium

Fungi	Day	Glucose (g L^{-1})	Xylose (g L^{-1})	Rhamnose (g L^{-1})	Xylose oligomers (g L^{-1})	Phenolic compounds (g L^{-1})
I. lacteus	3	0.14 ± 0.04	1.53 ± 0.23	0.62 ± 0.07	1.70	0.450 ± 0.010
	7	1.59 ± 0.21	0.70 ± 0.13	0.54 ± 0.08	NA	NA
	9	0.22 ± 0.07	0.55 ± 0.26	0.45 ± 0.21	1.50	0.101 ± 0.002
G. lucidum	3	0.21 ± 0.09	1.37 ± 0.14	0.68 ± 0.02	1.70	0.380 ± 0.010
	7	0.07 ± 0.02	0.09 ± 0.02	0.24 ± 0.03	NA	NA
	9	0.09 ± 0.01	0.09 ± 0.03	0.21 ± 0.02	1.61	0.124 ± 0.002

NA: not available

546 **Table 4:** Acute toxicity of the hemicellulose-rich liquor before and after WRF culture,
547 measured as the concentration of liquor (v:v) corresponding to 50% luminescence
548 inhibition (EC50) of *V. fischeri* after 5, 15 and 30 min exposure (95% confidence
549 range)

Sample	**EC50 5 min (v:v)**	**EC50 15 min (v:v)**	**EC50 30 min (v:v)**
Liquor	1.8%	1.5%	1.1%
Liquor (< 10 kDa)*	1.6%	1.4%	1.1%
Liquor treated by *I. lacteus* (< 10 kDa)	33%	27%	21%
Liquor treated by *G. lucidum* (< 10 kDa)	19%	13%	10%

550 * Liquor was ultrafiltrated to serve as control of the treated liquors, where enzymes were
551 recovered by ultrafiltration
552

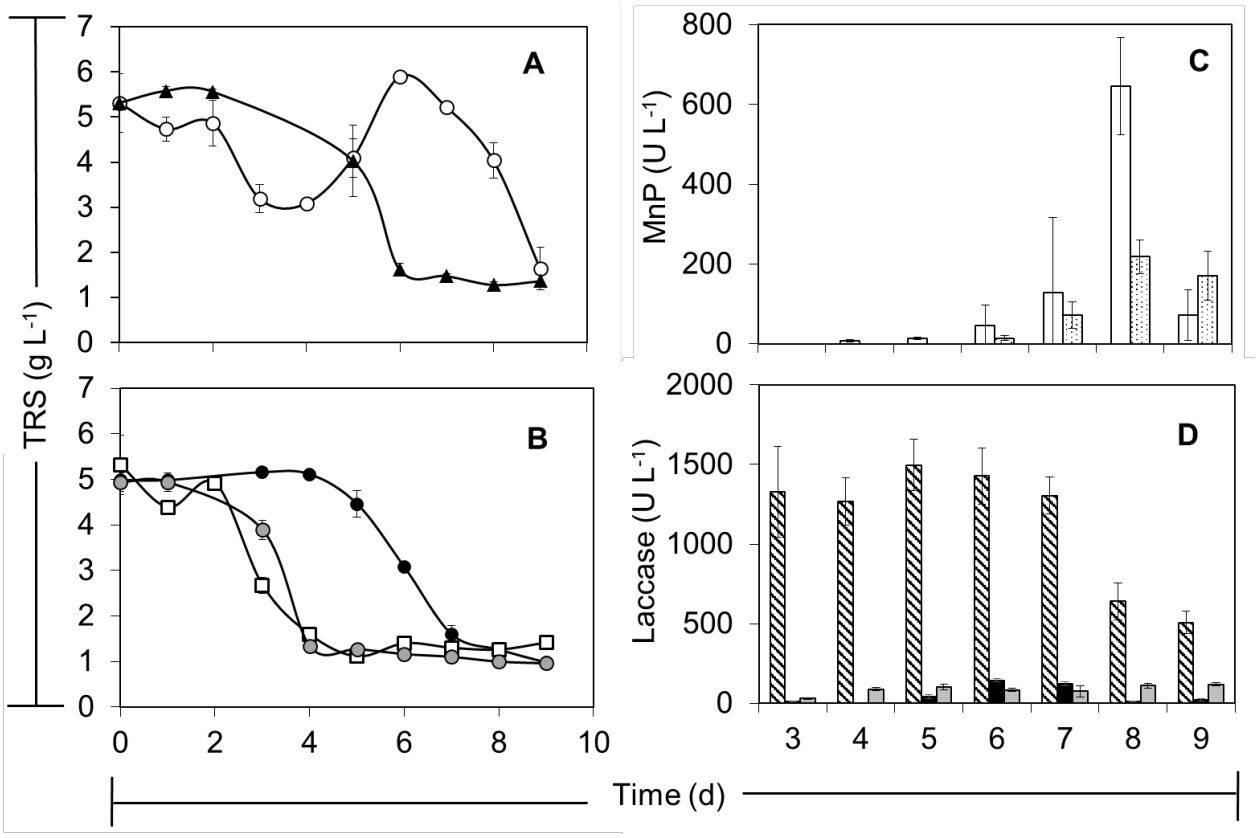
Figure captions

Figure 1: Fungal screening for LE production with *I. lacteus* (white circle and white bar), *Bjerkandera* sp. R1 (black triangles and dotted bar), *S. hirsutum* (black circles and black bar), *L. tigrinus* (grey circle and grey bar) and *G. lucidum* (white square and striped bar). In Fig. A and B, the TRS consumption is represented. Fig. C and D present MnP and laccase production, respectively

Figure 2: MnP production (**A**) and TRS consumption (**B**) by *I. lacteus* growing on different carbon sources: C_5 (white bar and circle), C_5+C_6 (dashed bar and cross), C_5+L (black bar and circle) and C_5+C_6+L (grey bar and circle)

Figure 3: Laccase production (**A**) and TRS consumption (**B**) by *G. lucidum* growing on different carbon sources: C_5 (white bar and circle), C_5+C_6 (dashed bar and cross), C_5+L (black bar and circle) and C_5+C_6+L (grey bar and circle)

Figure 4: MnP and laccase production by *I. lacteus* (**A**) and *G. lucidum* (**B**), respectively, from different carbon sources: X (black bar), X+C_6+L (dark grey bar), C_5-5g/L (light grey bar), C_5-10 g/L (white bar)

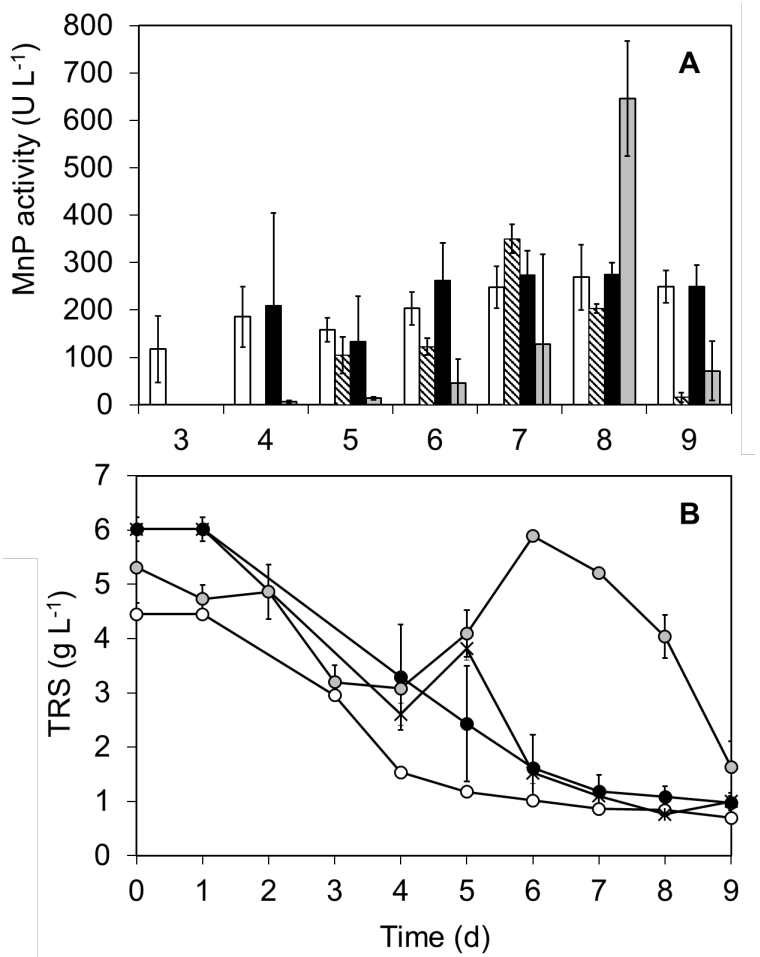


570

571 **Figure 1**

572

573

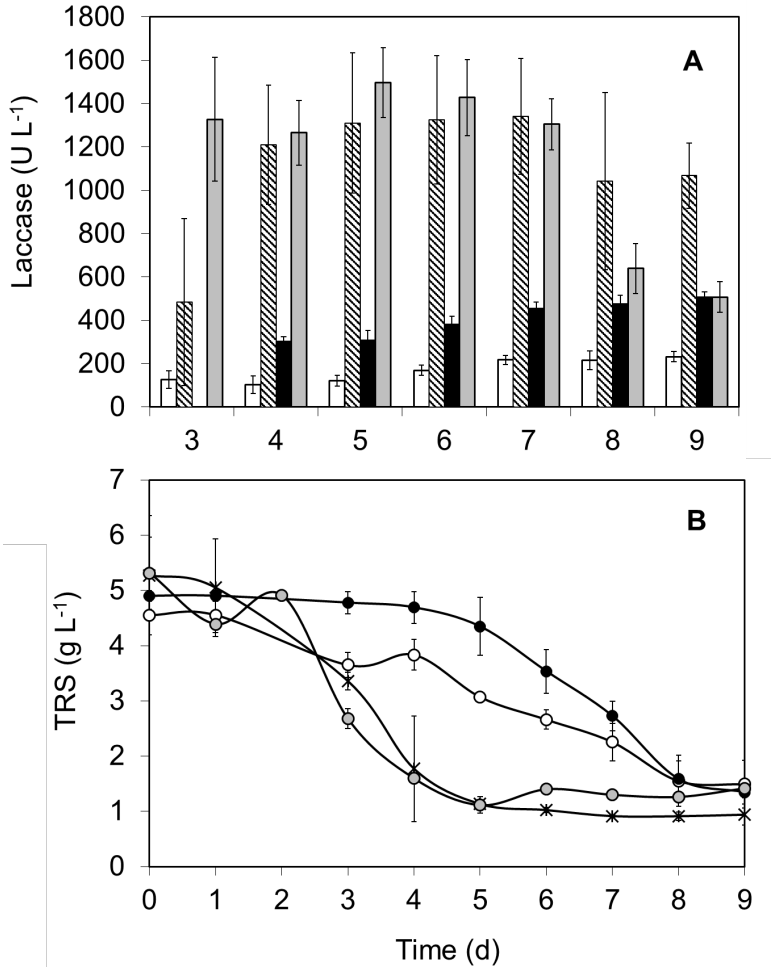


574

575 **Figure 2**

576

577



578

579 **Figure 3**

580

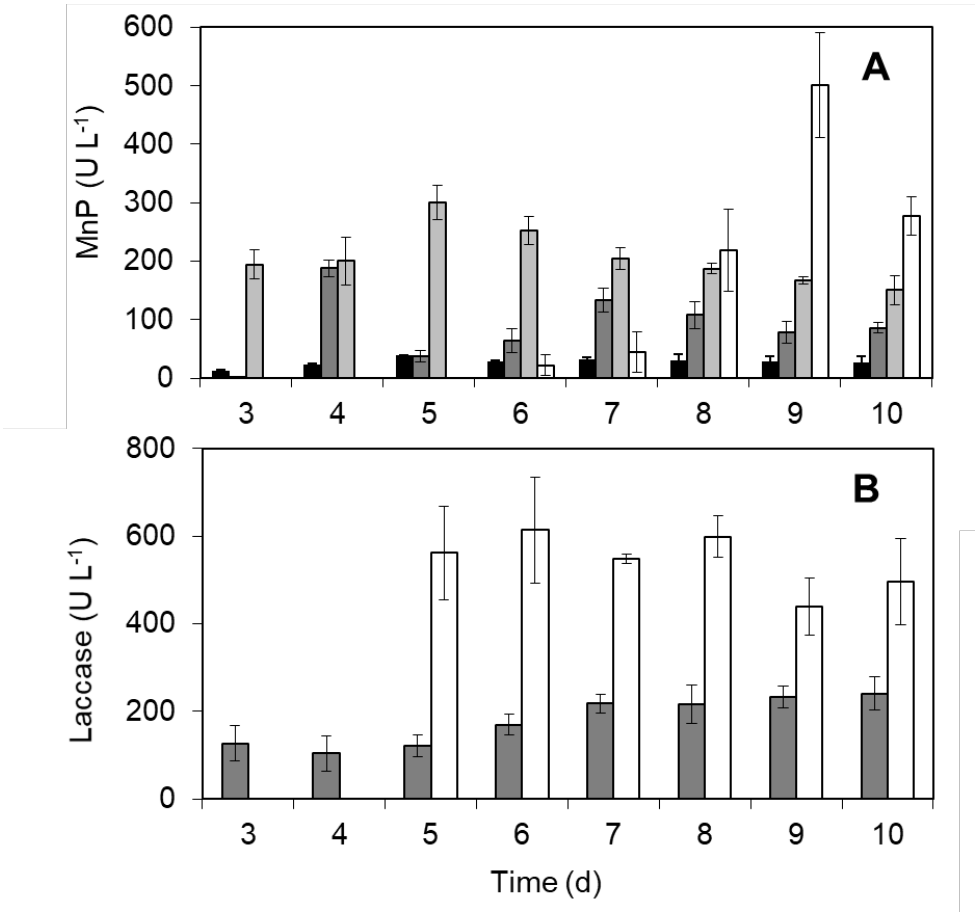

A
B
MnP (U L-1)
Laccase (U L-1)
Time (d)
0
100
200
300
400
500
600
800
3
4
5
6
7
8
9
10

Figure 4