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Operation of stirred tank reactors (STRs) and fixed-bed reactors (FBRs) with free and immobilized *Phanerochaete chrysosporium* for the continuous removal of pharmaceutical compounds

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ABSTRACT

Stirred tank reactors (STRs) and fixed-bed reactors (FBRs) were applied for the removal of diclofenac, ibuprofen, naproxen, carbamazepine and diazepam by *Phanerochaete chrysosporium*. The operation of STRs with free pellets and immobilized fungus attained stable operation for 50 days. Both bioreactors achieved high removal efficiencies for diclofenac, ibuprofen and naproxen while partial removal of carbamazepine and diazepam. The configuration of the FBR maintained a steady and feasible operation for 100 days with complete removal of diclofenac, ibuprofen and naproxen regardless of the aeration system (air or oxygen) and remarkably high removal percentages of carbamazepine and diazepam: 60–90%. The extraction of the target compounds from the biomass and the support showed low residual concentrations of all the compounds both on fungal pellets and on the support of the FBR. Only significant values were detected in the STR with immobilized mycelium, demonstrating partial adsorption. A tentative identification of the degradation products of the three anti-inflammatories was performed, indicating the presence of 4-hydroxy-diclofenac, 1-hydroxy-ibuprofen-, 6-O-desmethyl-naproxen, as the major degradation products of the three parent compounds.

1. Introduction

In the last century, the use of pharmaceutical compounds aided to improve the quality of life, but also contributed to their spread either from direct disposal or from the effluent of sewage treatment plants (STPs) [1]. These types of compounds are considered emerging pollutants. They are suspected to present remarkable impacts on the environment given their design to affect biochemical and physiological functions of humans and animals. Moreover, pharmaceuticals may cause increased aquatic toxicity and endocrine disruption [2]. Among the different classes of pharmaceuticals, some of the most commonly used worldwide are anti-inflammatory drugs, such as diclofenac, ibuprofen and naproxen, which contain analgesic, antipyretic and anti-inflammatory effects by inhibiting the synthesis of prostaglandin. Furthermore, two of the most recalcitrant compounds present in sewage effluents are carbamazepine and diazepam, a carboxamide-type anti-convulsant and a tranquilizer, respectively [3,4]. Significant concentrations of these compounds have been detected in the environment in a range from ng/L up to μg/L and they tend to bioaccumulate [5,6].

On-going initiatives rely on the use of white-rot fungi (WRF) for the oxidation of a wide range of recalcitrant compounds structurally similar to lignin, such as synthetic dyes, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pharmaceutical compounds and endocrine disrupting chemicals (EDCs) [7,8]. Regarding the degradation of pharmaceuticals by WRF, previous investigations have shown the ability of these microorganisms as well as their ligninolytic enzymes for the removal of this type of compounds during *in vivo* and *in vitro* experiments [9–14]; however, most of these experiments were conducted in batch mode. In the present study, an *in vivo* degradation system with different bioreactor configurations and two culture techniques was assessed to carry out the continuous degradation of this type of compounds. Two types of reactor configuration are preferred for culturing ligninolytic fungi: stirred tank reactors (STRs) and fixed-bed reactors (FBRs) [15–19]. An additional key issue besides the selection of the bioreactor is the choice of the culture type as free pellets or immobilized on a wide range of supports [15,16]. Pellet formation is considered as a natural immobilization process and occurs by the aggregation of free spores. Regarding immobilized cultures, the use of a support enables physical entrapment in the open pores of the reticulated matrix and provides a large surface area that improves oxygen and nutrient diffusion [20]. This culture technique

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restricts the growth of the fungus to the external and inner surface of the support. However, the use of a support for the degradation of certain compounds has to take into consideration the potential adsorption of these compounds onto the support surface, which may render for limited biodegradation and the need of an extraction stage to desorb them.

The aim of this study was to assess which is the best type of culture as free or immobilized mycelia as well as the bioreactor configuration: STR and FBR to carry out the continuous degradation of five pharmaceutical compounds belonging to different therapeutic groups: anti-inflammatory drugs (diclofenac, ibuprofen and naproxen), anti-epileptics (carbamazepine) and tranquilizers (diazepam). Furthermore, the influence of gas supply either as a continuous flow of air or as pulses of oxygen was evaluated in the operation of the FBR. Additionally, the identification of the major degradation products of the three anti-inflammatory drugs was conducted.

2. Materials and methods

2.1. Microorganism and pre-inoculum preparation

The white-rot fungus *Phanerochaete chrysosporium* (ATTC 24725) was obtained from the culture collection of the University of Santiago de Compostela (Spain). The pre-inoculum was cultured in static using Fernsbach flasks (1 L) with 100 mL of modified Kirk medium [21] and 6 plugs of malt extract agar (glucose, 10 g/L; agar, 15 g/L; malt extract, 3.5 g/L) with active fungus. Concerning the fungal immobilization, the support consisted of polyurethane foam cubes (volume, 0.5 cm^3; density, 20 kg/m^3; superficial area, $414 \pm 10\ m^2/m^3$) obtained from Copo Ibérica (Vigo, Spain) [11]. A support/liquid volume ratio of 0.015 g/mL was used. From the homogenized mycelium, 9 mL were added to Erlenmeyer flasks (250 mL) containing 90 mL of modified Kirk culture medium in order to form pellets or immobilize the fungus. Flasks were incubated at 30 °C and agitated at 150 rpm in an orbital shaker (C24 Incubator Shaker, New Brunswick Scientific, USA) for 3–5 days. Grown cultures of pellets or immobilized fungus were withdrawn by filtration using sterile gauze and used as inoculum for the bioreactors in a proportion of 10%.

2.2. Pharmaceutical compounds and chemicals

The target compounds considered were diclofenac (DCF), ibuprofen (IBP), naproxen (NPX) and carbamazepine (CBZ), all of them purchased from Sigma–Aldrich as pure grade. Diazepam (DZP) from Roche Pharma as pure grade was used. A stock solution prepared at three different concentrations: 1 mg/L (DCF, IBP and NPX), 0.5 mg/L (CBZ) and 0.25 mg/L (DZP) was used. 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. Comparison of a continuous stirred tank reactor (STR) using P. chrysosporium pellets and immobilized fungus for the degradation of DCF, IBP, NPX, CBZ and DZP

Degradation experiments were performed in a 2-L stirred tank fermenter Biostat B plus (Sartorius, Melsungen, Germany). The culture vessel consisted of a jacketed glass vessel designed for temperature control *via* a thermostated system. Dissolved oxygen concentration and pH were continuously monitored using pO_2 and pH electrodes and data were processed by software MFCS/DA 3.0 (Module operator service program, Sartorius sedimentation Systems, Germany). The bioreactor vessel was sterilized and filled with 1.5 L of modified Kirk medium (pH 4.5) previously sterilized [21] and pellets or immobilized fungus from Erlenmeyer flasks. The continuous operation was conducted for 50 days with a hydraulic residence time (HRT) of 24 h, feeding addition rates of glucose (125–300 mg/L h), ammonium tartrate (0.6–2.08 mg/L h) and continuous air flow (0.5–3 L/min) to maintain significant levels of dissolved oxygen and temperature of 30 °C. The feed medium was sterilized and spiked with the pharmaceutical compounds at different concentrations: 1 mg/L (DCF, IBP, NPX), 0.5 mg/L (CBZ) and 0.25–0.5 mg/L (DZP) were added in the upper port of the reactor vessel while the effluent was withdrawn from the bottom of the reactor. Samples were withdrawn daily for monitoring the main operational variables and twice per week to determine the residual concentration of the pharmaceutical compounds.

2.4. Operation of a continuous fixed-bed reactor (FBR) using P. chrysosporium immobilized in polyurethane foam for the degradation of DCF, IBP, NPX, CBZ and DZP

The continuous operation of the FBR was conducted for 100 days with a 24 h HRT, feeding addition rates of glucose (125–250 mg/L h) and ammonium tartrate (0.6–2.08 mg/L h) and temperature of 30 °C. The bioreactor configuration consisted of a glass jacketed column with an internal diameter of 4.5 cm and a height of 20 cm with two gas supply systems: continuous air flow (1 L/min) and pulsation of oxygen by means of a pulsing device consisting of an electrovalve located at the end of a flexible membrane tube (oxygen pulsation frequency of 0.062 s^{-1}) [22]. Dissolved oxygen was measured off-line by means of an electrode (Oxi 340i from Wissenschaftlich Technische Werk, Germany). Samples were withdrawn from the middle (every 10 days) and upper ports (twice per week) of the reactor.

2.5. Adsorption of the pharmaceutical compounds on the fungal biomass and on the polyurethane foam support

At the end of the operation of the STRs, a final extraction was carried out to determine the concentration of pharmaceuticals that could be adsorbed on the biomass and/or the support. For this purpose, 50 mL of blended mycelium (for pellets) or immobilized fungus was placed in an Erlenmeyer flask, supplemented with 50 mL of acetonitrile and agitated for 2 h at 150 rpm. In the case of the immobilized fungus from the FBR, samples were taken from the upper, the middle and the lower section of the column. After the agitation of the samples with acetonitrile, 4 mL of each sample were withdrawn and diluted in 100 mL of distilled water, to proceed with the solid phase extraction (SPE), stage required prior to gas chromatography-mass spectrometry.

2.6. Determination of the concentration of residual pharmaceutical compounds by gas chromatography-mass spectrometry (GC–MS)

Residual concentrations of DCF, IBP, NPX, CBZ and DZP were measured by gas chromatography-mass spectrometry (GC–MS). Prior to determination, a solid phase extraction (SPE) of the samples 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) to allow the determination of the soluble fraction of these compounds [23].

2.7. Identification of degradation products

The identification of the major degradation products of DCF, IBP and NPX was performed by GC–MS, using N-methyl-N-(tert.-butyldimehtylsilyl)trifluoroacetamide (MTBSTFA) as the