Evaluation of Two Operational Regimes: Fed-Batch and Continuous for the Removal of Pharmaceuticals in a Fungal Stirred Tank Reactor

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Two operational conditions were evaluated to maximize the removal of three anti-inflammatory drugs (diclofenac, ibuprofen and naproxen) and an antiepileptic (carbamazepine) in a stirred tank reactor (STR) operating with free pellets of *Phanerochaete chrysosporium*. First, the bioreactor was operated in a fed-batch mode (stage I) for 26 days and thereafter changed to continuous mode until day 70 (stage II). Time-course degradation experiments were carried out at days 0, 4, 7, 14 and 20 in order to compare the effect of the culture age on the degradation of the target compounds. The results showed that during stage I the fungus was able to completely remove the three anti-inflammatory drugs meanwhile only partial removal was attained for carbamazepine, between 30 % and 63 %. The maximum removal percentages were obtained on day 20, indicating that the culture age has an influence on the degradation efficiency. When the reactor operation was changed to a continuous feeding, the removal of the three anti-inflammatory drugs decreased until percentages in a range between 17 % up to 95 %; whereas, a recalcitrant compound such as carbamazepine achieved high removal percentage during this stage in comparison with the previous stage (93 % vs. 63 %).

1. Introduction

Some of the most commonly pharmaceuticals used worldwide are anti-inflammatory drugs such as diclofenac (DFC), ibuprofen (IBP) and naproxen (NPX). These compounds are used for the treatment of inflammation and pain caused for several illness such as arthritis, bursitis, gout or swelling (Adler et al., 2006). Moreover, an antiepileptic drug such as carbamazepine (CBZ) is one of the most recalcitrant compounds. All these compounds have been found ubiquitously in the aquatic environment at low concentrations (Fent et al., 2006). In the recent years different physicochemical and biological processes applied for the removal of pharmaceuticals have been investigated as most of these compounds are resistant to conventional treatments (Joss et al., 2006). Different white-rot fungi (WRF) have been used for the removal of these compounds, either by whole cultures or by the oxidative action of the enzymes produced (Marco-Urrea et al., 2009; Hata et al., 2010; Lloret et al. 2010). In this study, the fungal strain selected was *P. chrysosporium*, the best known WRF which produces two extracellular peroxidases: lignin peroxidase (LiP) and manganese peroxidase (MnP) during the secondary metabolism of the fungus (Wesenberg et al., 2003). The stability of a stirred tank reactor (STR) for the enzymatic production by WRF will depend on the operational conditions, which will be dependent on a number of variables such as culture age, medium composition, culture technique,

aeration supply and agitation rate, amongst others. This research work approaches a fungal STR operated with free pellets of *P. chrysosporium*, using two operational regimes (fed-batch and continuous) for the degradation of DCF, IBP, NPX and CBZ.

2. Materials and methods

2.1. Microorganism and inoculum preparation
The white rot fungus used in this chapter was *Phanerochaete chrysosporium* obtained from the culture collection of the University of Santiago de Compostela (Spain). The pre-inoculum for the STR was obtained according to Moreira et al. (2000).

2.2. Pharmaceutical compounds and chemicals
Pharmaceutical compounds (DCF, IBP, NPX and CBZ) were purchased from Sigma-Aldrich as pure grade. A range of solvents were used for the extractions and for the preparation of the pharmaceutical mixtures: acetone, ethyl acetate, acetonitrile, methanol and n-hexane; all of them purchased from J.T. Baker (all of them > 99.5 % pure).

2.3. Influence of the feed regime (fed-batch and continuous) on the operation of a stirred tank reactor for degradation of DCF, IBP, NPX and CBZ
Degradation experiments were performed in a 2-L stirred tank reactor (STR) (Sartorius, Melsungen, Germany). 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 Systems, Germany). Agitation was fixed at 200 rpm and temperature maintained at 30°C. In addition, the STR was continuously aerated for 70 d with a variable air flow to maintain the dissolved oxygen concentration close to saturation values. Two operational regimes were considered: a fed-batch and continuous feeding. During stage I (days 0-24), pulses of glucose (3-6 g/L) and pharmaceuticals were added sequentially while from day 25, the reactor operated with a continuous feeding of glucose and pharmaceutical compounds. During stage I, the monitoring of the main variables were controlled in a time course experiment at days 0, 4, 7, 14 and 20 to analyze the ability of the fungus to remove these compounds at different stages of the fermentation. To carry out these experiments, samples from the bioreactor were withdrawn immediately after the moment of the pharmaceutical compounds addition, then every half hour until 2 h, thereafter every 2 h until 8 h and finally after 24 h. In parallel and previous to the addition of the drugs, a sample was withdrawn from the bioreactor (~60 mL) and filtered to obtain the extracellular liquid free of biomass. Thereafter, it was placed in a flask and pulses of the pharmaceutical compounds were added to assess the capability of the extracellular fluid containing MnP to carry out the degradation of the target compounds. Additionally, abiotic controls were carried out using sterile distilled water to discard any volatilization of the compounds. When a continuous operation was performed (stage II), samples were taken twice per week to monitor the process.

2.4. Extraction of pharmaceuticals and determination of residual concentrations
Samples of 10 mL were withdrawn and 10 mL of acetonitrile was added for the extraction of pharmaceuticals. Flasks were sealed with Teflon and agitated for 2 h at 180 rpm in a shaker (Ika Labortechnik, HS 501 Digital, Germany). From the supernatant, 10 mL was withdrawn and placed in glass tubes sealed with Teflon and centrifuged at 5000 g for 15 min. An aliquot of 4 mL was taken from each tube, diluted in 100 mL water and then a Solid Phase Extraction (SPE) was carried out with 60 mg OASIS HLB cartridges (Waters closet, Milford, MA, USA) previously supplemented with 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of distilled water (pH 2). All the samples were derivatized using MTBSTFA (N-Methyl-N-(tert.-butyldmethylsilyl)trifluoroacetamide) as derivatizant agent. Finally, residual concentrations of DCF, IBP, NPX and CBZ were measured by gas chromatography-mass spectrometry (GC-MS) (Rodríguez et al., 2003).

2.5. Analytical techniques
Glucose concentration was analyzed with the dinitrosalicylic acid (DNS) method using D-glucose as a standard (Miller, 1959). Biomass concentration was determined as dry weight with 0.45 μm pore-size filters previously dried until constant weight. Enzymatic activity of manganese peroxidase (MnP) was

measured by the oxidation of dimethoxyphenol (DMP) in a spectrophotometer (Shimadzu UV-1603) as described by Field et al. (1992).

3. Results

During the fed-batch stage, the STR was fed with 10 g/L of glucose that was depleted at day 12; then, several pulses of glucose were added to the reactor (~2.5 g/L). Moreover, different glucose feeding rates (50-300 mg/L·h) were used during the continuous operation achieving high glucose consumption rates (from 30 mg/L·h up to 285 mg/L·h). The pH value was maintained in a range of 3.7 up to 5.3 during the experiment and a single enzymatic peak of 60 U/L was detected on day 20 (data not shown). Dissolved oxygen concentration during stage I was maintained below 4 mg/L. Contrary to this, when a continuous feeding was added, oxygen was maintained in a range between 2–8 mg/L.

The results of the time-course degradation experiments that were carried out on days 0, 4, 7, 14 and 20 are shown in Table 1. DCF was slightly removed at the beginning of the bioreactor operation (30 %); meanwhile, higher degradation percentages were detected in the following days (80 % - 94 %). IBP was efficiently degraded (above 95 %) throughout the experiment. Even more, 50 % of this compound was degraded after a very short period of time: 30 min on day 20 (data not shown). NPX was partially degraded (57 %) on day 0 and highly degraded during the rest of sampling days (~90 %). Contrary to the results shown above, CBZ was only partially degraded on days 4 and 7 (64 % and 32 %, respectively). No significant removal in the extracellular liquid was observed since the recovery of the added pulses was in the range of 70 % - 100 % for all the pharmaceutical compounds; however, low activities of MnP were detected throughout this stage, except on day 20 where 60 U/L of MnP were detected. In the case of abiotic controls more than 83 % of the compounds were detected after 24 h in all the experiments analyzed (data not shown).

Table 1. Residual percentage in the STR after 24 h

Day	Degradation percentages (%)			
	DCF	IBP	NPX	CBZ
0	30	95	57	0
4	87	97	89	64
7	83	95	82	32
14	76	95	88	0
20	94	100	94	0

Figures 1 and 2 show the feeding and degradation rates of DCF, IPB, NPX and CBZ during the continuous stage in this experiment. Feeding rates of pharmaceuticals were varied during the experiment. DCF feeding rate was in a range between 0.9 and 1.4 mg/L·d, except between days 40 and 47 when an increase up to 1.7 mg/L·d was applied. This compound was highly degraded during the period between days 26 and 54 (up to 90 %); then, a decrease in this degradation rate was observed between days 55 and 63 (34 % - 70 %). Finally, the degradation rate started to increase from day 66 until the end of the experiment (69 % - 82 %) (Figure 1, DCF).
IBP feeding rate was in a range of 0.8 - 1.2 mg/L·d with removal percentages above 94 % until day 54. Thereafter, a slight decrease was observed until the end of the experiment maintaining the removal percentages in a range between 65 % and 95 % (Figure 1, IBP). The feeding rates for NPX was in a range of 0.9 - 1.3 mg/L·d, showing a high removal in the period between days 26 and 54 (77 % - 94 %); then, the removal rate suffered a notorious decline during the rest of the experiment (Figure 2, NPX). On the other hand, CBZ feeding rate was maintained with a decreasing tendency from 2.2 mg/L·d until 1.0 mg/L·d throughout the experiment. The fungus was able to remove this compound in a range of 58 % - 93% until day 53; then, the removal rate decreased until only 5% on day 63. An improvement in the degradation rate (25 % - 44 %) can be observed during the last days of operation (Figure 2, CBZ).
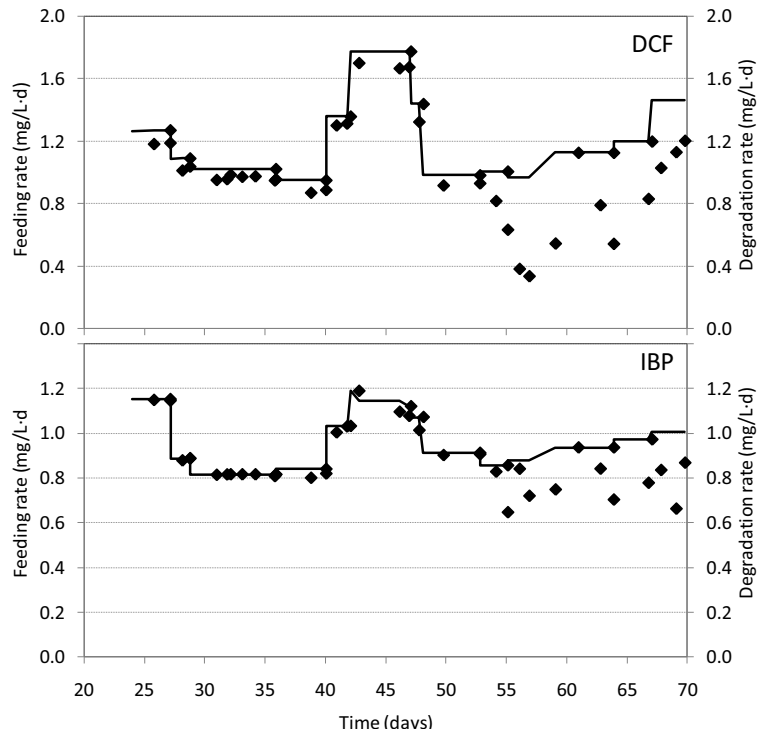
Figure 1. Feeding rates (—) and degradation rates (♦) of DCF and IBP during the continuous stage of the bioreactor with pellets of P. chrysosporium.
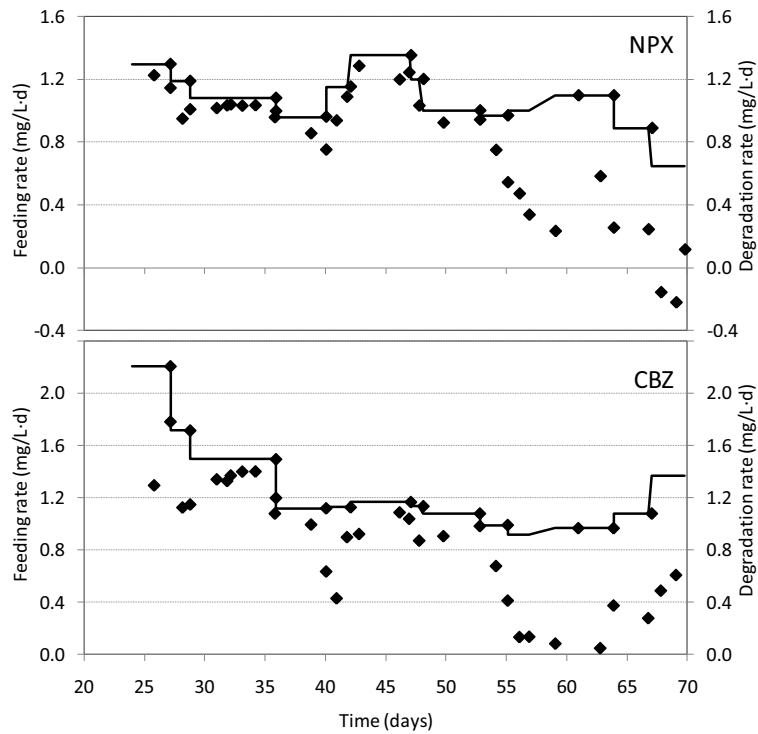

Figure 2. Feeding rates (—) and degradation rates (♦) of NPX and CBZ during the continuous stage of the bioreactor with pellets of P. chrysosporium.

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At the end of the experiment, a final biomass concentration of 7.8 g/L was measured. Also, high quantities of NPX and CBZ were detected per gram of biomass (up to 0.099 mg of drug); while around 0.022 and 0.039 mg of DCF and IBP were measured per gram of biomass. During the fed-batch stage, pellets were dense and compact; however a filamentous growth started to show after 3 weeks of experiment. Once the continuous feeding started, excessive growth of the fungus was observed; then hyphal branches and aggregates of pellets started to breakdown. New pellets with a compact aspect were formed from the fragments of fungal mycelia. A constant size throughout the experiment, in the range of 3.2 - 4.0 mm, was maintained. Despite the excessive fungal growth, no clogging problems were observed throughout the 70 days of operation in the STR.

4. Discussion

The operation of the STR remained stable during 70 days, despite the fact that it was operated with filamentous fungi which typically present excessive mycelium growth that might cause clogging problems. The fungus was able to achieve a great consumption rate of nutrients under both feeding strategies. It is important to ensure a high oxygen level, since it can improve the enzymatic production of MnP and also the removal of the pollutants; however, low enzymatic activities were detected during the experiment. Other authors have removed this type of compounds with low levels of enzyme suggesting that the degradation by fungal cultures could take place intracelularlly by the action of cytochrome P450 system (Hata et al., 2010). The degradation of DCF, IBP, NPX and CBZ by fungal action has been studied achieving high removal percentages; in fact, the use of ligninolytic enzymes such as Laccase enhances the removal of these compounds (Marco-Urrea et al., 2009; Lloret et al. 2010). When the bioreactor started with a fed-batch operation, DCF, IBP and NPX were totally removed while the removal efficiency decreased when a continuous feeding was added. Partial removal of CBZ was detected during the stage I; whereas under continuous feeding, a higher removal was achieved. Differences between the degradation percentages achieved for the considered pharmaceuticals may be related to their physicochemical properties. IBP is a readily biodegradable drug perhaps due to its chemical structure with one aromatic ring; while DCF has two aromatic rings connected by an N-H functional group as well as the presence of two chlorine atoms (Ziylan and Ince, 2011). Moreover, CBZ has a very small K_{biol} (< 0.01 L/g·SS·d) indicating that is hardly biodegradable compound (Joss et al., 2006). Adsorption of drugs onto biomass depends on the octanol-water partition coefficient (K_{ow}) and the acid dissociation constant (pKa) (Suárez et al., 2008). From the selected pharmaceutical compounds in this chapter, CBZ and DZP are more likely to be attached on the surface of solids.

5. Conclusions

In this study the degradation of DCF, IBP, NPX and CBZ by pellets of *P. chrysosporium* in a STR was analyzed under a fed-batch and a continuous operation. These compounds can be partially or totally removed during the fed-batch stage after a short period of time, depending on the culture age. When a continuous feeding was added, the removal of these drugs was maintained stable until day 55 but decreased in the final period of operation. Therefore, it can be concluded that both operational conditions can perform the efficient degradation of the target compounds provided that the fungal bioreactor maintains satisfactory viability; thus, prolonged operational periods, longer than 50 days, are not suitable for the operation of the free pellet reactor.

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