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ORIGINAL PAPER

Biotransformation of three pharmaceutical active compounds by the fungus *Phanerochaete chrysosporium* in a fed batch stirred reactor under air and oxygen supply

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Abstract White-rot fungi are a group of microorganisms capable of degrading xenobiotic compounds, such as polycyclic aromatic hydrocarbons or synthetic dyes, by means of the action of extracellular oxidative enzymes secreted during secondary metabolism. In this study, the transformation of three anti-inflammatory drugs: diclofenac, ibuprofen and naproxen were carried out by pellets of *Phanerochaete chrysosporium* in fed-batch bioreactors operating under continuous air supply or periodic pulsation of oxygen. The performance of the fungal reactors was steady over a 30-day treatment and the effect of oxygen pulses on the pellet morphology was evidenced. Complete elimination of diclofenac was achieved in the aerated and the oxygenated reactors, even with a fast oxidation rate in the presence of oxygen (77% after 2 h), reaching a total removal after 23 h. In the case of ibuprofen, this compound was completely oxidized under air and oxygen supply. Finally, naproxen was oxidized in the range of 77 up to 99% under both aeration conditions. These findings demonstrate that the oxidative capability of this microorganism for the anti-inflammatory drugs is not restricted to an oxygen environment, as generally accepted, since the fungal reactor was able to remove these compounds under aerated and oxygenated conditions. This result is very interesting in terms of developing viable reactors for the oxidation of target compounds as the cost of aeration can be significantly reduced.

Keywords Pharmaceutical · White-rot fungi (WRF) · Degradation · Diclofenac · Ibuprofen · Naproxen

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Introduction

Pharmaceutical compounds have been detected in natural water courses in a variable concentration range (Clara et al. 2005; Vogna et al. 2004; Suárez et al. 2008). Among the different classes of pharmaceuticals, some of the most commonly used worldwide are anti-inflammatory drugs, which possess analgesic, antipyretic and anti-inflammatory effects by inhibiting the synthesis of prostaglandin (Ikehata et al. 2006). It is important to highlight the potential impact of the release of pharmaceuticals into the environment, since these compounds are designed to affect biochemical and physiological functions of humans and animals. Moreover, this type of compounds may cause increased aquatic toxicity and endocrine disruption (Ikehata et al. 2006; Jjemba

2010). However, the real effect on the environment will depend on their concentration as well as other physicochemical factors such as adsorption coefficients, time of exposure, bioaccumulation and persistence (Esplugas et al. 2007).

There are three types of potential technologies for the degradation of pharmaceutical compounds in wastewater streams: conventional procedures (i.e., physical–chemical, activated sludge, nitrification–denitrification), advanced procedures (UV, ozone, anammox reactors) and finally, processes under development based on the application of white-rot fungi and their oxidative enzymes. In terms of overall efficiency, conventional treatments such as coagulation–flocculation and flotation, activated sludge and nitrification–denitrification attained variable degradation yields (from 10 to 70%) for the anti-inflammatory compounds (Carballa et al. 2004; Kosjek et al. 2007; Suárez et al. 2005). In the case of advanced technologies, higher removal efficiencies for anti-inflammatory compounds were achieved by using ozone (up to 100% for naproxen, NPX) or by a photo-Fenton system (80–100% for ibuprofen, IBP). However, other pharmaceutical compounds presented intermediate elimination percentages (50–70%) (Gagnon et al. 2008; Méndez-Arriaga et al. 2010). Emerging technologies, such as those based on the use of WRF or the ligninolytic enzymes have attained promising results, with degradation yields close to 100% for diclofenac (DCF) as pointed out by Lloret et al. (2010) and Marco-Urrea et al. (2010a). These degradation systems were based on the use of laccase as the biocatalyst, endogenously produced in batch fungal cultures for the in vivo degradation or exogenously added in combination with a suitable mediator, 1-hydroxibenzotriazole (HBT) (Cañas and Camarero 2010).

In the specific case of anti-inflammatory drugs, Marco-Urrea et al. (2010a) reported the degradation of DCF by the WRF *Trametes versicolor* and Rodríguez-Rodríguez et al. (2010) demonstrated the degradation of NPX by this fungus. The use of ligninolytic enzymes is largely based on laccase with the presence of mediators such as HBT (Cañas and Camarero 2010; Hata et al. 2010; Lloret et al. 2010). Although the in vitro application of oxidative enzymes is feasible, provided that cofactors and mediators are present, its use is beset by significant operational barriers: reusability, cost and denaturation of the enzyme (Cabana et al. 2007). Moreover, the requirement of the mediator implies a potential environmental impact because laccase mediators have been reported to be toxic or scarcely biodegradable (Cañas and Camarero 2010).

This research work presents a degradation approach based on the use of fungal reactors. Specifically, fed-batch stirred tank reactors with free pellets of the WRF *Phanerochaete chrysosporium* were considered for the degradation of three anti-inflammatory drugs: DCF, IBP and NPX. The activation of the ligninolytic system of this fungus has been reported to require high levels of oxygen concentration to promote the ligninolytic enzyme production (Moreira et al. 1996; Jiménez-Tobón et al. 1997; Rothschild et al. 1999). Additionally, it has been demonstrated that the pulsation of oxygen controlled the growth of the pellets and their shape (Moreira et al. 1996). Therefore, the bioreactors were operated under two aeration regimes: air or oxygen supply. The fungus selected produces two ligninolytic enzymes: lignin peroxidase (LiP) and manganese peroxidase (MnP). However, the operational conditions of the reactor used in this study were set to favor the extracellular production of the enzyme MnP and also to observe the effect of oxygen pulses on the morphology of *P. chrysosporium* pellets. Specifically, the fungal reactors were operated in fed-batch regime, with the controlled addition of glucose and anti-inflammatories. This operational strategy was planned to maintain the fungal culture active and to avoid overflow metabolism (formation of side metabolites) and nutrient deprivation.

Materials and methods

Chemicals

A set of pharmaceutical compounds were used in this study: DFC, IBP and NPX, all of them purchased from Sigma-Aldrich, pure grade. A stock solution of DCF, IBP and NPX (2,000 mg/l each) was prepared using methanol as the selected solvent. The following solvents were used in the extractions and for the preparation of the stock solution: acetone (J.T. Baker, 99.5%), ethyl acetate (J.T. Baker, 99.5%), acetonitrile (J.T. Baker, 99.8%), methanol (J.T. Baker, HPLC grade, 99.8% and Panreac, 99.5%) and *n*-hexane (J.T. Baker, 95%).

Microorganisms and inoculum preparation

The white rot fungus used was *P. chrysosporium* (ATTC 24725). This strain was previously grown in plates with agar (15 g/l), glucose (10 g/l) and malt extract (3.5 g/l) and then three agar plugs with active fungus were transferred to Fernbach flasks containing 100 ml of modified Kirk medium (Tien and Kirk 1988) and incubated for 5 days at 30°C. Thereafter, the grown mycelium was used as inoculum for Erlenmeyer flasks (250 ml) containing 90 ml of modified Kirk medium and 9 ml of homogenized mycelium. The flasks were cultured at 30°C and 150 rpm in an orbital shaker (C24 Incubator Shaker, New Brunswick Scientific, USA). After 5 days, pellets from five flasks were withdrawn by filtration and used as inoculum for the fed-batch reactor in a concentration of 1.2 g/l.

Operation of the stirred tank reactor with air and oxygen for the pharmaceuticals degradation

Experiments were conducted to study the degradation of DCF, IBP and NPX in a 2 l fermenter Biostat Bplus (Sartorius, Melsungen, Germany) stirred at 200 rpm and 30°C. The vessel was filled with 1.5 l of modified Kirk medium (pH 4.5) and fungal pellets. The hydraulic retention time (HRT) was 24 h, since in previous batch assays it was demonstrated that these pellets were able to eliminate more than 90% of the three anti-inflammatory drugs in less than 24 h (data not shown). Pulses of glucose and pharmaceuticals were added sequentially every 3–4 days in the aerated and oxygenated reactors. Daily additions of anti-inflammatory drugs were added during the 3rd week of the oxygenated assay. Dissolved oxygen concentration and pH were continuously monitored by pO_2 and pH electrodes and data were processed by software MFCS/DA 3.0 (Module operator service program, Sartorius sedimentation Systems, Germany). In the aerated reactor, the vessel was continuously aerated for 30 days with a variable air flow to maintain the dissolved oxygen concentration as high as possible. In the oxygenated reactor, an electrovalve located at the end of a flexible membrane tube (FMT) controlled by a cyclic timer was used to inject oxygen with a pulsing flow. The pulsing frequency is defined as the inverse of the sum of the opening and shutting times of the electrovalve (Moreira et al. 1996). Different pulsing frequencies were assayed to maintain the maximum concentration of dissolved oxygen (Table 1).

Table 1 Pulsing frequencies of the bioreactor operated with oxygen supply

Day	Opening time (min)	Shutting time (min)	Pulsing frequency (min^{-1})[a]
0–2	1.5	15	0.0606
2–6	1	15	0.0625
7–15	1	60	0.0164
15–20	1.5	60	0.0163
20–21	2	45	0.0213
21–30	1.5	60	0.0163

[a] Pulsing frequency $= 1/(t_o + t_s)$, where t_o corresponds to the opening time and t_s is the shutting time

Extraction of pharmaceuticals and gas chromatography-mass spectrometry (GC-MS) analysis

Samples of 10 ml were withdrawn during the bioreactor operation and 10 ml of acetonitrile were subsequently added for the extraction of pharmaceuticals. Flasks were sealed with Teflon and agitated in a shaker at 180 rpm for 2 h (Ika Labortechnik, HS 501 Digital, Germany). From the supernatant of each sample, a volume of 10 ml was withdrawn and placed in glass tubes sealed with Teflon and centrifuged at 7,000 rpm for 15 min. An aliquot of 4 ml was taken from each tube, diluted in 100 ml water and then extracted with 60 mg OASIS HLB cartridges (Waters closet, Milford, MA, USA). The cartridges were then dried with a nitrogen stream for 45 min and eluted with 3 ml of ethyl acetate. To determine the soluble fraction of DCF, IBP and NPX by GC-MS (Saturn 2100T, Varian, USA), an aliquot of 0.8 ml of the extract was taken and 200 µl of MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide) were added for the derivatization of the anti-inflammatory species (Reddersen and Heberer 2003; Rodríguez et al. 2003).

At the end of the operation of the aerated and the oxygenated reactors, a final extraction was carried out to determine the concentration of anti-inflammatory drugs that could be adsorbed on the biomass and also on the glass surface of the reactor vessel. For this purpose, the biomass was taken and blended in order

to homogenize the sample. Then, 50 ml of this blended mycelium was placed in an Erlenmeyer flask and 50 ml of acetonitrile were added. The samples were agitated for 2 h at 150 rpm. Thereafter, 4 ml were withdrawn and diluted in 100 ml of distilled water and then the solid phase extraction (SPE) was carried out to prepare the samples for the determination of the residual concentration of anti-inflammatory drugs by GC-MS as described above. Once the reactor was emptied, the vessel was washed with acetonitrile and aliquots of 4 ml were taken to determine the concentration of anti-inflammatory drugs by GC-MS.

Analytical techniques

Glucose concentration was analyzed with the dinitrosalicylic acid method using D-glucose as a standard (Miller 1959). Total organic carbon (TOC) concentration was measured in a Shimadzu analyzer (TOC 5000). Peroxide concentration was measured by a colorimetric method with test strips (Merckoquant, Merck, Germany). Enzymatic activity was measured by the oxidation of dimethoxyphenol (Field et al. 1992). Biomass concentration was determined as dry weight with 0.45 μm pore-size filters. Finally, the morphology and size of the pellets were measured by using image analysis with a stereomicroscope (Stemi 2000-C, Zeiss, Germany) with a digital camera (Coolsnap, Roper Scientific Photometrics, Germany). The images obtained were analyzed with the Image Pro Plus software (Media Cybernetics, Inc, USA).

Results

Evolution of key operational parameters in the free pellets reactor operated with air or oxygen supply

The time courses of the operation of the aerated and oxygenated reactors are presented in Fig. 1. The concentration of dissolved oxygen in the air reactor was variable, between 2 and 7 mg/l. On the other hand, the concentration of dissolved oxygen in the oxygen reactor was maintained between 22 and 29 mg/l; except for the period between days 3 and 6, when this concentration decreased to a range of

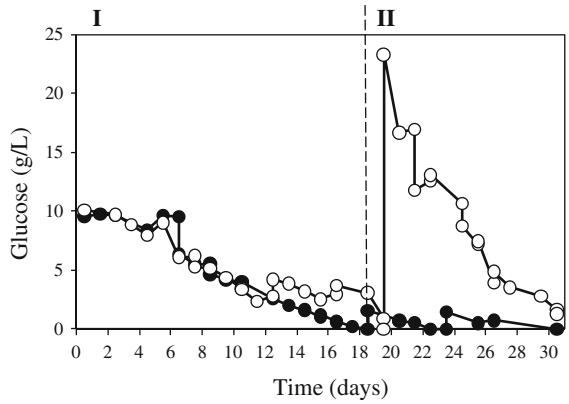


Fig. 1 Glucose concentration during the aerated (*open circle*) and oxygenated (*closed circle*) assays

7–8 mg/l due to a change to air supply (data not shown).

Regarding glucose depletion, glucose was added at the beginning of both experiments (10 g/l). It was depleted with similar consumption rates in the aerated and oxygenated reactors (0.5 g/l·day) (Fig. 1, stage I). During the second period of the aerated reactor, a pulse of 20 g/l of glucose was added ensuing into a high consumption rate of glucose: 1.7 g/l·day. Consequently, a high concentration of biomass was measured at the end of the experiment (6.5 g/l). This excessive growth of the fungal mycelium caused an evident increase of the broth viscosity. It is important to highlight that this assay was carried out until day 50. Nevertheless, for comparison purposes with the oxygenated reactor, data were depicted only until day 30. During the second stage of the oxygen reactor, two pulses of glucose ($\sim$2 g/l) were added at days 18 and 23, which led to a limited consumption of glucose: 0.3 g/l·day and restricted biomass growth (1.5 g/l) after 30 days of operation (Fig. 1, stage II). Thus, glucose consumption per gram of biomass was 0.26 g glucose/g biomass per day for the aerated reactor; while in the oxygenated reactor was 0.20 g glucose/g biomass per day. Similar trends were also found for the TOC, starting at a concentration of 4 g/l and decreasing up to values of 1.4 g/l. The addition of glucose in pulses reached maximum values of 10.5 and 2.6 g/l for TOC in the aerated and oxygenated reactor, respectively (data not shown). Finally, both peroxide concentration and MnP activity were detected at low levels, between 0.5 and 5 mg/l for H_2O_2 and 5–40 U/l for MnP (data not shown).

Morphological changes in pellets in the air and oxygen reactors

Figure 2 shows the photographs corresponding to the pellets from the aerated and oxygenated reactors at days 0, 7, 15 and 30. As expected, the pellet diameters from both reactors were similar at the beginning of both experiments: 2.8 mm. However, shortly after day 7, the size of the oxygenated pellet was smaller than the aerated fungus (2.3-fold approx.) and the pellet was dense and hyphae-free while the one of the aerated reactor presented filamentous growth. The aerated reactor, in the following days presented excessive growth of the fungus, which led to a series consequences: formation of hyphal branches and aggregates of pellets and their subsequent breakdown into fragments, which led to the release of free mycelia into the medium and the increase of the broth viscosity. New pellets of smaller size were formed from the free branches of mycelia. For the case of the oxygenated reactor, a large fraction of free pellets showed a constant size (2.8 mm), similar to that of the beginning of the assay, with a compact and spherical appearance, while a minor fraction started to form aggregates.

Biotransformation of anti-inflammatory drugs

During the operation of the aerated reactor, eight pulses of pharmaceuticals were added throughout the experiment. In the case of DCF, transformation percentages in the range of 65–99% were obtained (Fig. 3). A similar behavior was observed in the oxygenated reactor, where a considerable reduction of the initial concentration up to 93% was obtained after the addition of four pulses of pharmaceuticals during the first 2 weeks of the experiment. During the period of daily pulses of pharmaceuticals, DCF was transformed by 94% after a short period, between 6 and 18 h. Finally, during the last week of assay, this compound was totally degraded after each pulse (Fig. 3). Regarding IBP, a complete elimination was achieved in both reactors (Fig. 4). For the case of NPX, transformation percentages between 78 and 99% during the aerated assay were obtained. Similar results were obtained in the oxygenated reactor, where after the first 3 weeks of experiment, transformations up to 82% were achieved (Fig. 5); however, during the last week of incubation, an increase of this percentage was observed (up to 97%).

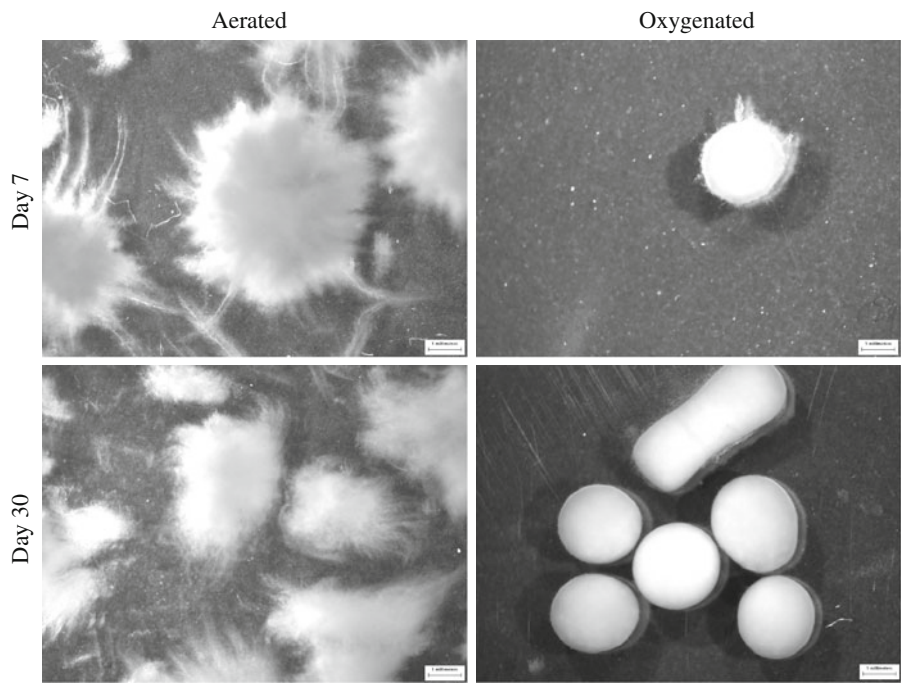


Fig. 2 Pellet morphology by stereomicroscope during the aerated and oxygenated assay

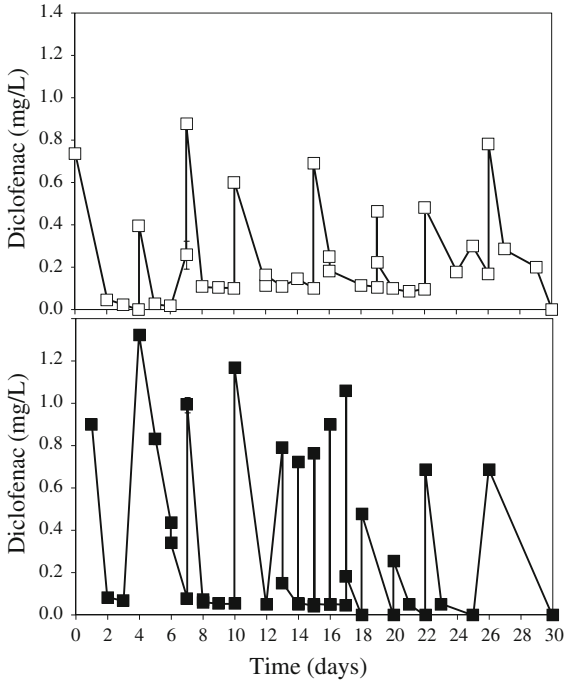


Fig. 3 Degradation of DCF in presence of air and oxygen. Symbols (*open square*) aerated assay (*closed square*) oxygenated assay

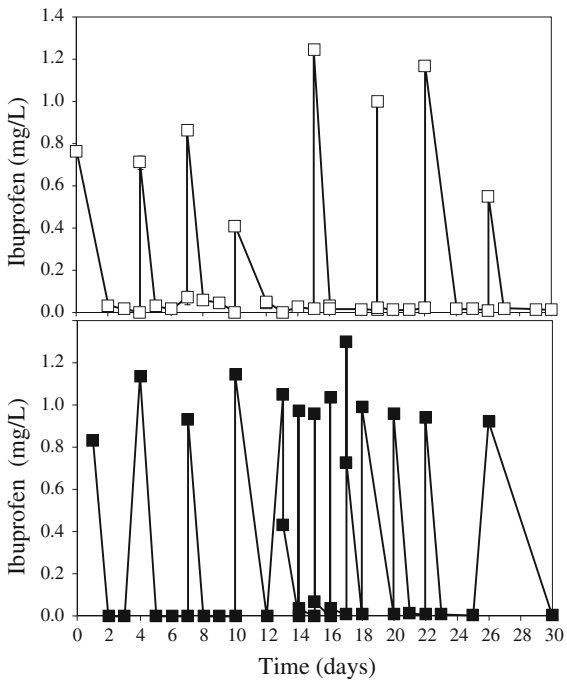


Fig. 4 Degradation of IBP in presence of air and oxygen. Symbols (*open square*) aerated assay, (*closed square*) oxygenated assay

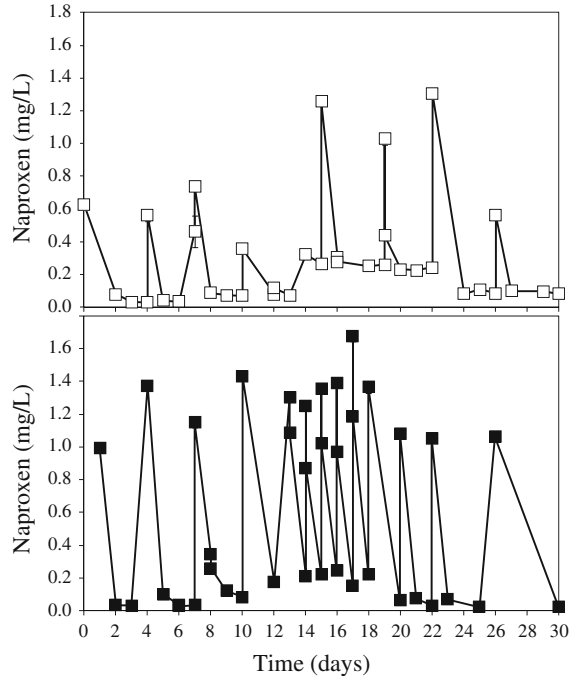


Fig. 5 Degradation of NPX in presence of air and oxygen. Symbols (*open square*) aerated assay, (*closed square*) oxygenated assay

In Fig. 6, a summary of the degradation kinetics obtained during the third week of the oxygenated assay can be observed. DCF was easily transformed in the short term, 77% after only 2 h while in the longer term, total elimination was observed after 23 h. In the case of IBP, this compound was transformed only by 30% after 2 h and completely transformed after 15 h. Finally, for NPX,

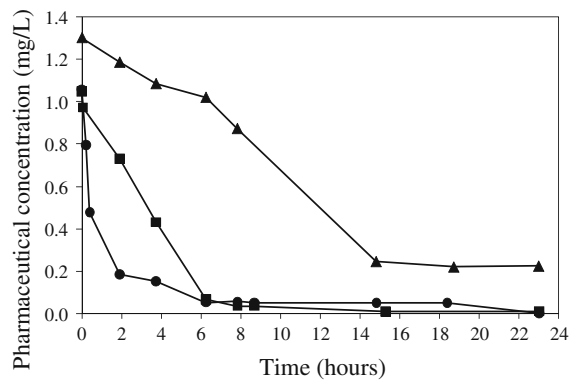


Fig. 6 Time-course of the degradation of DCF (*circle*), IBP (*square*) and NPX (*triangle*) during the third week of the oxygenated assay

Table 2 Residual concentration of anti-inflammatory drugs onto biomass and reactor vessel

	Anti-inflammatory concentration	
	Biomass (mg drug/g)	Vessel (mg drug/cm^2)
Aerated reactor		
DCF	0.011	1.0E−5
IBP	0.001	7.2E−7
NPX	0.005	6.5E−6
Oxygenated reactor		
DCF	nd	nd
IBP	nd	nd
NPX	0.017	8.8E−6

nd not detected

transformation percentages of 9 and 83% were achieved after 2 and 23 h, respectively. Regarding the residual concentration of the anti-inflammatory drugs adsorbed onto the biomass, Table 2 shows the results of the final extraction. Low concentrations of the three anti-inflammatory drugs were detected in the biomass and almost undetectable amounts were found in the reactor vessel.

Discussion

Physicochemical and biological treatments have been considered for the elimination of pharmaceutical compounds in the environment. For example, coagulation–flocculation and flotation processes have reached eliminations between 20 and 70% for DCF, IBP and NPX (Carballa et al. 2005); 80% for DCF, 40% for IBP and up to 90% for NPX in sewage treatment plants (STPs) (Carballa et al. 2004; Kosjek et al. 2007;); in the case of nitrifying–denitrifying biological reactors removal percentages up to 70% for IBP and NPX have been achieved while irrelevant (10%) rates have been identified for DCF (Suárez et al. 2005); membrane bioreactors (MBR) attained nearly complete degradation for IBP and NPX and variable degradation for DCF, in the range of 10–45% (Clara et al. 2005; Reif et al. 2008); and significant degradation for IBP and NPX was observed in extended sludge age bioreactors (Tsung-Hsien et al. 2009). Advanced oxidation processes (AOPs) based on the use of ozone (Gagnon et al. 2008), UV/H_2O_2 system (Vogna et al. 2004), photolysis and Fenton-type process (Ikehata et al. 2006) have also been considered as potential options with efficiencies up to 100% for NPX (ozone) and 80–100% for IBP (photo-Fenton system).

On-going research alternatives rely on the use of WRF, which have the ability to oxidize a wide range of organic pollutants such as synthetic dyes, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pharmaceutical and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) (Field et al. 1992; Ikehata et al. 2006; Wesenberg et al. 2003; Zeddel et al. 1993). This ability is related to the secretion of oxidative enzymes: LiP, MnP, versatile peroxidase (VP) and laccase (Lac) (Bao et al. 1984; Call and Mücke 1997; Tien and Kirk 1984). Different fungi (*T. versicolor*, *Phanerochaete sordida*, *P. chrysosporium*) have been used for the elimination of anti-inflammatory drugs (Marco-Urrea et al. 2009, 2010a; Rodríguez-Rodríguez et al. 2010). In the case of ligninolytic enzymes, recently Zhang and Geissen (2010) have carried out the degradation of DCF by LiP and they found that this compound could be totally eliminated only after 2 h. Also, Lloret et al. (2010) demonstrated a complete removal of DCF after 1 h using Lac-HBT mediator system.

In this study, a fungal reactor used for the elimination of DCF, IBP and NPX was developed and the results obtained showed that the fungus was able to eliminate these compounds under different aeration conditions during a prolonged operational period. Our approach consists of a straightforward system, based on the use of a wild fungal strain in a simple reactor configuration. The fungal reactor presented high capacity for the degradation of the target compounds and proved to be stable during the operation. Besides the fact that the pharmaceuticals were largely degraded after a short period of time, other relevant observations of this process include low energy requirements; low cost for aeration; and low consumption of chemical reagents (only glucose was added to maintain the culture active).

Concerning the specific data for DCF, transformations reaching 65% during the aerated assay and up to 93% during the oxygenated assay were achieved. In addition, *P. chrysosporium* was able to transform this compound to a significant extent after only 2 h (77%) (Fig. 6). Previous investigations have shown that this

compound can be degraded by 80% in STPs; nevertheless, these degradations were mainly in the range of 21–40% (Zhang et al. 2008). These results are higher than the elimination efficiencies achieved in a nitrifying–denitrifying plant where less than 10% of DCF was removed after 24 h (Suárez et al. 2005). Additionally, the use of MBR demonstrated an inefficient elimination of DCF after 12 h (Reif et al. 2008). Moreover, our results are similar to those obtained with Lac-producer fungus or in vitro degradation by Lac or lignin peroxidase (Lloret et al. 2010; Marco-Urrea et al. 2010a; Zhang and Geissen 2010).

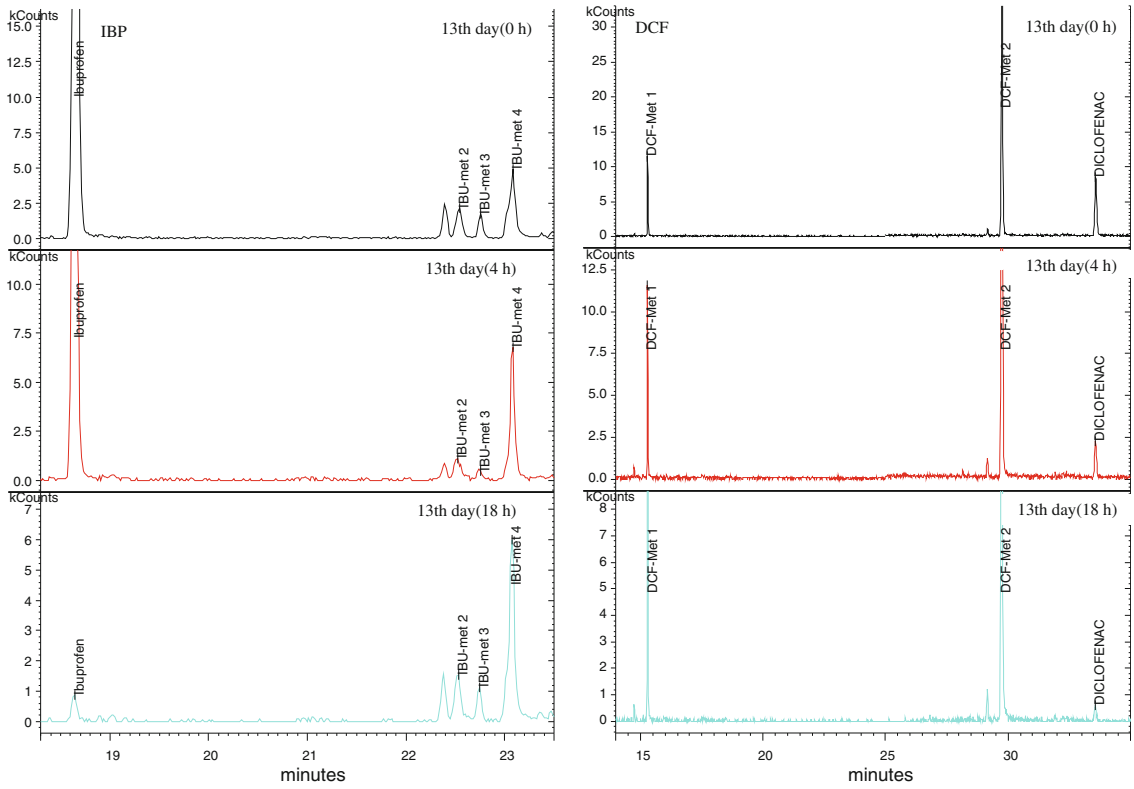
Regarding IBP elimination, several reports indicated that physicochemical processes such as coagulation–flocculation and flotation were inefficient for its elimination, achieving yields of 25% (Carballa et al. 2004), while biological processes attained significant levels of degradation: 70% in a STPs (Carballa et al. 2005), 82% in a nitrifying–denitrifying plant (Suárez et al. 2005) and 90% in a MBR (Clara et al. 2005; Reif et al. 2008). The use of AOPs such as photo-Fenton has been successful with removal percentages up to 80% (Méndez-Arriaga et al. 2010), while the use of ozone has only attained relative success: only 58% (Gagnon et al. 2008). In this study, the elimination yield for IBP was larger than that previously reported and the fungal reactor attained fast transformations for this compound with levels as high as 99% throughout the experiment.

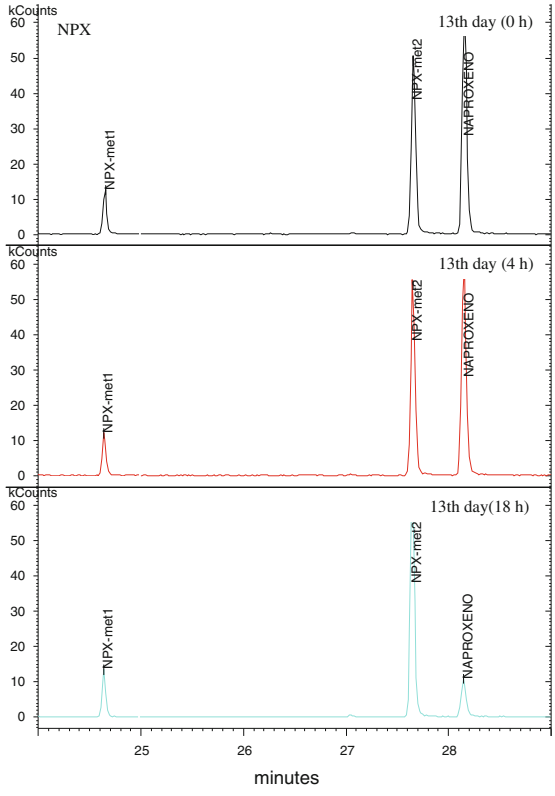
Regarding NPX, high transformation percentages close to 90% were obtained in a pilot wastewater treatment plant (Kosjek et al. 2007) and in a MBR (Reif et al. 2008). The use of AOPs for this compound is very efficient (Gagnon et al. 2008), much better than coagulation–flocculation and flotation processes: 30–42% (Carballa et al. 2004). Related to the use of WRF, Rodríguez-Rodríguez et al. (2010) used bio-slurry and solid phase systems with *T. versicolor* fungus for the elimination of NPX and the results showed elimination percentages in the range of 47–99% after 3 days of incubation. In the current study, this compound was transformed to a large extent during the operation of both reactors; nonetheless, the monitoring of NPX showed that this compound required a longer period to be degraded, a feature that should be taken into account when selecting the bioreactors' residence time. As can be seen in Fig. 5, a slight accumulation of this compound takes place between days 12 and 22 in the aerated and oxygenated reactor. There is no clear evidence that explains this fact, but it may be due to a decline in the glucose consumption observed in this period resulting from a slowdown in the fungus metabolism. Moreover, it may also be attributed to an accumulation of either NPX or its degradation products, since Lindqvist et al. (2005) have reported higher concentration of this compound in effluents of STPs than in the influents, which is attributed to a possible hydrolysis of the metabolites, forming the parent compound.

Fig. 7 GC-MS chromatograms obtained from the degradation ▶ of IBP, DCF and NPX

On the one hand, concerning the absorption of anti-inflammatory drugs, the three compounds were detected in the biomass of the aerated reactor in concentrations below 0.011 mg of drug per gram of biomass. On the other hand, concentrations of NPX in the oxygenated reactor were in the order of 0.017 mg of drug per gram of biomass. Finally, almost undetectable concentrations of these compounds were detected in the reactor vessel. Pharmaceutical concentrations in the biomass were similar for both types of pellets, but DCF and IBP were not detected in oxygenated pellets since aerated pellets posse a larger area where the drugs can be adsorbed. In general, anti-inflammatory drugs are negatively charged molecules that will not adsorb, due to their liphophilic character (K_{OW}) and acid dissociation constant (pKa) (Suárez et al. 2008).

It is important to note that the differences between pellets from the aerated and oxygenated reactor depend on several factors, being one of them the aeration system: continuous air flow and oxygen pulses. Fungal growth under high oxygen level conditions controls the pellets morphology and enzyme production and was found to be optimal for lignin degradation (Moreira et al. 1996; Rothschild et al. 1999; Miura et al. 2004). Other authors found that oxygen pulses control the size of the pellets and its morphology. For instance, Moreira et al. (1996) obtained spherical shape and hyphae-free pellets when applying oxygen pulsations, favouring in this way fungal morphology. Otherwise, when a continuous air flow was used an excessive fungus growth was observed. The pellets tend to breakdown followed by the release of mycelium fragments on the


IBP
13th day(0 h)
Ibuprofen
TBU-met 2
TBU-met 3
TBU-met 4
13th day(4 h)
13th day(18 h)
Tbuprofen
IBU-met 4
kCounts
minutes
DCF
DCF-Met 1
DCF-Met 2
DICLOFENAC


NPX
13th day (0 h)
NPX-met1
NPX-met2
NAPROXENO
13th day (4 h)
13th day(18 h)
kCounts
minutes

medium which in turn forms aggregates with a final increase in the viscosity in the reactor. In accordance with this, the results of this research showed that the application of oxygen pulses controls the excessive growth of pellets of *P. chrysosporium* (Fig. 2).

With respect to the enzymatic activity, throughout this assay only low levels of the MnP were detected 40 U/l (data not shown). Despite this, it was possible to eliminate the pharmaceutical compounds during aerated and oxygenated assays. This behavior was also observed in previous batch experiments in which the elimination of these compounds was carried out in static fungal cultures with low levels of the enzyme MnP (20 U/l) (Rodarte-Morales et al. 2011). Other authors carried out the in vivo elimination of these anti-inflammatory drugs by *T. versicolor* and *P. sordida* with low levels of the enzyme MnP (5–48 U/l) achieving high percentages of removal. They suggest that the degradation by fungal cultures could take place intracellularly by the action of cytochrome P450 system (Hata et al. 2010; Marco-Urrea et al. 2009, 2010a). In a recent study by Eibes et al. (2011) the in vitro elimination of several compounds with low and high doses of the enzyme VP was carried out. They achieved a complete elimination of DCF after 25 min using only 10 U/l of enzyme. Additionally, using 200 U/l of VP a removal of 80% of NPX was observed after 7 h. These results demonstrate that low ligninolytic enzyme activities may be enough to carry out the degradation of anti-inflammatory drugs.

Residual pharmaceuticals and their metabolites were detected by GC-MS analysis. Figure 7 shows the chromatograms of a sample obtained just at time 0, 4 and 18 h. It is noteworthy to mention that four drug pulses were added before the 3rd week of assay. This may explain the presence of peaks that could correspond to possible metabolites and/or degradation products. In the case of IBP, the peak corresponding to the parental compound decreases while other peaks appear; these compounds were named as metabolites 2, 3, 4 and 5. In the case of DCF, the presence of two metabolite peaks was detected; both of them were observed at time 0. Finally, during the degradation of NPX the presence of two peaks was constant during the 18 h of assay. The metabolite peaks detected at the addition time could be due to residual metabolites from the previous pulses of anti-inflammatory drugs. It is important to mention that the quantification of the metabolites detected was not possible, since the authentic compounds of these metabolites were not available. Hence, the estimation was carried out from the relative area of the metabolite compound after 18 h of the addition (t18), and the relative area of the parental compound at time zero (t0). In Table 3, the calculated relative area of these compounds is shown. The identification of anti-inflammatory drug metabolites via fungal transformation is a novel research line and scarce investigations are currently found in literature. For example, Marco-Urrea et al. (2009) carried out the identification of 1,2-hydroxy ibuprofen as the major metabolite of IBP degradation by the fungus *T. versicolor* after 7 days of incubation. Compared to diclofenac, the results in the identification of its degradation products match two previous studies (Hata et al. 2010; Marco-Urrea et al. 2010a) which, managed to identify 4-hydroxy diclofenac as the major metabolite and small amounts of 5-hydroxy diclofenac and 4,5-hydroxy diclofenac. Finally, the degradation products of the anti-inflammatory NPX were identified by Marco-Urrea et al. (2010b) using the fungus *T. versicolor*. Results showed the presence of the metabolite 6-desmethyl, a derivate of NPX. Moreover, they also conducted an in vitro assay with the so-called Lac-mediator system enabling the identification of the metabolite 1-(6-methoxynaphthalen-2-yl) ethanone. The mineralization of this compound was also analyzed after 6 h of incubation, since it was not possible to detect neither NPX nor its metabolites. Finally, Marco-Urrea et al. (2010b) carried out a toxicity assessment of NPX and its metabolites, finding that only the parental compound could be toxic, but not its metabolites. The elimination of three anti-inflammatory drugs by the fungus *P. chrysosporium* was proven in this research study.

Table 3 Relative area of the metabolites of the anti-inflammatory drugs

	Relative area (t18)/(t0)		
	IBP	DCF	NPX
Met 1	–	0.45	0.16
Met 2	0.03	2.68	0.98
Met 3	0.03	–	–
Met 4	0.02	–	–
Met 5	1.22	–	–

Nevertheless, the identification of its metabolites was not possible. It is important to highlight that the presence of several anti-inflammatory metabolites was identified when examining the chromatograms obtained in the study. However, in order to carry out a broader identification of these compounds and the analysis of their toxicity, further research is necessary.

Conclusions

According to the obtained results, the addition of oxygen pulses that can control the growth of fungal biomass, thus facilitating the operation of the reactor with high elimination percentages for the pharmaceutical compounds (80–99%), was demonstrated. Meanwhile, the aerated reactor presented excessive growth, with elimination rates of the three anti-inflammatories in the range of 65–99%. In further detail, IBP was the pharmaceutical compound that showed the highest elimination rates in a bioreactor with free pellets of *P. chrysosporium* in presence of continuous air flow and oxygen pulses. Elimination of NPX was similar in the aerated and oxygenated reactor, showing a slight accumulation of the compound during the 3rd week of operation. Finally, the elimination percentages of DCF are higher in presence of oxygen pulses than in presence of continuous air flow. Therefore, the elimination of the three evaluated anti-inflammatory drugs by the fungus *P. chrysosporium* was demonstrated. Additionally, the presence of several anti-inflammatory metabolites was demonstrated; however, to carry out a thorough identification and quantification of these compounds a broader investigation is necessary. These findings prove that the oxidative capability of this microorganism for the anti-inflammatory drugs is not restricted to an oxygen environment, as generally accepted, since the fungal reactor was able to remove these compounds under aerated and oxygenated conditions. This result is very interesting in terms of developing viable reactors for the transformation of target compounds as the cost of aeration can be significantly reduced.

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