DOI 10.1016/j.biortech.2017.07.055

Comparison between several reactors with *Trametes versicolor* immobilized on lignocellulosic support for the continuous treatments of hospital wastewater

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Abstract

Hospital wastewater is a major source of pharmaceutically active compounds (PhACs), which are not all removed in conventional wastewater treatment plants. White rot fungi can degrade PhACs, but their application has been limited to non-sterile conditions due to the competition with other microorganisms for growth. In this study, immobilization of *Trametes versicolor* on different lignocellulosic supports was studied as strategy to ensure fungal survival under continuous treatment conditions. A fluidized bed reactor and a trickling packed-bed reactor with *T. versicolor* immobilized on pallet wood were employed for the removal of ibuprofen, ketoprofen and naproxen. Best results were obtained with the trickling packed-bed reactor, which operated for 49 days with high removal values in real hospital wastewater.

Keywords: fungal bioreactor; lignocellulosic support; pharmaceutically active compounds

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1. Introduction

Water pollution by micropollutants that result from predominantly human activities has become one of the most critical problems in recent decades. Pharmaceutically active compounds (PhACs) are increasingly detected in the surface of different environmental water compartments (Benotti et al., 2009; Watkinson et al., 2009), as not all are removed in conventional wastewater treatment plants (Joss et al., 2008).

Many studies have reported the ability of white-rot fungi (WRF) to degrade PhACs due to their unspecific oxidative enzyme system (Prieto et al., 2011; Marco-Urrea et al., 2009, 2010a, 2010b,). The key issue, in practice, for applying the white-rot technology is to design and establish a suitable reactor. Different configurations such as a stirred-tank reactor (Rodarte-Morales et al., 2012), an airlift (Zhou et al., 2006), bubble column (Cerrone et al., 2012), fluidized bed reactor (Badia-Fabregat et al., 2017; Mir-Tutusaus et al., 2017; Cruz-Morató et al., 2014), trickling packed-bed bioreactor (Ehlers and Rose, 2005), packed bed bioreactor (Li et al., 2015) and MBR (Nguyen et al., 2013) have been used for wastewater treatment. The present research focuses on an air-pulsed fluidized bed bioreactor and a trickling packed-bed bioreactor.

T. versicolor has also been proven to degrade PhACs in real wastewater (Badia-Fabregat et al., 2016; Cruz-Morató et al., 2014; Cruz-Morató et al., 2013; Zhang and Geißen, 2012). A significant decline in the removal performance of the continuously operated bioreactor under non-sterile conditions, especially with a real matrix, commonly occurs. This performance deterioration is generally caused by the overgrowth of bacteria, which impose an inhibition on fungal growth and enzyme production (Yang et al., 2013; Zhoue et al., 2013).

Many authors have studied some strategies to foster fungal growth such as using nitrogen-limiting conditions, maintaining an acidic pH, using immobilized or encapsulated mycelium, pretreating wastewater and employing selective carbon sources (Gao et al., 2008; Libra et al., 2003; Mir-Tutusaus et al., 2016).

In addition, due to the low concentration of PhACs in wastewater, it is necessary to add other carbon sources to perform their degradation with WRF in order to maintain the fungus viability, irrespective of whether their degradation is cometabolic or not. Easily biodegradable carbon sources fostered bacterial growth, while lignocellulosic sources could be a selective carbon source that allow white rot fungi to outcompete bacteria.

Lignocellulosic materials have already been proven to successfully sustain fungal biodegradation (Ehlers and Rose, 2005; Rodriguez-Rodriguez et al., 2010). Therefore, this strategy to prevent bacterial contamination in real wastewater treatment is studied in this paper.

Moreover, the use of lignocellulosic material could bring other advantages: they are readily available, economical (Saba et al., 2016) and represent a physical support for immobilized cultures, which are more resilient to environmental perturbations and exposure to toxic chemical concentrations than suspension cultures (Rodríguez-Couto et al., 2004; Shin et al., 2002).

The objective of the present work is to establish a white-rot fungus reactor and determine the best operational conditions for the continuous treatment of real hospital wastewater. The overgrowth of bacteria is the main problem for the fungal survival when working with a real matrix. To solve this issue, different strategies are studied in this paper: fungal immobilization, control of pH, wastewater pretreatment and using different reactors. With the best reactor´s configuration it is expected to create the conditions to ensure the growth

and survival of *T. versicolor* during wastewater treatment, maintaining good levels of PhAC removal.

2. Materials and methods

2.1 Fungal strain

Trametes versicolor ATCC 42530 was obtained from the American Type Culture Collection and was maintained by subculturing on petri dishes in malt extract (2%) and agar (1.5%) medium at 25°C (pH 4.5). Blended mycelial suspension was prepared according to Blánquez et al. (2004).

2.2 Lignocellulosic material, culture condition and pellet formation.

Several lignocellulosic substrates were employed for the culture of *T. versicolor:* pine bark, nutshell, hazelnut shell and wood pallet. Each lignocellulosic substrate was autoclaved at 120°C during 30 min immersed in tap water and then strained to remove excess water. Cultures were performed in Schott bottles (250 ml, 95 x 105 mm; Duran, Inc) equipped with 1 port screw cap opened, using a 0.45 um filter as passive air intake. Under sterile condition, 10 g of each sterile substrate (with 100% of the water holding capacity) were placed in each bottle and inoculated with 3 mL mycelia suspension. Cultures were incubated in static condition at 25°C.

For the pellets with a wood core production, the method previously described by Blánquez et al. (2004) was modified, adding 5 g of woods pre-culture with *T. versicolor* instead of mycelial suspension, in Erlenmeyer flask with malt extract. Defined medium (Borràs et al., 2008) was used when the pellets formation was done inside the bioreactor, adding 30 $g.L^{-1}$ of woods pre-culture with *T. versicolor*.

2.3 Chemical

Naproxen, Ketoprofen and Ibuprofen selected in this study were purchased from Sigma-Aldrich (USA). Thiamine hydrochloride was adquired from Merck (Barcelona, Spain), peptone and yeast extract from Scharlau (Barcelona, Spain) and glucose, ammonium chloride and other chemicals were purchased from Sigma –Aldrich (Barcelona, Spain). All chemical used were of analytical grade.

2.4 Synthetic water and Hospital Wastewater

For the experiments two types of water were used: synthetic tap water and hospital wastewater (HWW) collected directly from sewer manifold of Sant Joan de Deu Hospital (Barcelona, Spain).

In the case of the HWW, a coagulation-flocculation pretreatment was applied in order to reduce the bacterial contamination as previously reported Mir-Tutusaus et al. (2016). No pretreatment was employed with the synthetic water due to the tap water low microbial content.

The coagulation-flocculation pretreatment was carried out in a jar-test apparatus (Flocculator W1 from Stuart Scientific, Staffordshire, UK). Coagulant HyflocAC50 and flocculant HimolocDR3000 were provided by Derypol S.A. (Barcelona, Spain). The jar test involved 2min of coagulation at 200rpm, 15 min of flocculation at 20 rpm and 30 min of settling (Mir-Tutusaus et al., 2016).

2.5 Experimental procedures

With the objective to determine the best operational conditions to ensure the survival of the fungi during the continuous treatment, different experiments were proved working first with synthetic water and then with HWW.

Synthetic water and HWW were spiked with ibuprofen, ketoprofen and naproxen to a final concentration of 20 $mg.L^{-1}$. Naproxen was spiked only in HWW. Taking into account

that PhACs are found in concentrations in the order of $\mu g.L^{-1}$ in HWW (Verlicchi et al., 2012), a higher concentration is used in this work for analytical purposes. This increase does not imply important differences since the degradation of these contaminants do not depend on their initial concentration.

Thus, in order to evaluate the reactor's performance, the degradation of ibuprofen, ketoprofen and naproxen were monitored along the treatment. These compounds were selected due to the well know ability of *T. versicolor* to degrade them involving different metabolic pathways (Marco-Urrea et al., 2009, 2010a, 2010b)

Air-pulsed fluidized bed bioreactor

Different kinds of experiments were carried out in a glass air fluidized bed bioreactor (Blánquez et al., 2006). The bioreactor was filled with 1.5 L of synthetic tap water (SW) or flocculated HWW depending on the experiment.

The temperature was set up at 25°C, and the pH was controlled to be constant at 4.5 by addition of 1M HCl or 1M NaOH. The hydraulic retention time (HRT) was 3 days. Fluidized conditions in the reactor were maintained by air pulses generated by an electrovalve. The cycling timer of the electrovalves and the air flow depended on the experiment and always worked at the minimum fluidization velocity. In all cases, no nutrients were added to the reactor. Samples were collected for laccase and PhAC analysis and heterotrophic plate counting. For these experiments, pallet wood was chosen as the best substrate for the growth of the fungi.

To study the best growth conditions, *T. versicolor* was added in two different ways: cultures growing directly on the wood and on pellets with a wood core. The effects of different wood sizes and times of incubation were tested. The experiments were carried out until the pharmaceutical removal was less than 75%.

Trickle-bed bioreactor

The cylindrical bioreactor was made of glass with a 160 cm^3 working volume, 3 cm diameter and 25 cm height. Humidified air was introduced in the top of the reactor. The pH was controlled in the reservoir bottle at 4.5 by adding 1M HCL or 1 M NaOH. A magnetic stirrer was used to mix the reservoir bottle. The reactor was provided with an external bottom-to-top recirculation loop. In a continuous process, the HWW flew into the reservoir bottle with a hydraulic retention time of 3 days, which is the same period that was used for the air fluidized bed bioreactor. The total volume of the process was 0.25 L.

2.6 Analytical methods

Ergosterol quantification.

Ergosterol was measured in a homogeneously-mixed samples of lignocellulosic cultures and extraction was performed as previously described (Rodríguez-Rodríguez et al., 2010).

PhACs analysis

Samples were filtered through a Millipore Millex-GV PVDF 0.22 µm membrane and placed in ambar vials. Analyses were carried out using a Dionex Ultimate 3000 HPLC system equipped with a UV detector. The separation was achieved on a GraceSmart RP 18 column (250mm x 4.6mm, particle size 5 um). The mobil phase consist of milliq water adjusted pH to 3.5 with methane sulfonic acid (Pump A) and acetonitrile (Pump B). The flow rate was 1.5 $mL.min^{-1}$ and the eluent gradient started at 15%B and increased to 50% from 0 to 15 min; the gradient decreased to 15-5 B from 15 to 16 min and remained at 15-5B from 16 to 20 min. A sample volum of 20 µl was injected from Dionex autosampler and the detection was carried out at 210 nm. All determinations were performed at 30°C.

Routine analysis

157 Laccase activity was measured through the oxidation of 2.6-dymetoxyphenol (DMP) by
158 the enzyme laccase (Cruz-Morató et al., 2013). Activity units per liter ($UA.L^{-1}$) are defined
159 as the amount of DMP in micromoles per liters which are oxidized per minute (µmol DMP.
160 L^{-1}. min^{-1}).
161 A Microtox bioluminescence assay with *Vibrio fisheri* was used to perform acute toxicity
162 test. Effluent toxicity was expressed in toxicity units (TU) and an effluent was considered
163 toxic when its TU was over 25 as it is set by local sewage disposal regulation (Badia-
164 Fabregat et al., 2016).
165 The conductivity was determined by a CRISON MicroCM 2100 conductometer, and the
166 absorbance at 650 nm was monitored by UNICAM 8625 UV/VIS spectrometer.
167 Heterotrophic plate count (HPC) was analyzed per triplicate according to APHA (1995),
168 The N-NH4 concentration and chemical oxygen demand (COD) were analyzed by using
169 commercial kits LCH303 and LCK114 or LCK314m respectively (Hach Lange, Germany).

170 **3. Results and discussion**

171 **3.1 Colonization of substrates by *T. versicolor*: screening**

172 An initial colonization screening of *T. versicolor* was performed in order to identify the
173 most suitable substrates for fungal growth using the visual observation and ergosterol
174 content as an indicator of active fungus (Bååth, 2001; Barajas-Aceves et al., 2002).
175 Ergosterol is a sterol found in cell membranes of fungi and microalgae; an advantage of
176 using ergosterol is that it indicates only viable biomass since it is quickly degraded after
177 cell death (Gutarowska and Zakowska, 2009).
178 Growth on pallet wood and pine bark yielded high amounts of biomass, with maximum
179 values of 0.028 and 0.031 $mg.g^{-1}$ DW after 9 days. According to Planinić et al. (2016), *T.*
180 *versicolor* grows until the ninth day, whereupon it reached a stationary phase. Otherwise,

181 0.011 and 0.016 mg of ergosterol per g^{-1} DW were obtained with growth on hazelnut shell
182 and nutshell, respectively.

183 Ergosterol values ranging from 0.025 to 0.05 $mg.g^{-1}$ DW have been reported for
184 *Trametes versicolor* grown on wheat straw and maize stalks, while bigger values were
185 obtained with agricultural wastes that were processed for animal feeding (Borràs et al,
186 2011).

187 Pallet wood and pine bark yielded the highest concentrations of ergosterol, so a fluidized
188 bed reactor was loaded with the substrates and tap water to determine the best conditions of
189 fluidization (data no show). In the reactor with the pine bark, the water was totally brown
190 after two days, probably due to the extractives (Ramos et al., 2013); this phenomenon has
191 been a problem for PhAC analysis. Consequently, the pine bark was discarded because its
192 use increased the colour of the water; using this water would require the application of
193 another type of treatment to remove the added colour. Based on these results, pallet wood
194 was chosen for the degradation experiment.

195 **3.2 Fluidized bed bioreactor**

196 *3.2.1 Treating synthetic water with T. versicolor culture growing on wood*

197 The bioreactor was filled with spiked synthetic water and inoculated with *T. versicolor*
198 that grew directly on the wood (30 $g.L^{-1}$). To study the best growth conditions in
199 immobilized cultures, growth with two sizes of wood (0.5 x 0.5 cm and 2 x 1 cm) and two
200 incubation times (9 or 30 days) were tested. Fig. 1 presents the results of ibuprofen and
201 ketoprofen removal in each reactor. In all cases, after 6 days of treatment, the fungus
202 detached from the wood and started to accumulate on the glass walls of the bioreactor
203 before it achieved a stationary state. The best results were obtained with the smaller size of
204 wood and with 9 days of incubation, as previously reported (Planinić et al., 2016).

205 These results demonstrated that growing *T. versicolor* grown directly on the wood is not
206 a good immobilization strategy for its subsequent use as inoculum in the bioreactor since it
207 was observed that the fungus was removed from the wood, and consequently, the removal
208 of the PhACs decreased.

209 *3.2.2 Complex wood-pellet formation in Erlenmeyer flasks and the reactor*
210 *performance treating synthetic water.*

211 Taking into account that the previous results, when *T. versicolor* was directly pregrown
212 on wood, were not good, one step of wood core pellet formation was added before
213 continuous treatment was started in order enhance the adherence of the fungus to the wood.
214 The wood size in a pellet core was 0.5 x 0.5 cm, and the pellets were obtained after 6
215 days of incubation in Erlenmeyer flasks with malt extract. The reactor was filled with 30 g
216 of wood core pellets (complex pellets) and synthetic tap water that was spiked with
217 ibuprofen and ketoprofen. High removal of ibuprofen was obtained during all treatments
218 and over 80% ketoprofen removal was achieved from day 8 to the end (Fig. 2). Laccase
219 was detected in all treatments, peaking at the beginning.
220 Good removal was obtained using complex pellets. Similar results were previously
221 reported for complex pellets of sawdust and activated carbon with *Anthracophyllum*
222 *discolor* for the biological treatment of wastewater contaminated with Reactive Orange 165
223 (Elgueta et al., 2012).

224 *3.2.2 Complex wood-pellet formation inside a bioreactor. Comparison between*
225 *simple and complex pellet-treated SW and HWW.*

226 Based on the good results obtained using complex pellets produced in Erlenmeyer flasks,
227 pellets were directly produced in the bioreactor with defined medium under sterile
228 conditions in order to make the process scalable (Borràs et al., 2008). For the pellet

229 formation, two bioreactors were set up in parallel: one inoculated with 9 days wood
230 cultures (30 $g.L^{-1}$) (complex pellets), and the other was inoculated with a mycelial
231 suspension (4 $mL.\ L^{-1}$) as a control (simple pellets). After 6 days, when the pellets were
232 grown, the medium was withdrawn from both reactors. In the case of simple pellets, the
233 pellets were maintained inside the reactor. Meanwhile, the complex pellets were manually
234 separated from the simple pellets that were also produced. However, complete separation is
235 not possible, in which cases a mixture of complex and simple pellets was returned to the
236 reactor.
237 Different experiments testing PhAC removal were carried out in parallel with simple and
238 complex pellets in order to compare their reactor performance. The experiments were first
239 done with synthetic tap water, and then flocculated HWW was employed.
240 The results of the continuous treatments are presented in Fig. 3. In experiment A (with
241 synthetic water and complex pellets (A-I)), almost 100% of the ibuprofen was degraded,
242 while ketoprofen degradation rates were greater than 80% for 31 days, after which they
243 started to decrease until they reached 70% on day 41. Meanwhile, in the control reactor
244 with simple pellets (A-II), the degradation of ibuprofen and ketoprofen were almost 100%
245 and 90%, respectively, for only 8 days, after which they decreased until day 12. In both
246 reactors, low laccase production was observed. The differences in the degradation profiles
247 suggest that *T. versicolor* is able to use the pine wood as the only source of nutrients
248 (Valentín et al., 2009), and the immobilization could be a good strategy for the continuous
249 treatment.
250 In the experiments with flocculated HWW (B), the reactor that was inoculated with
251 complex pellets (B-II) operated in continuous mode for 28 days. The degradation profiles
252 were ibuprofen, 90% for 18 days; ketoprofen, 75% for 14 days; and naproxen, 80% until

253 day 21, at which point the degradation started to decrease. In the control reactor (B-II), the
254 degradation was below 60% after 7 days, when it was stopped.
255 The laccase profile, in both cases, showed a maximum peak and then started to diminish.
256 In B-I, the laccase activity decreased from 60 $UA.L^{-1}$ (day 3) to very low levels (less than 1
257 $UA.L^{-1}$). On the other hand, in B-II, the peak was at day 5 (89 $UA.L^{-1}$) and decreased to 20
258 $UA.L^{-1}$ at the end of the treatment (day 7). There is not a direct relationship between the
259 laccase values and the PhAC´s removal, since the fungus can act as a multi-enzymatic
260 reactor. In addition, when working with real wastewater, laccase detection confirms the
261 fungus activity, on the contrary the low level of the enzyme is not an indication of fungus
262 inactivity (Mir-Tutusaus et al., 2017).
263 In both cases (with SW and HWW), the complex pellets presented better removal than
264 the simple pellets in long-term treatments. Since no external nutrients were added to the
265 treatment, the complex pellets can use the wood as a carbon source, while the simple pellets
266 lost their structure, probably due to a lack of nutrients. The lignocellulosic materials have
267 been demonstrated as suitable substrates for the growth and survival of *T. versicolor*
268 (Walter et al., 2004; Maciel et al., 2013).
269 Many studies demonstrated the advantages of using fungi immobilized on lignocellulosic
270 substrates in different treatments (Bending et al., 2002). Elgueta et al. (2016) evaluated the
271 atrazine dissipation in a biobed system that was inoculated with white-rot fungi that were
272 immobilized on a pelletized support.
273 The results in this work indicated that pallet wood could be used as a single specific
274 source of nutrients and support material for the formation of complex pellets. This kind of
275 pellets could be used in the continuous treatment of PhACs in a fluidized bed reactor for
276 long time periods, achieving high removal for more than 40 days in SW and 25 days in

277 HWW. A decrease in the duration of the HWW treatment resulted, probably due to the
278 accumulation of free mycelia in the reactor, which promotes bacterial contamination. The
279 free mycelia are derived from the complex pellets that lose their structure, taking into
280 account that superficial hyphae could not easily access the carbon source (wood) that is in
281 the centre of the pellet. Additionally, free mycelia come from simple pellets that were not
282 separated at the beginning of the treatment. It was observed that these pellets broke and
283 accumulated in the reactor.

284 The lower level of removal that was obtained in HWW is in accord with previous studies.
285 Zhang and Geißen (2012) operated a plate bioreactor with WRF immobilized on polyether
286 foam for the degradation of carbamazipine and demonstrated a significant decrease in the
287 performance when they employed a real effluent from a municipal wastewater treatment
288 plant. Hai et al. (2009) demonstrated that the worse performance under non-sterile
289 conditions in a decolourization reactor is cause by bacterial disruption, fungal morphology
290 and enzyme washout in a continuous fungal reactor.

291 Complex pellets could be a good strategy for working with HWW, but the scaling up of
292 the process is very complicated because after the formation of the pellets inside the reactor,
293 a manual step is needed to separate the complex pellets from the simple pellets that were
294 formed at the same time. Additionally, the separation is not complete, and some simple
295 pellets are introduced into the reactor.

296 **3.3 Packed-bed bioreactor treating HWW**

297 Previous results obtained with a fluidized bed reactor shows that the immobilization on
298 the wood and pellets that were formulated with a wood core represented a good strategy to
299 ensure the fungus survival in wastewater treatment, but the scaling up is complicated and
300 the performance decreased in HWW. A packed-bed bioreactor was studied as an alternative

301 reactor configuration in examining the HWW treatment employing *T. versicolor*
302 immobilized on the wood.
303 Under sterile conditions, the reactor was packed with 60 g of 9-day wood culture of *T.*
304 *versicolor* (size: 2 x 1 cm). When the reactors started, in order to adapt the biomass to the
305 new environment, sterile tap water (pH 4.5) was continuously recirculated to ensure the
306 humidity conditions. After 5 days of adaptation, the continuous process was initiated under
307 non-sterile conditions. Flocculated HWW at pH 4.5 that was spiked with naproxen,
308 ketoprofen and ibuprofen was feed from the influent tank.
309 A first reactor was set up with the objective to determine the best recycling ratio (RR) for
310 the continuous treatment. The RR can be defined as the ratio of the recirculated flow rate to
311 the main influent flow rate. Four different RR (86,173,432 and 877) were tested for 45
312 days, and an increase in degradation was obtained with the highest RR (data no show).
313 Based on these results, the recycling ratio of 877 (recirculation flow/influent flow) was
314 chosen for the next experiments. High PhACs removals were obtained employing this RR,
315 therefore we decided to use the results of this preliminary study. For future work it will be
316 necessary to carry out exhaustive studies to fix this parameter.
317 Two bioreactors were set up in parallel, one packed with 9-day wood culture and the
318 other with sterile wood as a control. After 5 days of adaptation, the bioreactors were
319 continuously operated for 49 days to treat flocculated HWW.
320 The results are presented in Fig. 4. In the inoculated reactor (I), the following stable
321 removal values were obtained throughout the treatment: ibuprofen 90%, ketoprofen 80%
322 and naproxen 60%. The laccase profile was at its maximum until day 10 and then decreased
323 to the end of the experiment, in concordance with previous studies (Ehlers and Rose, 2004).

As mentioned before, the low laccase level obtained is not an indicator of the fungal inactivity.

In the control reactor (II), until day 9, the removal value was over 50% of removal, probably due to the adsorption on to the wood. After day 9, the removal value started to decrease. In the case of ketoprofen and naproxen, the removal was very variable depending on the bacteria present in water. Finally, from day 30 till the end the removal values were less than 30%. Regarding ibuprofen, high removal rates (>80%) were achieved from day 22 until the end of the treatment. As it has already been reported, ibuprofen can be easily degraded (Garcia-Rodriguez et al., 2014).

The results obtained with the trickling packed bed-bioreactor indicate that *T. versicolor* grew well when it was attached to the wood, and their particle sizes allowed the movement of wastewater and air along the reactor to give suitable aeration for *T. versicolor*. The high porosity of the reactor (37.5%) avoided clogging problems and ensured good surface contact. Moreover, the wood was not visibly degraded during the whole process. Altogether, it makes this support a very suitable material for this type of processes.

In addition, the use of wood as a support and a substrate presents several advantages such as the reduction in production cost, due the wood's double function as a place of attachment and a source of nutrients. Rodríguez-Couto et al. (2003; 2004) already indicated that the use of a natural support provides the fungus an environment that is similar to its natural habitat and offers the possibility of reusing waste.

Excessive growth of bacteria was avoided by limiting the nutrient supply and HWW pretreatment and pH control, making possible a long-term operation. Cruz-Morató et al. (2013) already reported the need to maintain pH values for fungal survival in wastewater treatment.

Böhmer et al. (2006) obtained good bioreactor performances decolourizing textile dyes with *T. versicolor* that was immobilized on pine wood chips under temporary immersion conditions. Jonstrup et al. (2012) achieved high decolourization by treating industrial textile wastewater in a packed-bed bioreactor with a *Bjerkandera* sp. that was immobilized on wood and reported a drop in decolourization efficiency after 17–19 days of continuous operation, which was caused by reactor contamination. Additionally, Li et al. (2015) found improved efficiencies of removing naproxen and carbamazepine when WRF was immobilized on wood chips, employing a fixed bed bioreactor for the treatment of synthetic water. Meanwhile, with real hospital wastewater, Mir-Tutusaus et al. (2016) reported wastewater treatment using a fluidized bed bioreactor with coagulated flocculated non-spiked HWW for 56 days. Therefore, with this system, we can combine the following strategies in order to promote fungus survival: coagulation-flocculation pretreatment, use of lignocellulosic material and controlling the pH value.

It is remarkable that the system in this work was able to operate in a continuous mode with high PhAC removal and without operational problems for 49 days while treating real wastewater. This result indicates the suitability of this system to treat flocculated hospital effluents under non-sterile conditions.

Physicochemical and biological parameters

Table 1 shows the physicochemical parameters and biological characterization of the hospital wastewater before and after the coagulation-flocculation pretreatment and after the biological treatment. Most of the parameters of the raw HWW are in the same range as those of other hospital effluents (De Oliveira et al., 2017).

After the coagulation-flocculation pretreatment, an important reduction of the absorbance, HPC, TSS and COD was achieved, as previously reported Mir-Tututsaus et al.

372 (2016). A reduction of three orders of magnitude was obtained for the bacterial
373 concentration and almost 80% of reduction in the case of TSS.
374 Additionally, Table 1 shows the characterization of the final effluent after the trickling
375 bed-bioreactor treatment and the control reactor treatment. An increase in the COD
376 concentration was observed in both effluents, probably due to by-products or metabolites
377 that were released during wood degradation by fungus (Palli et al., 2016). As previously
378 reported by Badia-Fabregat et al. (2017), *T. versicolor* cannot remove wastewater COD,
379 and it should be included as a pretreatment process only for PhAC removal.
380 On the other hand, the number of bacterial colonies in the final effluent increase by two
381 orders of magnitude compared with that of the initial flocculated water; however, this value
382 was lower than that of the initial raw water. In addition, the number of bacterial colonies in
383 the fungal and control reactor remained stable at a value of less than 1.10^7 during all
384 treatment.
385 Additionally, the absorbance and TSS values that were obtained in the final effluents did
386 not change after treatment. These parameters, together with the HPC, allow the conclusion
387 that bacterial growth was successfully limited by the operation strategies to be made.
388 The results of the Microtox analysis showed no toxicity (0 TU) in hospital wastewater
389 before and after the treatment, demonstrating that the system does not increase the toxicity
390 of the effluents. Taking into account that hospital wastewater may contain a wide variety of
391 contaminants, with this test it was confirmed that other contaminants were not altered.

392 **4. Conclusions**

393 The immobilization of *T. versicolor* on wood is a good strategy to ensure fungus survival
394 in HWW treatment. Complex pellets with a wood core were formed in a fluidized bed-
395 reactor; these pellets exhibited high PhAC removal efficiencies when they treated

flocculated HWW for 28 days. However, the system was not scalable. A packed bed bioreactor with *T. versicolor* that was immobilized on wood was successfully operated to remove ibuprofen, ketoprofen and naproxen from flocculated HWW for 49 days. Different strategies—immobilization on a lignocelullosic support, controlling the pH and using a coagulation flocculation pretreatment—were combined in this system to result in good removal without operational problems.

Acknowledgements

This work has been funded by the Spanish Ministry of Economy and Competitiveness (project CTM 2013-48545-C2-1-R) and partly supported by the Generalitat de Catalunya (Consolidated Research Group 2014-SGR-0476). The Department of Chemical Engineering of UAB is member of the Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya. J. Torán acknowledges the predoctoral grant from UAB.

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Figure Captions

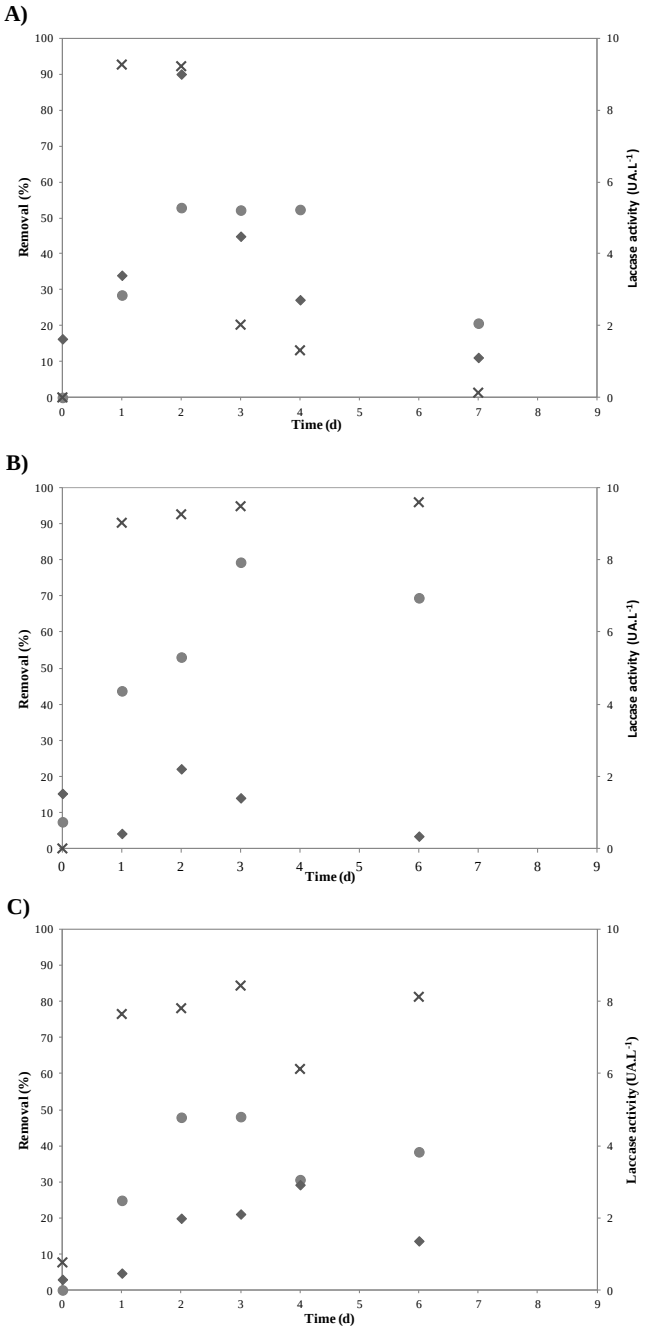
Fig. 1. Fluidized bed bioreactor with pre-grouth culture over wood. 9 days culture over wood with big size (A) and small size (B). 30 days culture over small size wood (C). Removals of ibuprofen (cross) and ketoprofen (circle) with synthetic water under non-sterile conditions. Laccase activity is represented with rhombus.

Fig. 2. Removals of ibuprofen (cross) and ketoprofen (circle) in fluidized bed bioreactor with synthetic water. Treatment with complex pellets obtained in Erlenmeyer flask. Laccase activity profile is represented with rhombus.

Fig. 3. Removals of ibuprofen (cross), ketoprofen (circle) and naproxen (triangle) in fluidized bed bioreactor with synthetic water (A) and HWW (B). Treatment with complex wood pellets (I) and simple pellets as a control (II). Laccase activity profile is represented with rhombus.

Fig. 4. Removals of ibuprofen (cross), ketoprofen (circle) and naproxen (triangle) in pinewood-packed reactor inoculated with *T. versicolor* (I) and non-inoculated as a control (II). Laccase activity profile is represented with rhombus.

627 **Tables and Figures**



628

629 **Fig. 1.** Fluidized bed bioreactor with pre-grouth culture over wood. 9 days culture over

630 wood with big size (A) and small size (B). 30 days culture over small size wood (C).

631 Removals of ibuprofen (cross) and ketoprofen (circle) with synthetic water under non-

632 sterile conditions. Laccase activity is represented with rhombus.

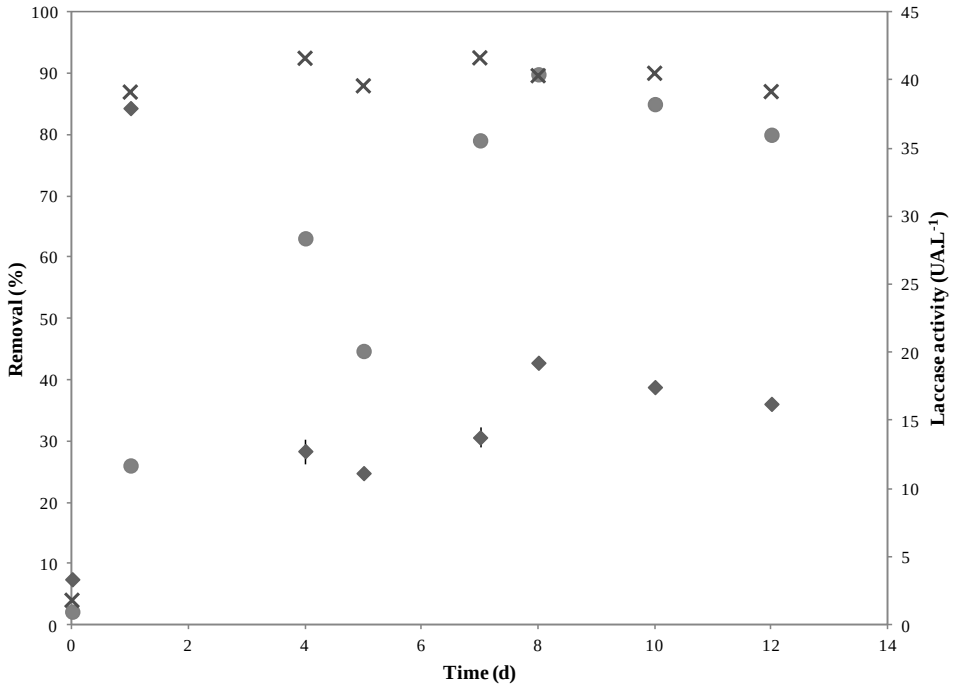
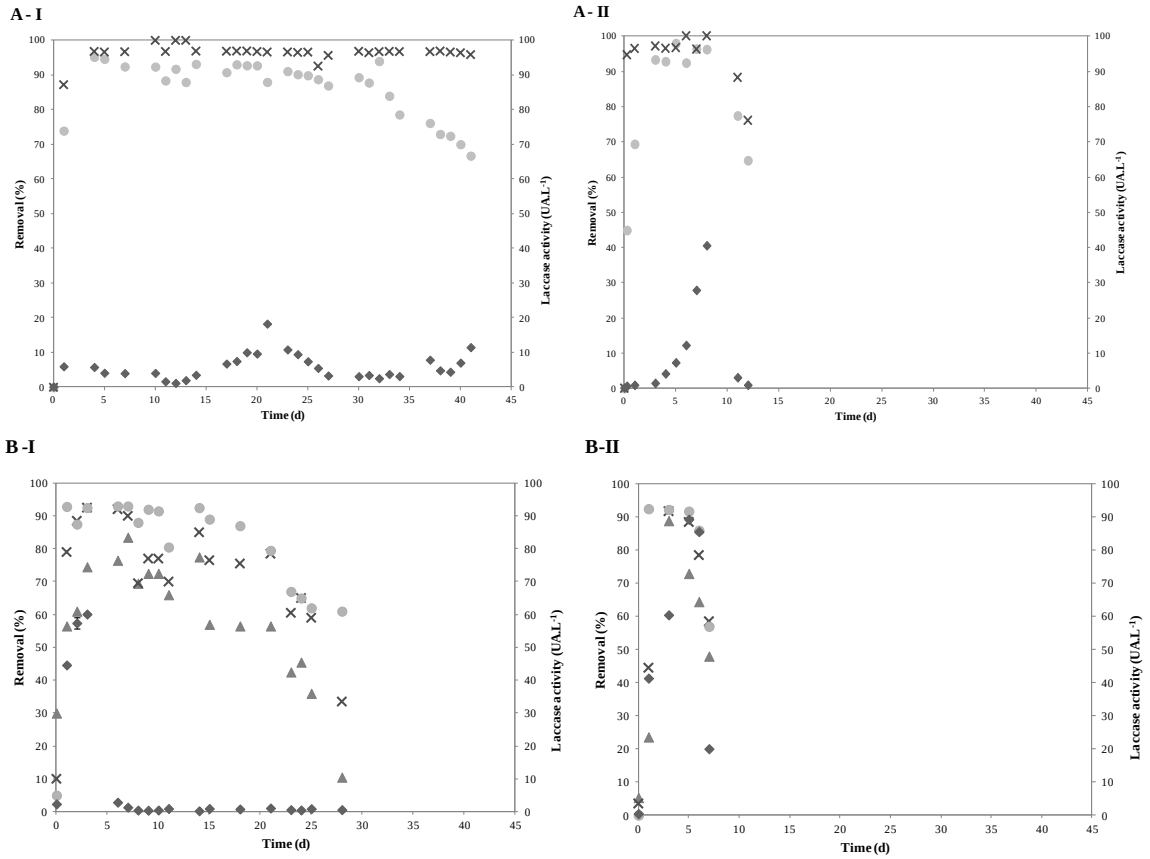


Fig. 2. Removals of ibuprofen (cross) and ketoprofen (circle) in fluidized bed bioreactor with synthetic water. Treatment with complex pellets obtained in Erlenmeyer flask. Laccase activity profile is represented with rhombus.



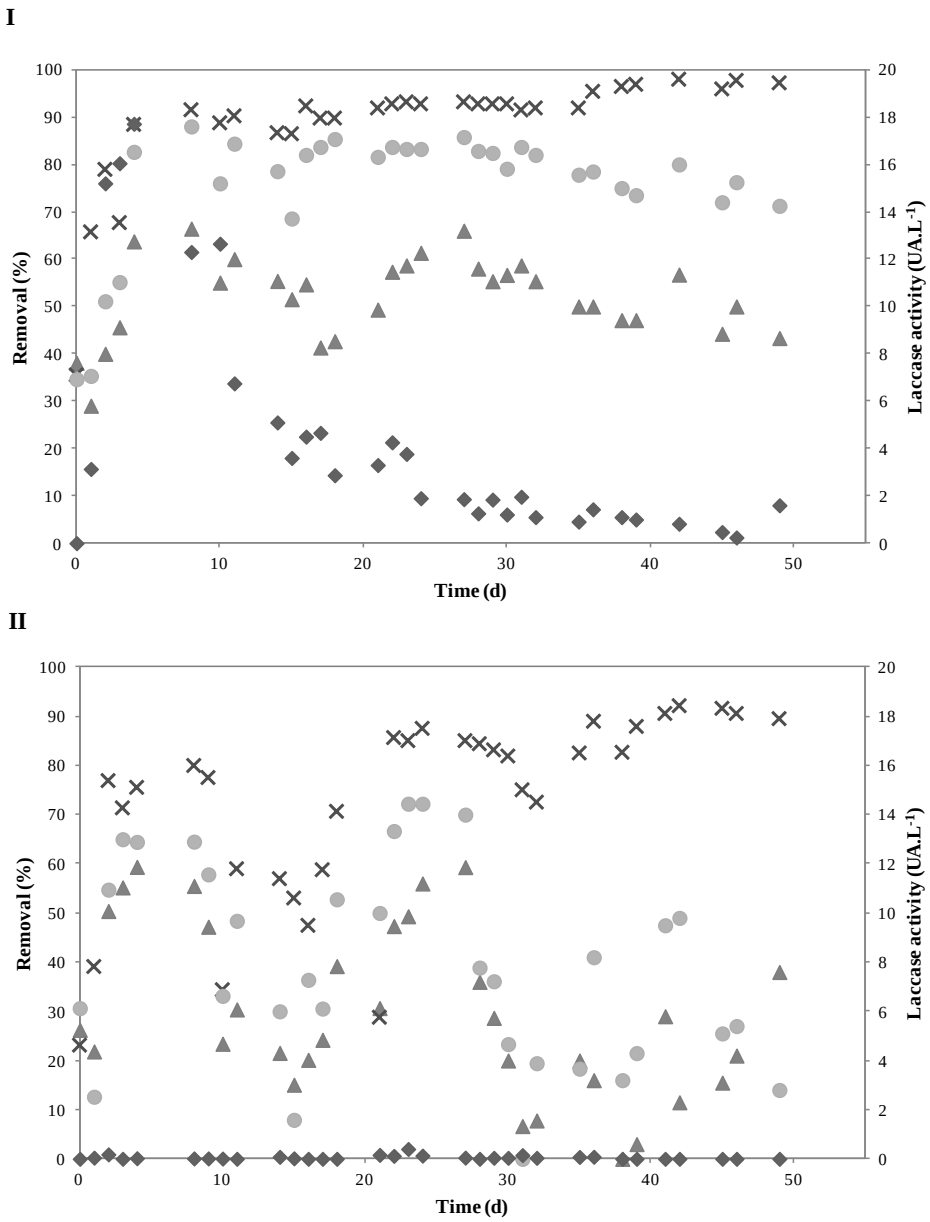
647

648 **Fig. 3.** Removals of ibuprofen (cross), ketoprofen (circle) and naproxen (triangle) in

649 fluidized bed bioreactor with synthetic water (A) and HWW (B). Treatment with complex

650 wood pellets (I) and simple pellets as a control (II). Laccase activity profile is represented

651 with rhombus.



652

653 **Fig. 4.** Removals of ibuprofen (cross), ketoprofen (circle) and naproxen (triangle) in

654 pinewood-packed reactor inoculated with *T. versicolor* (I) and non-inoculated as a control

655 (II). Laccase activity profile is represented with rhombus.

656

657

Table 1. Physicochemical characterization of raw HWW, flocculated HWW, final effluent from tricked bed bioreactor and control reactor.

	Raw HWW	Flocculated	Reactor	Control
Absorvance at 650 nm	0.3	0.009	0.019	0.015
Heterotrophic plate count ($CFU \cdot mL^{-1}$)	$5 \cdot 10^7 \pm 3 \cdot 10^7$	$3.5 \cdot 10^4 \pm 1.2 \cdot 10^4$	$6 \cdot 10^6 \pm 1 \cdot 10^6$	$5 \cdot 10^6 \pm 1 \cdot 10^6$
TSS ($mg \cdot L^{-1}$)	122	22	29.57	36.9
COD ($mg\ O_2 \cdot L^{-1}$)	178	109	351	273