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Biodegradation of pharmaceuticals by *Trametes versicolor*.

PhD Thesis

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Bellaterra, 27 de Setembre de 2013

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Abstract

Pharmaceutical active compounds (PhACs) are an important group of emerging contaminants that have raised an increasing interest in the scientific community due to their ubiquitous presence in the environment and their difficult degradation. Some of these drugs are extensively used as non-prescription drugs and after their intake, are excreted with urine and faeces either as active substances or metabolites. These substances come into wastewater treatment plants where some compounds are not efficiently removed, being able to reach surface, groundwater and subsequently, drinking water.

The present work assesses the feasibility of PhACs bioremediation by white-rot fungi (WRF). WRF have the potential to degrade a wide range of xenobiotic and recalcitrant contaminants due to their unspecific enzymatic system, able to act on diverse substrates through the action of intracellular (i.e. cytrochrome P450 system) and extracellular (i.e laccases and peroxidases) enzymes. The fungus *Trametes versicolor* has been chosen to carry out the degradation study of some analgesics and anti-inflammatory (ketoprofen and diclofenac), anti-epileptics (carbamazepine), lipid regulators (clofibric acid), antibiotics (ofloxacin) and a X-ray contrast agent (iopramide).

The first step in the research deals with the preliminary assessment of the individual PhACs degradation by *T. versicolor* at Erlenmeyer scale and sterile conditions. To obtain further insights in the mechanism of PhACs degradation by the fungus, the transformation products were identified as well as the enzyme responsible for the degradation of the parent compound with the aim of proposing a degradation pathway. In addition, an assessment of the toxicity of the broth, where transformation products were present, was included.

Furthermore, with the aim of scale up the PhACs degradation process, a fluidized bed bioreactor was employed for the degradation of carbamazepine and clofibric acid, operated in both continuous and batch mode. Results also include the identification of transformation products and the toxicity assessment.

On the other hand, due to the great number of publications about the degradation of pharmaceuticals by white-rot fungi that appeared over the course of this thesis, it was decided to include a literature review to evaluate the current state of the art in this topic.

Finally, in an attempt to scale up the process to real approaches and thus provide a better estimation of the potential environmental impact of the application of such process, *T. versicolor* was used in a non-sterile batch bioreactor treatment for the removal of pre-existent PhACs from urban and hospital wastewater, where many contaminants and other microorganism are present. In preliminary experiments with urban wastewater, it was found the necessity of an extra source of carbon and nitrogen to maintain the activity of the fungus in the wastewater. Moreover, an important removal was observed for almost all drugs detected in both urban and hospital wastewater, together with a remarkable reduction of the overall toxicity.

Resum

Els fàrmacs (PhACs) són un important grup de contaminants emergents que degut a la seva presència en el medi ambient i la seva difícil degradació han aixecat un gran interès en la comunitat científica. Alguns d'aquests PhACs són àmpliament utilitzats sense recepta i després de la seva ingestió son excretats per la orina i els fems, ja sigui en forma de compost actiu o com a metabòlit. Aquests productes entren a les estacions depuradores d'aigües residuals, on alguns d'aquests compostos no són eliminats eficaçment, sent capaços d'arribar a les aigües superficials, subterrànies i, posteriorment, a l'aigua potable.

El present treball avalua la viabilitat de la bioremeïació dels PhACs per fongs de podridura blanca. Aquests fongs tenen el potencial de degradar una àmplia gamma de contaminants xenobiòtics i recalcitrants degut al seu sistema enzimàtic inespecífic, capaç d'actuar sobre diversos substrats a través de l'acció d'enzims intracel·lulars (com el citocrom P450) i extracel·lulars (com la lacasa i peroxidasas). De tots els fongs, s'ha escollit *Trametes versicolor* per dur a terme l'estudi sobre la degradació d'analgèsics i anti-inflamatoris (ketoprofen i diclofenac), antiepilèptics (carbamazepina), reguladors de lípids (àcid clofibric), antibiotics (ofloxacina) i un agent de contrast per rajos X (iopromida).

El primer pas en la investigació va consistir en el estudi de la degradació de PhACs de forma individual per *T. versicolor* a escala Erlenmeyer i en condicions estèrils. Per tal d'obtenir més coneixements en el mecanisme de degradació dels PhACs pel fong, es va estudiar en detall la transformació dels fàrmacs anteriorment esmentats i en alguns casos es va proposar la via de degradació. Paral·lelament es va estudiar els enzims implicats en la degradació dels PhACs. També es va avaluar la toxicitat del brou de cultiu, on els productes de degradació estaven presents i d'aquesta manera poder observar si els compostos produïts son més tòxics que el propi fàrmac.

Posteriorment, amb l'objectiu d'escalar el procés de la degradació de PhACs, es va utilitzar un bioreactor de llit fluïditzat per la eliminació de la carbamazepina i de l'àcid clofibric, operat tant en continu com discontinu. En els resultats també es va incloure

la identificació dels productes de transformació, junt amb l'avaluació de la toxicitat dels efluents.

D'altra banda, a causa del gran nombre de publicacions sobre la degradació de PhACs individuals per fongs publicats durant el transcurs d'aquesta tesis, es va realitzar una revisió bibliogràfica sobre aquest camp de recerca.

Per últim, en un intent d'escalar el procés a nivells més reals i per tant proporcionar una millor estimació del possible impacte ambiental de l'aplicació d'aquest procés, es va tractar una aigua residual urbana i d'hospital en un bioreactor operat en discontinu. El tractament de l'aigua residual es va fer en condicions no estèrils, on altres microorganismes estan presents, i a les concentracions preexistents dels contaminants, és a dir, sense afegir cap contaminant. En un experiment preliminar amb l'aigua residual urbana, es va observar la necessitat d'afegir una font addicional de carboni i nitrogen per mantenir l'activitat del fong. Per altra banda, els resultats obtinguts van ser positius ja que es va observar l'eliminació de gairebé tots els PhACs detectats en les aigües urbanes i d'hospital, juntament amb una notable reducció de la toxicitat global després del tractament, el que fa concloure que pot ser un tractament adequat i cal seguir investigant en altres aspectes per desenvolupar i optimitzar el procés abans de implementar-lo a escala real.

SECTION 1:

GENERAL ASPECTS

Chapter I:

BACKGROUND AND INTRODUCTION

Chapter I:

BACKGROUND AND INTRODUCTION

I.1. Emerging contaminants in the environment: pharmaceuticals

Until last decades of the twentieth century the study of the environmental contamination was mainly focused on the impact of chemical pollutants discharged at high concentrations, such as pesticides and industrial wastes which persist in the environment. Lately an important group of chemicals have been considered as potential contaminants for the environment, named emerging contaminants. They are chemicals or products that are being detected in the environment and characterized by a lack of information concerning their harmful pollution. They may be candidates for future regulation depending on the research about their health effects and their presence in the environment. Among the emerging contaminants, pharmaceuticals and personal care products are of special concern.

Pharmaceuticals active compounds (PhACs) are a large and diverse group of compounds designed to prevent, cure and treat disease and improve health. They have long been used in significant quantities throughout the world. Their usage and consumption are increasing consistently due to the discoveries of new drugs, the expanding population and the continuous improving in the quality of life, as well as due to expiration of patents that makes drugs more affordable (Van der Aa et al., 2011). As evidence, in 1992 the annual consumption in Spain of anti-inflammatory drugs like acetylsalicylic acid, paracetamol, ibuprofen and diclofenac was approximately 757 tons, which increased up to 1495 tons in 2006 (García and de Abajo, 2007). Similarly, the annual consumption of psychiatric drugs, like the antiepileptic drug carbamazepine or diazepam, was also increased more than doubled (from 74 to 175 tons) in the same period (de la Fuente and García, 2007). Meanwhile the use of antibiotics in Spain has been kept around 500 tons between 1994 and 2009 with little fluctuations. This fact can be explained by the increase in consumption of new antibiotics which outweighed the decreased use of obsolete antibiotics (Lázaro et al., 2010).

The main source of the pharmaceutical contamination is the households, livestock farms, hospitals and veterinary effluents and pharmaceutical manufactures (Sim et al., 2011). After intake, PhACs undergo metabolic processes in organism. A significant fraction of the parent compound is excreted as unmetabolized form but also as metabolites (active or inactive) into raw sewage and wastewater treatment systems (Mompelat et al., 2009). Disposal of drug leftovers to sewage and trash is another source. In Germany, it was estimated that amounts of up to 16000 tons of pharmaceuticals were disposed every year from human medical care and 60-80 % of them were either flushed down the toilets or disposed of with normal household waste (Scheytt et al., 2006).

In addition, several published reports reveal the inefficiency of conventional wastewater treatment plants in the removal of PhACs (Gao et al., 2012; Ratola et al., 2012; Verlicchi et al., 2012). These chemicals are not completely degraded and are either removed by sorption, which means deposition to the final sludge (Jelić et al., 2011) or discharged onto a surface water body, if they remain in the wastewater effluent stream. The latter fraction is the most concerning, since it has been shown to be readily bioavailable to living organisms, able to enter the food chain and hence ultimately exposing humans (Katsoyiannis and Samara, 2007). Thus, during the last 10 years several studies reported the occurrence of numerous PhACs in both ground and surface water throughout the world (Caliman and Gavrilescu, 2009). On the other hand, the biosolids produced in wastewater treatment also constitute a possible source of contamination, given the fact that PhACs remained adsorbed in the sludge and the latter is reused in agriculture as soil amendment or disposed to landfill.

The possible negative ecotoxicological effect provoked by the presence of PhACs in the environment is an issue of environmental concern. Consequently, several researchers have focused their attention on the potential risk of the presence of PhACs in different water compartments, which were recently reviewed by Santos et al., 2010, de Jong et al., 2012 and Stuart et al., 2012. Although, chronic ecotoxicity data are scarce compared to acute studies, accumulative effects have been shown to damage some ecosystems (Daughton and Ternes, 1999).

Consequently, the development of processes to remove PhACs is required. Among the different alternatives, the biological treatments are more desired because they are sustainable and environmental friendly. Therefore, a new biological treatment must be studied in order to get a wastewater free of PhACs, which would help to reduce the environmental impact of these chemicals in the environment.

I.2. White-rot fungi

A fungus is a member of a large group of eukaryotic organisms. Many of them are filamentous which means that they consist of hyphae, surrounded by a cell wall. The hyphae grow at their tips and branch periodically, which creates a network of hyphae called mycelium. All fungi are heterotrophs, and due to their rigid cell wall they excrete extracellular enzymes to break down complex polymers and then absorb simple nutrients (Hanson, 2008).

White-rot fungi (WRF) have an important role in the environment as degraders of cellulose, hemicelluloses and lignin (Kirk and Fenn, 1982). Lignin is the second most abundant renewable organic compound in the biosphere, behind cellulose, and has a molecular structure very heterogeneous and complex, as shown in figure I.1 (Boominathan and Reddy, 1992). Due to the fact that lignin is an insoluble polymer, the initial steps in its biodegradation are catalyzed extracellularly by fungi. (Kirk and Farrell, 1987).

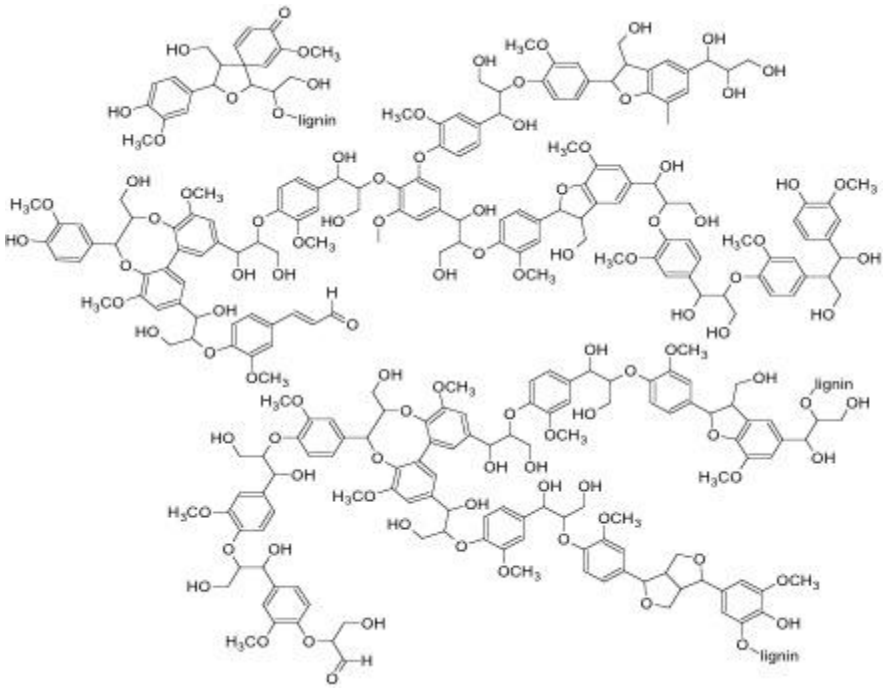

Figure I.1: Representative structure of lignin

Lignin-degrading fungi are classified into three major categories based on the type of wood decay caused by these organisms: white-rot fungi, brown-rot fungi and soft-rot fungi. Among these three groups, WRF are the most effective lignin degraders and have been the most extensively studied group. Taxonomically WRF comprise a heterogeneous collection of several hundreds of species of basidiomycetes (Ainsworth et al., 1973) and some ascomycetes (Eaton and Hale, 1993). WRF present an extracellular oxidative enzymatic system employed in the primary attack of lignin and its posterior mineralization (Martinez et al., 2005) in a non-specific and non-selective mechanism. This enzymatic system includes lignin-modifying enzymes (LMEs), which are extracellular and metal-containing oxidoreductases, especially peroxidases and laccases. The catalyzed reactions include lignin polymerization and dimethoxylation, decarboxilation, hydroxylation and breakdown of aromatic rings (Harms et al., 2011).

I.2.1. Enzymatic system of WRF

WRF secrete mainly two different groups of LMEs, laccases and lignin-modifying peroxidases (LMPs), particularly lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP), which act synergistically during lignin degradation (Lundell et al., 2010). The main difference is the electron acceptor, O_2 for laccases and H_2O_2 for peroxidases. The lignin degrading system is induced when starvation of C or N occurs. The secretion pattern is specie-dependent. Moreover, agitation and temperature can significantly affect the levels of these enzymes (Gao et al., 2010). On the other hand, WRF possess the cytochrome P450 system, an intracellular enzymatic system involved in the the degradation of several organic pollutants (Cerniglia, 1997).

I.2.1.1. Laccase

Fungal laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper blue phenoloxidases. It is a glycosilated protein expressed in multiple forms and shows high molecular weight variability, ranging from 59 to 110 KDa. Laccase is expressed as multiple isoenzymes being both constitutive and inductive (Dittmer et al., 1997; Svobodová et al., 2008). The enzyme contains four copper atoms (Cu), in different states of oxidation (Thurston 1994) which play an important role in the catalytic mechanism. Laccase presents low specificity to electron-donating substrates. The catalytic cycle of laccase (figure I.2) comprises one-electron transfers from the four substrate molecules containing copper to one molecule of O_2 which is reduced to water. With this mechanism, laccases generate phenoxy radicals from phenolic compounds. These radicals are highly reactive and produce new oxidations, either spontaneous or by enzymatic ways (Thurston, 1994).

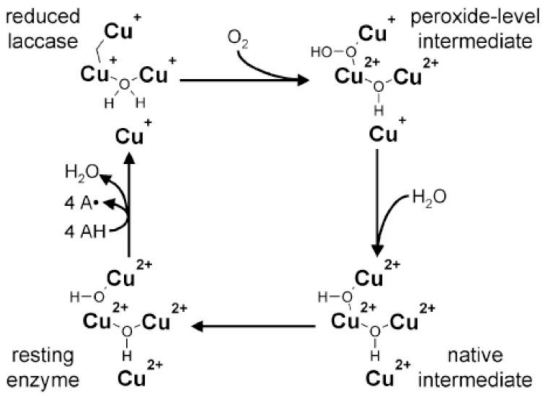

Figure I.2: Catalytic cycle of laccase (Wesenberg et al., 2003)

Due to the high redox potential of these enzymes, they are able to oxidize a broad range of aromatic compounds (phenols, polyphenols, methoxy-sustituted phenols, aromatic amines, benzenethiols). Other enzymatic reactions catalyzed by laccase include decarboxylations and demethylations (Nyanhongo et al., 2007). However, laccase only oxidize non-phenolic compounds in the presence of small molecules capable to act as electron transfer mediators (Rodríguez-Couto and Toca-Herrera, 2006, Call and Mücke, 1997). For example, various fungal metabolites such as N-hydrozyacetanilide (NHA), N-(4-cyanophenyl)acetohydroxamic acid (NCPA), 3-hydroxyanthranilate, syringaldehyde, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate (ABTS), 2,6-dimethoxyphenol, 2,2,6,6-tetramethylpipperidin-N-oxide radical (HBT), acetovanillone (AV), acetosyringone and acetohydroxamic acid perform the role of mediators of laccase and hence, their presence enhance pollutant degradation (Asgher et al., 2008).

I.2.1.2. Lignin peroxidase

Lignin peroxidase (LiP, E.C. 1.11.1.14) was the first ligninolytic enzyme discovered in the WRF *Phanerochaete chrysosporium* (Tien and Kirk, 1983). Later it was also found in other basidiomycetes. LiP is a glycoprotein that contains iron protoporphyrin IX (heme) as a prostetic group and requires H_2O_2 for catalytic activity (Hofrichter et al., 2010). It is expressed in multiple isoenzymes with molecular weights ranging from 38 to 47 kDa. It is capable of oxidizing recalcitrant phenolic and non-phenolic lignin model substrates. The catalytic cycle (figure I.3) starts when H_2O_2 oxidizes LiP to form a two-

electron intermediate (LiP-compound I). The latter oxidizes substrates by removing one electron leading to the formation of another intermediate (LiP-compound II), which subsequently oxidize substrates by one electron completing the cycle. However, LiP-compound II can react with H_2O_2 with low substrate concentration, leading to the formation of an inactive form of the enzyme (LiP-compound III).

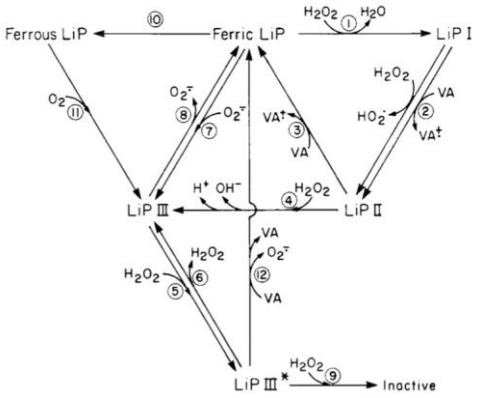

Figure I.3: Catalytic cycle of lignin peroxidase (Wariishi and Gold, 1990)

I.2.1.3. Manganese peroxidase

Another type of heme peroxidase, manganese peroxidase (MnP, E.C. 1.11.1.13), was later found in the culture media of the fungus *P. chrysosporium* (Kuwahara et al., 1984). It is an extracellular, glycosylated heme-containing peroxidase (Paszczynski et al., 1988). The enzyme presents multiple isoforms ranging from 40 to 50 kDa (Hofrichter, 2002).

The catalytic cycle (figure I.4) of MnP resembles those of other heme peroxidases such as LiP, and includes the native ferric enzyme as well as the reactive intermediates compound I and compound II. But in contrast to other peroxidases, MnP uses Mn^{2+} as the preferred substrate (electron donor). Then, Mn^{2+} is oxidized to highly reactive Mn^{3+}, which is stabilized by fungal chelators such as oxalic acid. The product Mn^{3+} forms a complex with organic acids and diffuses away from the enzyme to oxidize other materials.

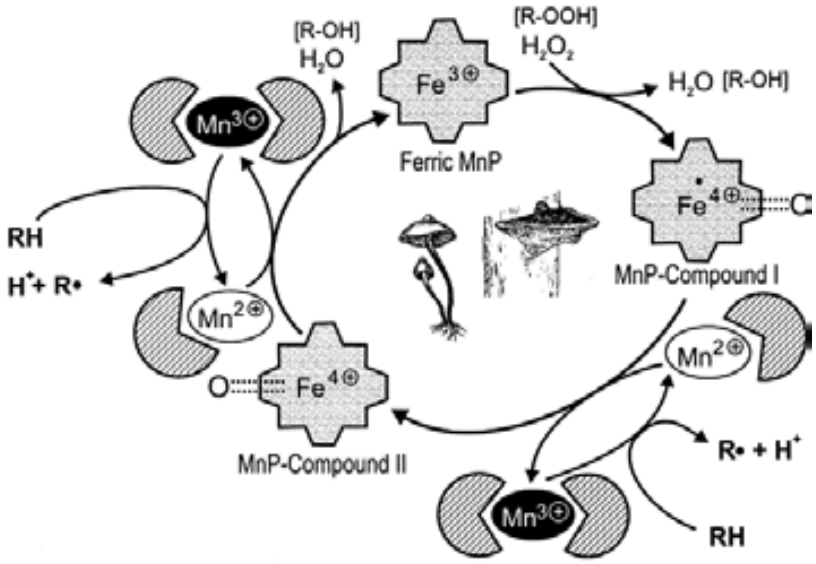

Figure I.4: Catalytic cycle of manganese peroxidase (Hofrichter, 2002)

I.2.1.4. Versatile peroxidase

Versatile peroxidase (VP, E.C. 1.11.1.46) is present in geners such as *Pleurotus, Bjerkandera* and *Trametes*, and share the catalytic properties of MnP and LiP (Hofrichter et al., 2010). As MnP, exhibits a high affinity for Mn^{2+} and catalyzes the oxidation of Mn^{2+} to Mn^{3+}. However, in Mn^{2+} absence, VP can also oxidize aromatic-phenolic and non-phenolic substrates, like LiP. Thus, its catalytic cycle is constituted by the sum of the catalytic cycles of both LiP and MnP (Gómez-Toribio et al., 2001).

I.2.1.5. Cytochrome P450 system

The ability of WRF to degrade pollutants was first ascribed to the LMEs in nitrogen limiting conditions, particularly LiP and MnP from *P. chrysosporium*. However, it was demonstrated afterwards that some xenobiotics such as 2,4,5-trichlorophenoxacetic acid could be degraded in nitrogen-rich media, without the expression of LiP and MnP (Yadav and Reddy, 1992), thus suggesting the role of alternate enzymatic systems. Further research demonstrated that the intracellular cytochrome P450 system exerts a leading role in the degradation of organic contaminants by WRF, as reviewed in Cerniglia (1997). Evidence is based on the induction pattern of cytochrome P450 codifying genes in response to exposure to organic pollutants (Doddapaneni and

Yadav, 2004) and also on the important reduction in the extent of degradation in the presence of cytochrome P450 inhibitors (Bezalel et al., 1996; Marco-Urrea et al., 2006, 2008). The main reactions catalyzed by the intracellular cytochrome P450 monooxygenases are hydroxilations, heteroatom oxygenation, dealkylation, epoxidation of C=C bonds, reduction and dehalogenation (Bernhardt 2006). Cytochrome P450 was suggested to play an important role in the mineralization of ligninolitic metabolites produced during lignin despolymerization by LMPs (Subramanian and Yadav, 2008)

I.2.2. Trametes versicolor

The fungus *T. versicolor* (figure I.5) is a filamentous WRF and belongs to the family polyporaceae. It is an obligate aerobic fungus commonly found year-round on dead logs, stumps, tree trunks and branches. The fungus occurs all over the wooded temperate zones of Europe, Asia, and North America. It is supposed to be the most common shelf fungus in the northern hemisphere. It plays an important role as wood degrader and a minor as tree parasite. Many different names have been used in the literature including *Agaricus versicolor, Boletus versicolor, Polyporus versicolor, Polystictus versicolor* and *Poria versicolor*, inter alia, and more than 120 strains of *T. versicolor* are known (Gerhardt et al., 2000).

Figure I.5: *Trametes versicolor* fruit bodies in the environment.

In agitated submerged culture the filamentous fungi grows as dispersed or pelleted mycelium (figure I.6), but neither fruiting body nor spores are formed. The morphology obtained during growth depends on the composition of the medium and the growth conditions. Hence, the carbon source, the C/N ratio, pH and ionic strength of the medium determine their morphology, but also the inoculum level, aeration, the type and speed agitation are crucial in the growth process. The morphology of the fungus determines in many cases the industrial application. For example, it has been observed that the production of some secondary metabolites is closely related to the morphology of growth (Smith and Lilly, 1990). In addition, the cultures of filamentous microorganisms growing as dispersed mycelium are characterized by getting very high viscosity and non-newtonian behavior of the culture broth (Sarrà et al., 1996). This significantly influences the processes of mass and energy transfer. On the other hand, cultures growing in pellets form do not experience changes in the viscosity of the broth, which is comparable to the water. However, the structure of pellets usually led to micronutrient limiting conditions and oxygen supply is mandatory to avoid diffusion problems. These limitations depend strongly on the size of the pellet.

In this thesis, *T. versicolor* strain ATCC#42530 was used in all the experiments. Previous characterization of LMEs production by this strain showed that secretion of LiP, MnP and laccase was produced at different extent depending the composition of the medium (Acebes, 2008). Moreover, the fungus enzymatic machinery includes cytochrome P450 that can be implied in degradation mechanisms (Ichinose et al., 2002).

Figure I.6: *Trametes versicolor* grown in pelleted morphology.

I.3. Bioremediation by white rot fungi.

Past production and improper disposal of large quantities of environmentally persistent and toxic chemicals generated very legitimate public health concern. Widespread contamination of soils as well as groundwater and surface water has brought this problem to forefront. However, cleanup of environmental pollution present a serious economic burden to society. Therefore, it becomes apparent that cost-effective and efficient methods of decontamination are vital to our success in solving the hazardous waste problem (Asgher et al., 2008). Since this problem is known, bioremediation became increasingly popular. The use of indigenous or introduced microorganisms to decontaminate waste sites provides a very attractive economic solution to many of our hazardous pollution problems (Barr and Aust, 1994). Most studies in bioremediation have focused on bacteria as degraders because of their rapid growth, and their usual ability to employ the pollutants as carbon and energy source. Nevertheless, research concerning the use of white-rot fungi in pollutants removal applications has increased over the last decades. The characteristics of white-rot fungi make them attractive candidates for use in bioremediation applications, but the complexity of their enzymatic system has also made the technology slow to emerge as viable approach for bioremediation. One distinct advantage of the use of WRF against bacteria is their extraordinary versatility to degrade a large variety of complex and recalcitrant contaminants in mixtures and single chemicals due to the non-specific enzymatic system. Another interesting aspect for their application is that WRF do not require preconditioning to a particular pollutant, while bacteria must be pre-exposed to a pollutant to allow the enzymes that degrade the pollutant to be induced. In addition, the pollutant must also be present in a significant concentration; otherwise induction of enzyme synthesis will not occur in bacterial system (Harms et al., 2011). Therefore, there is a finite level to which pollutants can be degraded by bacteria. In contrast, the induction of the ligninolytic enzyme system in WRF is not dependent on the chemical and allows to degrade xenobiotics to near non-detectable

levels (Barr and Aust, 1994). In addition, bacteria usually employ the pollutants as nutrient sources (C and N), while the degradation of pollutants by WRF becomes a co-metabolic process in which additional C and N sources are required (Pointing, 2001). This capacity also represents an advantage respect bacteria as it prevents the need to internalize the pollutant into the cell, thus permitting to attack low-soluble compounds and avoiding toxicity problems. However, an economic liability appears because of the possible extra source of nutrients required.

First studies about the use of WRF and their LMEs for the removal of recalcitrant xenobiotics appeared in 1980s. These studies demonstrated the ability of WRF in the degradation of very toxic compounds as pesticides, including the organoclorines dichlorodiphenyltrichloroethane (DDT) and its very toxic metabolite dichlorodiphenyldichloroethylene (DDE) (Bumpus et al., 1987; 1993). Subsequently, several studies appeared demonstrating the ability of WRF and their LMEs in the removal of hazardous contaminants to the environment as polychlorinated biphenyls (PCBs) at different degrees of chlorine substitution (Zeddel et al., 1993; Yadav et al., 1995; Novotny et al., 1997), even some of them getting mineralization (Dietrich et al., 1995; Beaudette et al, 2000), diverse polycyclic aromatic hydrocarbons (PAHs) in liquid media and contaminated soils or in complex mixtures such as creosote (Field et al., 1992; Yadav and Reddy, 1993; Lamar et al., 2002; Byss et al., 2008;), components of munitions wastes including 2,4,6-trinitrotoluene (TNT) and its metabolite 2,4-dinitrotoluene (DNT) (Bumpus and Tatarko, 1994; Donelly et al., 1997; Hawari et al., 1999; Jackson et al., 1999), nitroglycerin (Bhaumik et al., 1997), and cyclotrimethylenetrinitramine (RDX) (Bayman et al., 1995), among others reviewed elsewhere (Higson, 1991; Pointing, 2001; Rodríguez-Couto and Toca-Herrera, 2006; Asgher et al., 2008; Gao et al., 2010; Majeau et al., 2010).

Since the treatment of industrial effluents such as olive mill wastewater, bleach plant effluents and textile, paper, plastic, cosmetic and food industries effluents containing different dyes and pigments, are ineffective by conventional biological wastewater treatments (Ahmadi et al., 2005), they have also been subjected to WRF-mediated bioremediation studies. The treatment of olive mill wastewater, containing

high organic load, acidic pH and recalcitrant and toxic substances such as phenolic and lipidic compounds, by WRF has shown chemical oxygen demand reduction, detoxification (Blánquez et al., 2002; Dhouib et al., 2006), decolorization (Jaouani et al., 2006) and dephenolization (García et al., 2004). Likewise, studies of the treatment of highly toxic bleach plant effluents delivered from the pulp and paper industry, have reported its decoloration, dechlorination and detoxification (D'Souza et al., 2006; Font et al., 2006; Minussi et al., 2007), as well as degradation and decoloration of industrial synthetic dyes have also been widely documented (Blanquez et al., 2008; Asgher et al., 2008; Casas et al., 2009).

Once demonstrated the ability of the WRF in the biodegradation of hazardous pollutants (PHAs, pesticides, industrial effluents, etc.), which are typically released at high concentrations and their maximum value in environmental matrices are commonly regulated, research about the application of WRF was focused in the removal of the so called emerging contaminants. These chemicals are of concern because their widespread occurrence and the risk associated to their toxicity and potential endocrine disruptor effects. Therefore, the number of publications dealing with the degradation of such chemicals by WRF increased dramatically in the last years, as for example brominated flame retardants (Uhnáková et al., 2009, 2011; Zhou et al., 2007), UV filters (Gago-Ferrero et al., 2012) as well as pharmaceuticals, such as analgesics (Eibes et al., 2011; Marco-Urrea et al., 2009, 2010), antibiotics (Accinelli et al., 2010; Rodarte-Morales et al., 2011; Schwarz et al., 2010; Rodríguez-Rodríguez et al., 2012), psychiatric drugs (Hata et al., 2010; Zhang and Geiβen, 2012) and lipid regulators (Tran and Kusakabe, 2010), among others.

Despite the high potential described for WRF in terms of degrading ability, most of the reports refer to Erlenmeyer scale and at sterile conditions, and should be noted that in several of this studies contaminants were spiked in the matrix instead of being pre-existent. The use of real contaminated samples can provide a better estimation of the potential environmental impact of the application approaches. Therefore, the research must be focused on the application of fungus under non-sterile conditions, were several microorganisms are competing, as well as in the scale-up of the process, which would allow consider the real application.

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Chapter II:

OBJECTIVES AND CONTENT OVERVIEW

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OBJECTIVES AND CONTENT OVERVIEW

II.1. Objectives

The main goal of the present thesis is to demonstrate the degradation of pharmaceuticals in liquid media by the WRF *T. versicolor* from first stages (Erlenmeyer scale) to non-sterile bioreactor treatments in order to approach real applications. To achieve the general objective, the work comprised in the thesis has been organized according to fit the following specific objectives:

- To determine the ability of *T. versicolor* to degrade individual pharmaceuticals spiked in defined liquid medium under sterile conditions.

- To scale up the process to lab bioreactor operating in batch and continuous mode.

- To identify transformation products of the pharmaceuticals degradation and to assess their acute toxicity.

- To analyze the effect of the nutrients addition and pH changes in the activity of the fungus in non-sterile real wastewater.

- To treat a non-sterile urban and hospital wastewater by *T. versicolor* in a fluidized bed bioreactor to remove pharmaceuticals at their pre-existent concentrations in order to approach real applications.

II.2. Content overview

The low removal efficiency of PhACs reached in conventional wastewater treatments plants led their subsequent detection in the environment. Therefore, research of alternate biological treatments in order to bioremediate these emerging contaminants are mandatory. Due to the known capability of the WRF in the removal of hazardous contaminants, it was studied the possibility to use them in the elimination of PhACs. Screenings of some PhACs degradation by different WRF (*Irpex lacteus, Ganoderma lucidum, P. chrysosporium* and *T. versicolor*) were firstly reported by Marco-Urrea et al (2009) and showed the high potential of *T. versicolor* as bioremediation agent for PhACs but also for other pollutants such as TCE and PCE (Marco-Urrea et al., 2006), endocrine disruptors (Blánquez and Guinesse, 2008) and synthetic dyes (Bánquez et al., 2004). Therefore, based on these previous evidences, *T. versicolor* was selected to carry out the experiments in the present thesis.

The present work is divided in 3 sections. In the first section called general aspects is placed the two first chapters: in chapter one the state of the art of the presence of PhACS in the environment, the morphology of WRF and and their use as bioremediation agent is described. In the second chapter, the objectives and the structure of the thesis are presented.

The following section (section 2) reports the research work either as published or submitted articles, or still as manuscript draft. Section 2 is divided in two single sections. Section 2.1 shows the degradation of individual PhACs (chapters from III to VII) and section 2.2 shows the treatment of urban and hospitals effluents contaminated with PhACs by enzymes and *T. versicolor* (chapters from VIII to XI). Finally, in section 3 are found the conclusion and future prospects.

The first step of the research work was focused on the thorough study of the degradation of singles PhACs. In chapters III and IV are presented the degradation of both anti-inflammatory drugs ketoprofen and diclofenac, respectively, in Erlenmeyer flasks at different concentrations. The study of the degradation of these two PhACs

also included the identification of their transformation products, identification of the enzymes responsible, and the assessment of toxicity.

Subsequently, the degradation of other PhACs, as the recalcitrant psychiatric drug carbamazepine and the blood lipid regulator clofibric acid, was studied (chapters V and VI, respectively). The process was scaled up in a fluidized bed bioreactor performed in batch and continuous mode. In addition the transformation products were identified and toxicity was assessed.

A comprehensive review of the state of the art regarding degradation of PhACs by WRF and the main enzymatic mechanisms involved constitutes Chapter VII.

Once demonstrated the degradation of several PhACs by *T.versicolor* and the possibility to scale up the process, the removal of pre-existent PhACs present in urban wastewaters at non-sterile conditions, where several pollutants along with their metabolites and other active microorganism may be present, was the next challenge (section 2.2). Chapter VIII presents the process to make a novel combined enzyme aggregate and the subsequent application in the treatment of urban and hospital wastewater. Chapter IX describes firstly the study of the growth pattern of *T. versicolor* in a non-sterile urban wastewater treatment and the evaluation of the nutrient requirements to find the optimal conditions for its application. Afterwards, a fluidized bed bioreactor was used to remove PhACs at their pre-existent concentration in a non-sterile treatment, including assessment of the toxicity through the treatment to demonstrate the feasibility to scale up the process.

In Chapter X is described the degradation of the X-ray constrast agent iopromide and the fluoroquinolone antibiotic ofloxacin by *T. versicolor*, selected because they are ubiquitous PhACs in hospital wastewaters. In addition, a hospital wastewater was treated in a batch bioreactor treatment in order to removed iopromide and ofloxacin at their pre-existent concentration at non-sterile conditions. The identification of the transformation products of both PhACs in the hospital wastewater through the treatment was also attempted.

Chapter XI focused in the non-sterile hospital wastewater treatment by *T. versicolor* in a fluidized bed bioreactor, in order to remove PhACs at their pre-existent concentration and looking the possibility to treat specific effluents with concentrated loads of PhACs.

Finally, section 3 summarizes the general conclusions of the thesis (chapter XII) and future prospects are discussed (chapter XIII).

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SECTION 2:

RESEARCH WORK

SECTION 2.1:

Degradation of individual pharmaceuticals

Chapter III:

White rot fungus –mediated degradation of the analgesic ketoprofen and identificaiton of intermediates by HPLC-DAD-MS and NMR

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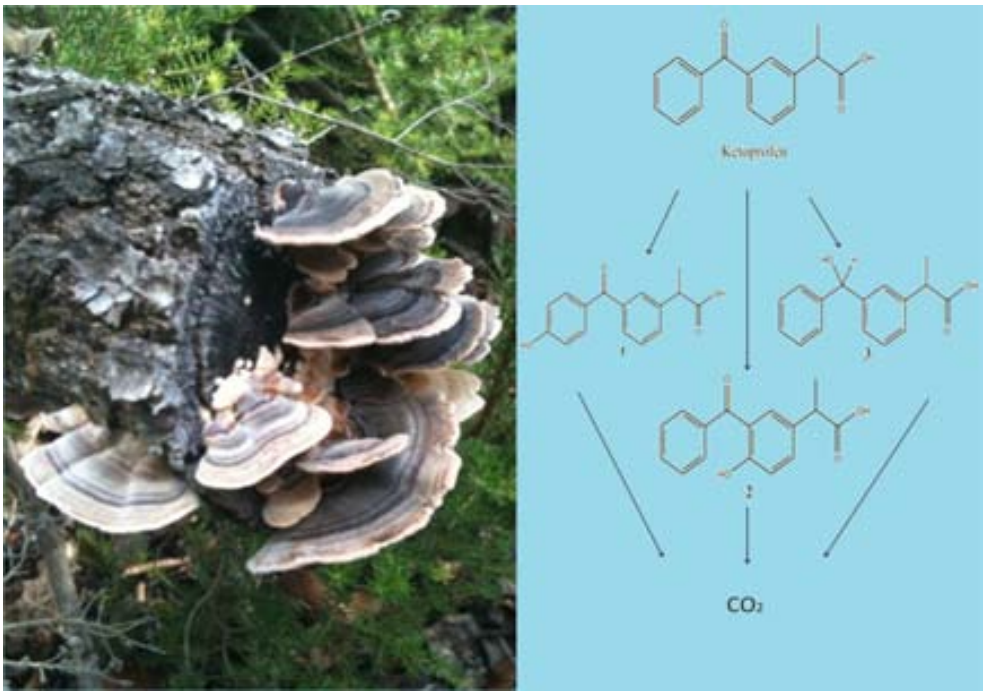

Abstract

Ketoprofen is a nonsteroidal anti-inflammatory drug that has been detected in the environment in the range of ng L^{-1} to µg L^{-1} due to its low degradability in some wastewater treatment plants. In this study, the use of the white-rot fungus *Trametes versicolor* to effectively degrade ketoprofen in a defined liquid medium was assessed. The fungus eliminated ketoprofen to non-detectable levels in 24 h when it was added at 10 mg L^{-1} whereas at low concentration of 40 µg L^{-1} it was almost completely removed (95%) after 5 h. Low extracellular laccase activity was detected in the *T. versicolor* cultures but the addition of the laccase-mediator system did not lead to ketoprofen oxidation. The cytochrome P450 inhibitor 1-aminobenzotriazole reduced ketoprofen oxidation. These data suggest that the first oxidation step is cytochrome P450 mediated. During time-course degradation experiments, three intermediates were structurally elucidated and quantified by HPLC–DAD–MS and NMR: 2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid, 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid, and 2-(3-benzoyl-4-hydroxyphenyl)-propanoic acid. The latter was reported for the first time in biological systems. After 7 d of incubation, only small amounts of 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid (0.08 mg) remained in the liquid medium in comparison with the initial ketoprofen dose (1.0 mg), suggesting possible mineralization of ketoprofen.

Keywords: Ketoprofen, biodegradation, Trametes versicolor, HPLC-DAD-MS, NMR.

III.1. Introduction

The presence of pharmaceuticals in the environment has received more attention over the last decade due to the potential adverse environmental and human health effects. A remarkable group of such pharmaceuticals comprises non-steroidal anti-inflammatory drugs (NSAID), which exhibits anti-inflammatory, analgesic and antipyretic activities.

Ketoprofen (2-(3-benzoylphenyl)-propanoic acid) is a type of NSAID, extensively used as non-prescription drug, which has been detected in surface waters in concentrations ranging from ng L^{-1} up to μg L^{-1} (Metcalfe et al., 2003; Tixier et al., 2003).

After intake of ketoprofen in humans, it is primarily metabolized by acyl glucuronidation and subsequently excreted in the urine for more than 80% of the given doses (Foster et al., 1988; Skordi et al., 2004). Once glucuronide conjugates reached wastewater treatment plants (WWTPs), they can be cleaved by enzymatic processes releasing ketoprofen. Great variability in the ketoprofen removal efficiencies in WWTPs were reported by several authors, ranging from 38% to almost 100% (Lindqvist et al., 2005; Santos et al., 2007). Results obtained by Quintana et al. (2005) using activated sludge as inoculum under aerobic conditions indicated that ketoprofen, added at 20 mg L^{-1}, was partially mineralized as a sole source of carbon and energy by microorganismsin WWTPs. Other authors suggested direct phototransformation and biodegradation as the main elimination processes of ketoprofen in the environment (Tixier et al., 2003; Matamoros et al., 2009). In any case, ketoprofen is not completely removed in most of sewage treatment and it is detected in both sewage sludge and effluent from WWTPs (Radjenovic´ et al., 2009).

For pharmaceuticals like NSAID, bioremediation can be a feasible removal process to be applied in wastewaters as well as sewage sludge. Recently, Marco-Urrea et al. (2009a) showed the capability of white-rot fungi (WRF) to degrade the anti-

inflammatory ibuprofen, the lipid regulator clofibric acid and the antiepileptic carbamazepine, the two latter considered very poor biodegradable in WWTPs (Radjenovic´ et al., 2007). From a biodegradation point of view, WRF are considered an interesting group of microorganisms due to the non-specificity of their extracellular ligninolytic enzymes, that include high redox potential peroxidases (lignin peroxidase, manganese peroxidase and versatile peroxidase) and laccases (Martínez et al., 2005). Alternatively, WRF have the potential to metabolize xenobiotics by the cytochrome P450 monooxygenases, a mechanism typically found in mammal systems that incorporate one atom of oxygen into the substrate (Doddapaneni and Yadav, 2004). Since these enzymatic mechanisms have wide substrate specificity, they can simultaneously attack a broad range of organic compounds, which is relevant for pharmaceutical remediation purposes. In this regard, degradation of pharmaceuticals contained in real sewage sludges by *Trametes versicolor* in solid-phase and bioslurry systems is now underway in our research group and preliminary assays revealed promising results.

In this work, the feasibility of ketoprofen degradation by a white-rot fungus, *T. versicolor*, in a liquid medium was studied for the first time. Obviously pharmaceuticals are found in the environment in a complex mixture of organics but single substance degradation studies allow a better mechanistic assessment of xenobiotic degradation. The enzymatic system responsible for ketoprofen degradation and the identification of degradation intermediates were also assessed.

III.2. Materials and methods

III.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 d.

Pellets of *T. versicolor* were produced by inoculating a 1 L Erlenmeyer flask containing 250 mL of malt extract medium with 1 mL of a mycelial suspension and shaking (135 rpm, r = 25 mm) at 25 ºC for 5 d (Marco-Urrea et al., 2009a). Pellets formed by this process were washed with sterile deionised water.

Ketoprofen was obtained from Sigma–Aldrich (Barcelona, Spain). Purified laccase from *T. versicolor* was obtained from Fluka (Barcelona, Spain). The following chemicals were used in the analyses for identification and quantification of the metabolites: CH_3CN supergradient HPLC grade, H_2O LC–MS grade, CH_3CO_2H (96%, v/v) reagent grade (Scharlau, Barcelona, Spain), CD_3OD (99.80% D) (Euriso-top, Saint-Aubin Cedex, France), and 3-(Trimethylsilyl)- 1-propanesulfonic acid sodium salt (DSS sodium salt, 97%) (Sigma–Aldrich, Barcelona, Spain).

All other chemicals used were of the highest available purity and were purchased from Sigma–Aldrich (Barcelona, Spain).

III.2.2. Experimental procedures

III.2.2.1. In vivo degradation experiments

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing appropriate amounts of mycelial pellets in a total volume of 25 mL of a chemically defined medium (pH 4.5) described elsewhere (Marco-Urrea et al., 2009a). Degradation experiments included uninoculated controls containing 25 mL of defined medium as well as heat-killed controls that consisted of autoclaved cultures performed under conditions identical to those of the experimental cultures. When required, controls containing 10 mM of sodium azide were included.

Ketoprofen (20 µL) was added into the flasks to give the desired final concentration (approximately 11 mg L^{-1} except where specified) from a stock solution in ethanol. After ketoprofen addition, flasks were incubated under shaken conditions (135 rpm) at 25 ºC. To obviate the possible influence of light on ketoprofen stability, all the experiments were carried out in the dark.

In time-course experiments, 1mL sample was withdrawn at each time point, which was filtered through a Millex-GV (Millipore, Barcelona, Spain) 0.22 µm filter and subsequently analyzed by HPLC. Degradation at a specified interval was calculated by comparing ketoprofen concentration in the uninoculated controls with that in the

experimental flasks. All the degradation values were corrected for the sorption values determined in either heat-killed or sodium azide control flasks.

When ketoprofen was added at low concentration (40 µg L^{-1}, we proceed as stated above but Erlenmeyer flasks contained a total volume of 50 mL of defined medium instead of 25 mL. Entire flasks were sacrificed at each experiment time and they were filtered through 0.45 lm glass fiber filter from Whatman (Barcelona, Spain). The target compound in the liquid medium was extracted in one step by solid-phase extraction with Oasis HLB cartridges (60 mg adsorbent, Waters, Barcelona, Spain) as is described elsewhere (Radjenovic et al., 2007). Briefly, the cartridges were reconditioned sequentially with 5 mL of methanol and 5 mL of deionized water. After that, the sample was passed through the cartridge and it was dried under vacuum. Then, the adsorbed compounds were eluted with methanol (2 x 2 mL) and subsequently concentrated to dryness under a gentle nitrogen stream. The extracts were reconstituted with 0.5 mL 25:75 (v/v) methanol-water. Extraction efficiency of ketoprofen, evaluated by recovery experiments, was 91.5%.

III.2.2.2. Experiments with cytochrome P450 inhibitors and purified laccase

Laccase-mediated degradation experiments were performed in serum bottles containing 10 mL of a purified laccase solution at a final enzyme activity of 10 000 AU L^{-1} (pH 4.5). Effect of the laccase mediator ABTS (2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) was evaluated adding 0.8 mM ABTS to the reaction mixture. The bottles were incubated on an orbital shaker (135 rpm) at 25 ºC for 20 h.

For those microcosms that were tested with the cytochrome P450 inhibitor, 1-aminobenzotriazole was added to a final concentration of 5 mM in experiments performed as described in Section 2.2.1. Heat-killed and inhibitor-free controls were included in duplicate.

In all the cases, ketoprofen was added at a final concentration of 10 mg L^{-1}.

III.2.3. Analytical procedures

III.2.3.1. Analysis of ketoprofen

Analysis of ketoprofen was performed using a Dionex 3000 Ultimatehigh performance liquid chromatography (HPLC) (Barcelona,Spain) equipped with a UV detector at 230 nm. The column temperature was 30 ºC and a sample volume of 20 µL was injected from a Dionex autosampler (Barcelona, Spain). Chromatographic separation was achieved on a GraceSmart RP 18 column (250 mm x 4 mm, particle size 5 µm). The mobile phase consisted of 6.9 mmol L^{-1} acetic acid in water adjusted to pH 4 (by NaOH) with 35% v/v acetonitrile. It was delivered isocratically at 1 mL min^{-1} as was described elsewhere (Stafiej et al., 2007). The detection limit was >0.125 mg L^{-1}.

III.2.3.2. Identification and quantification of metabolites

i. HPLC-diode array detection-electrospray ionization mass spectrometry (HPLC-DAD-MS) analyses.

HPLC–DAD–MS analyses were performed in order to follow the degradation of ketoprofen and the formation of derivate metabolites in the degradation experiments. The MS data contributed in the identification of the metabolites. HPLC–DAD–MS measurements were carried out using an Agilent 1200 liquid chromatograph, with an autosampler (Agilent Technologies Deutschland GmbH, Böblingen, Germany), coupled via a split unit (Bruker NMR MS interface, BNMI) to a Bruker diode array detector (Bruker Biospin, Karlsruhe, Germany) and to a Bruker Esquire 6000 octopole ion-trap mass spectrometer, equipped with an electrospray ionization (ESI) source (Bruker Daltonik, Bremen, Germany). Chromatographic and MS data were collected and treated using Bruker HyStar 3.2 and Bruker Daltonics 3.2 software (Barcelona, Spain).

The analyzed samples consisted of aliquots of time-course degradation experiment described in Section 2.2.1 at experimental times of 0, 1, 3, 6 and 24 h. Separation was performed on the Grace-Smart RP C18 column described before and the mobile phase consisted of 65% of (A) 1.0 mM acetic acid in water and 35% of (B) acetonitrile. The flow rate was 1 mL min^{-1}, the chromatography was run under isocratic conditions during 40 min and the injection volume was 100 µL. The system was

operated at 25 ºC. The HPLC flow was split 95:5 (DAD:MS). The chromatogram was recorded at 230 nm. ESI–MS data were acquired in the negative mode over a scan range between 50 and 1000 Da. The electro spray conditions were the following: temperature of the ESI interface heated capillary set to 300 ºC, nebulizer gas (N_2) pressure of 2.5 bar (37 psi) and dry gas (N_2) flow of 6 L min^{-1}.

ii. Nuclear magnetic resonance (NMR) experiments.

To identify and quantify the major metabolites of ketoprofen, 80 mL of defined medium were added to 500 mL Erlenmeyer flasks and subsequently they were inoculated with 20 g of wet pellet of *T. versicolor*. Each flask contained ketoprofen at a final concentration of 10 mg L^{-1} and they were incubated under shaking conditions (135 rpm) at 25 ºC. Uninoculated flasks were included as controls. At each time point (0, 1, 24 h and 7 d) one flask was sacrificed, filtered through a 0.45 µm glass fiber filter (Whatman, Barcelona, Spain) and cooled immediately. Extraction and preconcentration was performed by solid-phase extraction as described in Section 2.2.1 but the extracts were reconstituted in 0.6 mL of CD_3OD after evaporation to dryness under nitrogen.

^{1}H (500.13 MHz) and ^{13}C (125.76 MHz) NMR spectra of the samples were recorded on an AVANCE 500 Bruker spectrometer equipped with a high-sensitivity cryogenically cooled triple-resonance TCI probehead (Bruker Biospin, Karlsruhe, Germany). The structural characterization of all compounds was carried out with the aid of 2D NMR correlations, such as COSY (Correlated Spectroscopy), NOESY (Nuclear Overhauser and Exchange Spectroscopy), HSQC (Heteronuclear Single-Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation). The ^{13}C NMR spectroscopic peaks were assigned by HSQC and HMBC experiments.

To quantify the identified metabolites and the ketoprofen at each experimental time, 0.283 mg (1.26 µmol) of 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS sodium salt) were added in each NMR sample and they were completely dissolved. After that, a ^{1}H NMR spectrum of each sample was performed with 64 number of scans and a relaxation delay of 2 s. The ratio between the integration of the

DSS peaks and the peaks corresponding to every metabolite provided the µmols and mg (due to their identified structure and therefore known molecular weight) of them in each sample.

III.2.3.3. Other analyses

Laccase activity was assayed in 100 mM sodium phosphate buffer, pH 5, using 10 mM DMP as substrate and measuring the production of coerulignone (e469 = 27 500 M^{-1} cm^{-1}, when referred to DMP concentration) (Martínez et al.,1996). Manganese-peroxidase activity was determined as described elsewhere (Camarero et al., 1999).

Mycelial dry weights were determined by vacuum filtering the cultures through preweighed glass filters (Whatman, Barcelona, Spain). The filters containing the mycelial mass were placed in glass dishes and dried at 100 ºC to constant weight.

III.3. Results and discussion

III.3.1. Degradation of ketoprofen by *T. versicolor*

Time-course degradation experiment of ketoprofen is shown in figure III.1A. The experiment was carried out with the fungus growing in a defined medium and besides the uninoculated control two different treatments were added to discard the role of sorption in analgesics removal: cultures containing sodium azide and heat-killed controls. As can be observed, removal of ketoprofen appeared to be almost immediately after its addition to the cultures. Near the half of ketoprofen was eliminated after the sixth hour and after 24 h of incubation it was completely eliminated from the liquid medium. The fast degradation rate observed for ketoprofen is not in accordance with previous published reports regarding degradation of other environmental pollutants by *T. versicolor* under the same culture conditions tested here, in which a lag phase of at least one day was commonly observed before starting degradation (Marco-Urrea et al., 2009b).

Our results revealed that ketoprofen concentration in both sodium azide and uninoculated flasks was almost the same through the studied period. In the case of mammals, sodium azide is applied to inhibit ATPase activity of cells that allows discriminating between energy-dependent transports of drugs across the cell membrane (virtually inhibited by sodium azide) such as active carrier-mediated transport or vesicular pathways, and passive uptake, which is usually associated to physico chemical processes (Gabor and Wirth, 2008). Interestingly, degradation of pollutants by fungi can be carried out in two phases: a surface binding to the fungal cell of physicochemical nature (biosorption), usually fast, reversible and energy-independent; followed by a metabolism-dependent phase, whereby the compound is transported across the membrane into the cell (Jarosz-Wilkolazka et al., 2002; Verdin et al., 2005). Based on these evidences, our results suggest the possible involvement of an energy-dependent transporter of ketoprofen into the cell, where degradation may take place. This active transport would be impeded in sodium azide cultures because of a lack of intracellular ATP, leading to ketoprofen concentrations in the medium similar to those observed in uninoculated controls (figure III.1A). However, more research is needed in the future to confirm this initial finding.

Removal percentage of ketoprofen in heat-killed controls was near 15% in average, but it could be ascribed solely to sorption. It is known that fungal cells exposed to heat treatments alter the physicochemical properties of the cell wall leading to a greater, equivalent to or less bioadsorptive capacities than that of living cells depending the pollutant (Arica et al., 2003). In any case, degradation metabolites of ketoprofen were observed neither in heatkilled nor in the sodium azide controls indicating that disappearance of ketoprofen from experimental flasks was due to degradation.

To assess the relevance of using *T. versicolor* for ketoprofen degradation in regards to the normal concentrations in which the pharmaceuticals are found in the environment, a degradation experiment was performed at 40 μg L^{-1}. As shown in figure III.1B, after 5 h of incubation ketoprofen was almost completely removed (95%) and it

was not detected at 24 h. In this case, adsorption levels in heat-killed controls were not relevant.

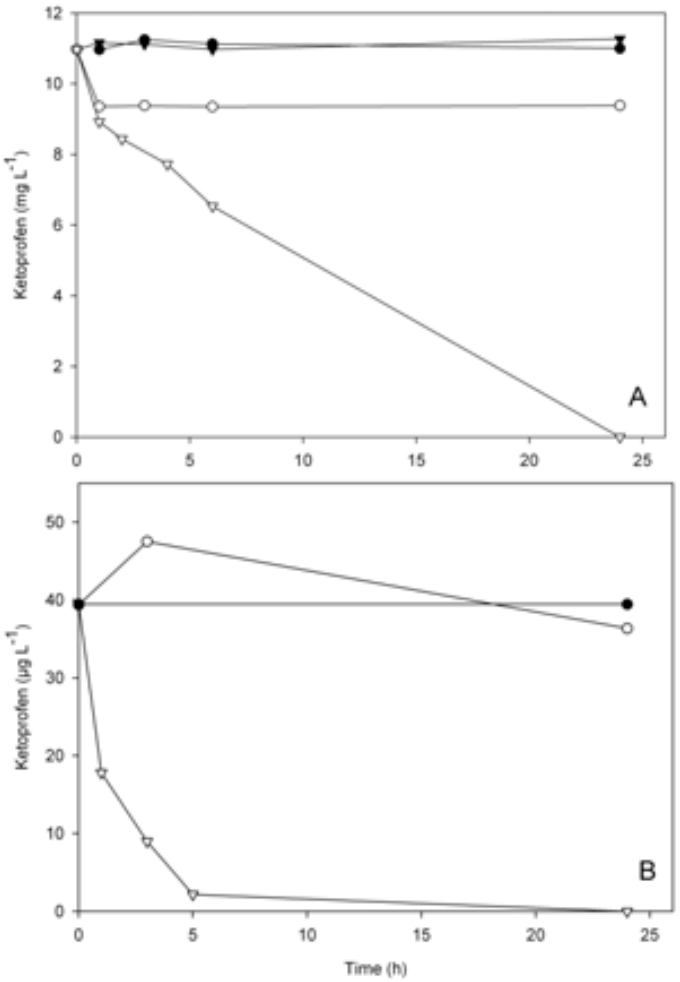

Figure III.1: Time-course degradation of ketoprofen added at 11 mg L^{-1} (A) and 40 µg L^{-1} by *T. versicolor*. Symbols: uninoculated controls (●), experimental cultures (▽), heat-killed (○) and sodium azide controls (▼). Values plotted are means for duplicate cultures. The initial mycelial pellets dry weight added to each flask was 124.2 ± 5.2 mg (A) and 233.2 ± 8.4 mg (B).

III.3.2. Effect of laccase-mediator system and cytochrome P450 inhibitor on degradation of ketoprofen

During ketoprofen degradation experiments, extracellular activities of laccase were detected in experimental flasks (peak of 94.3 AU L_1 at 24 h) whereas peroxidase activities were not observed. Thus, possible involvement of laccase in ketoprofen

degradation was investigated adding purified laccase and the well known laccase redox mediator ABTS but ketoprofen was not oxidized in any of these treatments (data not shown). The effect of 1-aminobenzotriazole, a cytochrome P450 inhibitor, on the fungal degradation of ketoprofen was also studied (see Supplementary material). Inhibitor-free cultures and those containing 1-aminobenzotiazole showed similar percentages of ketoprofen removal for the first 6 h. After this period, degradation was inhibited in cultures with 1-aminobenzotriazole whereas ketoprofen in inhibitor free-cultures was almost totally degraded after 24 h. These data combined with the data showing no participation of laccase in ketoprofen degradation (see above) suggest the involvement of P450-type catalyst(s) in the degradation of this anti-inflammatory drug. Furthermore, they additionally support our hypothesis of the involvement of an energy-dependent transport in ketoprofen degradation. Thus, the disappearance of ketoprofen in cultures containing 1-aminobenzotriazole as well as in those without the cytochrome P450 inhibitor the first 6 h may be due to the active transport of the pharmaceutical into the cell, a mechanism that was previously shown to be blocked in sodium azide controls. Once inside the cell, 1-aminobenzotriazole may inhibit the action of the intracellular cytochrome P450 system impeding ketoprofen degradation, whereas ketoprofen degradation took place in free-inhibitor cultures.

III.3.3. Identification of degradation products of ketoprofen

During ketoprofen degradation, three new metabolites of relatively high intensity were detected and tentatively identified by means of HPLC–DAD–MS and NMR analyses. HPLC–DAD–MS chromatograms reveal the formation of three metabolites, peaks 1–3 in figure III.2A; peak 4 corresponds to ketoprofen. Figure III.2A shows the base peak chromatograms at three experimental times: before starting the experiment (which consists in the uninoculated sample) (a), after 3 h of experiment (b) and after 6 h of experiment (c). The negative- ion ESI–MS spectra for the four compounds are also presented (figure III.2B).

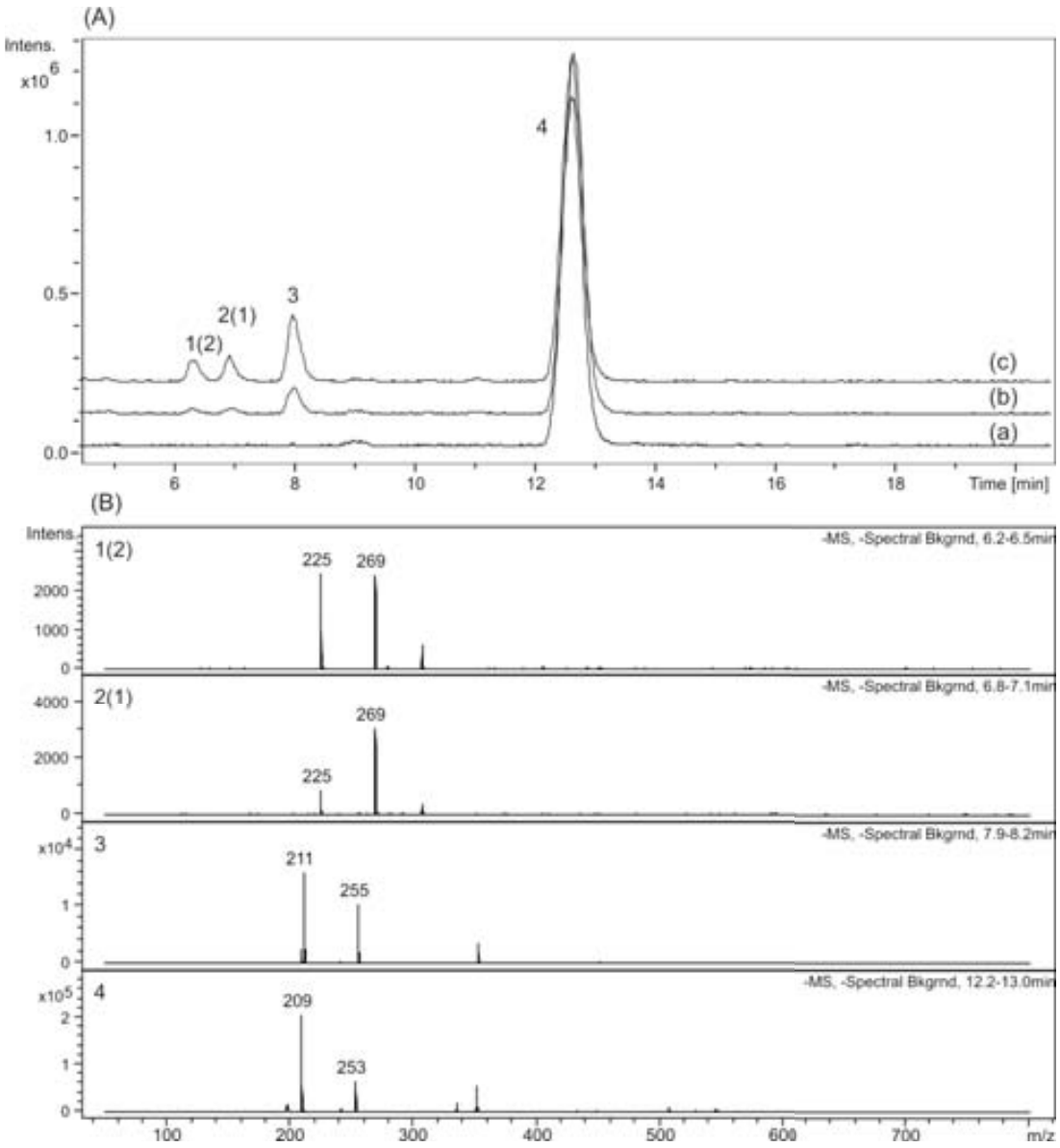

Figure III.2: (A) Base peak chromatogram of HPLC-DAD-MS samples at experimental times 0 h (a), 3 h (b) and 6 h (c). (B) Negative-ion ESI-MS spectra of peaks 1-4.

The negative-ion electrospray ionization mass spectrum of ketoprofen, 4, shows a molecular ion $[M–H]^-$ at *m/z* 253 and a fragment ion $[M–CO_2]^-$ at *m/z* 209. Compounds 1 and 2 present a mass spectra with two major ions at *m/z* 269 and 225, which would correspond to a molecular ion $[M–H]^-$ and to the fragment ion $[M–CO_2]^-$. These molecular masses could correspond to the addition of a hydroxyl group to two different sites of the ketoprofen molecule, being 1 and 2 constitutional isomers.

The negative-ion ESI mass mass spectrum of compound 3 shows, as well, two major ion peaks at m/z 255 and 211 corresponding to the molecular ion $[M–H]^-$ and to the fragment $[M–CO_2]^-$ respectively. These molecular masses could correspond to a reduction of a carbonyl group of the ketoprofen molecule, being transformed to a hydroxyl group and being the molecular mass of compound 3 the sum of the mass of ketoprofen +2. In order to corroborate the above hypotheses and to identify the final structures of the three metabolites, NMR analyses were needed.

Samples at experimental times 0, 1, 24 h and 7 d were prepared for the NMR analysis as described in Section 2.3.2.2. ^{1}H NMR spectra of each sample were recorded and they were compared. Sample at 1 h showed ketoprofen as the major compound and, contrary to the 0 h sample, some weak aromatic peaks were observed in the 7.1–7.4 ppm region. In the 24 h sample these peaks correspond to the major compound and some weak aromatic peaks corresponding to ketoprofen and to other compounds not identified then, were observed. After 7 d of experiment the proton NMR spectrum showed practically no peaks at the aromatic region, only very weak signals corresponding to the major compound at 24 h.

One hour and twenty-four hour samples were studied more in detail. With the aid of 2D NMR experiments, such as COSY, HSQC and HMBC, ^{1}H and ^{13}C NMR resonances of ketoprofen were assigned from sample at 1 h (Table III.1 and figure III.3). As in sample at 1 h, different 2D NMR experiments were performed in the 24 h sample in order to characterize and identify the three metabolites. COSY, NOESY, HSQC and HMBC experiments were acquired.

Table III.1: Description of ^{1}H and ^{13}C NMR chemical shifts (δ) and H,H-coupling constants ($^{3}J_{H,H}$) of metabolites 1 and 3 and ketoprofen. Data were obtained from samples dissolved in CD_3OD and spectra were acquired at 25 ºC and at a magnetic field of 500 MHz.

Atom	Ketoprofen		1		3	
	δ (^{1}H) [ppm] and $^{3}J_{H,H}$ [Hz]	δ (^{13}C) [ppm]	δ (^{1}H) [ppm] and $^{3}J_{H,H}$ [Hz]	δ (^{13}C) [ppm]	δ (^{1}H) [ppm] and $^{3}J_{H,H}$ [Hz]	δ (^{13}C) [ppm]
a		177.8		177.8		178.1
b	3.82 (q, *J* = 7.l, 1H)	46.2	3.82 (q, *J* = 7.5, 1H)	46.1	3.69 (q, *J* = 7.l, 1H)	46.3
c	1.49 (d, *J* = 7.1, 3H)	18.7	1.50 (d, *J* = 7.5, 3H)	18.8	1.42 (d, *J* = 7.1, 3H)	18.8
d		142.9		142.8		142.8
e	7.75 (s, 1H)	129.9	7.67 (s, 1H)	129.9	7.37 (s, 1H)	126.6
f		138.7		139.2		146.1
g	7.66 (d, *J* = 7.3, 1H)	129.4	7.60 (d, *J* = 8.5, 1H)	129.1	7.25 (d, *J* = 7.4, 1H)	129.1
h	7.50 (t, *J* = 7.8, 1H)	129.5	7.48 (m, 1H)	129.4	7.23 (m, 1H)	126.2
i	7.62 (d, *J* = 7.8, 1H)	132.7	7.57 (d, 1H)	132.0	7.20 (d, *J* = 7.3, 1H)	127.1
j		198.2		197.7	5.76 (s, 1H)	76.6
k		138.7		a		145.3
l	7.78 (d, *J* = 7.8, 1H)	130.7	7.72 (d, *J* = 8.8, 1H)	133.7	7.36 (d, *J* = 7.3, 1H)	127.5
m	7.54 (t, *J* = 7.8, 1H)	129.3	6.89 (d, *J* = 8.8, 1H)	116.0	7.30 (t, *J* = 7.4, 1H)	128.9
n	7.64 (m, 1H)	133.5		163.4	7.22 (m, 1H)	127.9
o	7.54 (t, *J* = 7.8, 1H)	129.3	6.89 (d, *J* = 8.8, 1H)	116.0	7.30 (t, *J* = 7.4, 1H)	128.9
P	7.78 (d, *J* = 7.8, 1H)	130.7	7.72 (d, *J* = 8.8, 1H)	133.7	7.36 (d, *J* = 7.3, 1H)	127.5

[a] Not detected, possibly overlapped.

Figure III.3 shows the proton NMR spectrum of both samples. The structures of ketoprofen and of metabolites 1, 2 and 3 are presented and their most important peaks are indicated on the spectra. Table III.1 describes the ^{1}H and ^{13}C NMR assignments of ketoprofen, metabolite 1 and metabolite 3. Although metabolite 2 was identified by NMR, its complete ^{1}H and ^{13}C characterization by NMR was not possible, due to the overlap of its signals. The study of the 24 h sample allowed the complete ^{1}H and ^{13}C characterization of the derivate 3 (major metabolite) and derivate 1. As shown, the aromatic protons of compound 3 are high field shifted with respect to those of the ketoprofen.

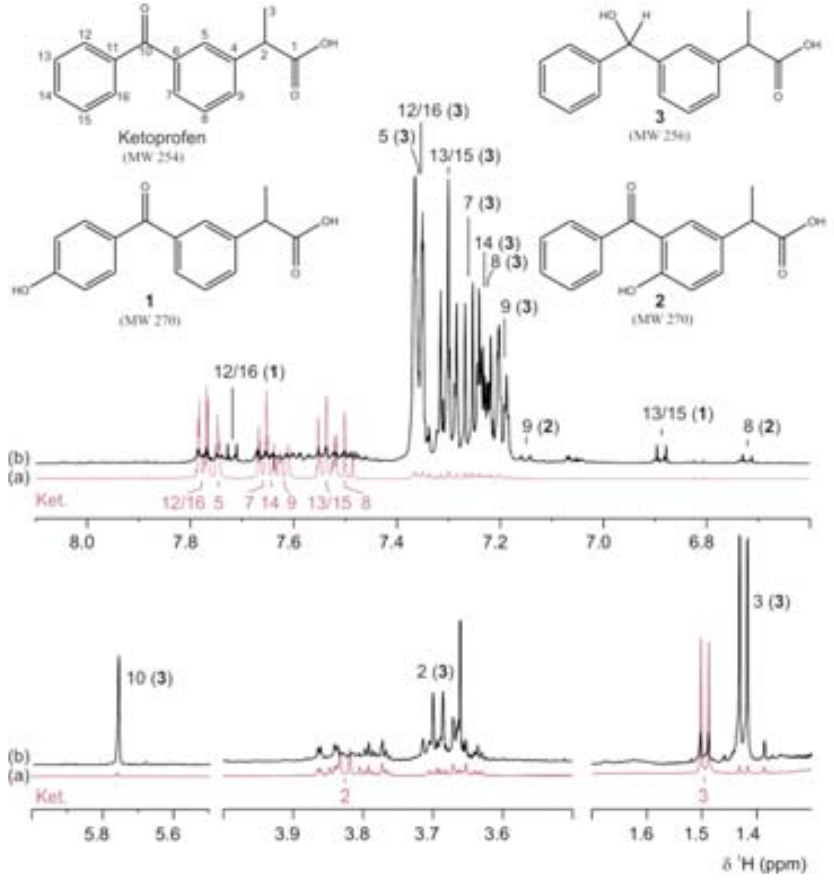

Figure III.3: Structure of ketoprofen and its metabolites 1–3 and some important regions of the ^{1}H NMR spectra of NMR sample at 1 h (a) and sample at 24 h (b). Most characteristic peaks of each compound are indicated. Both samples were dissolved in CD_3OD and the spectra were acquired at 25 ºC and at a magnetic field of 500 MHz.

A HMBC correlation between the singlet at 5.76 ppm and He, Hg, Hl and Hp of compound 3 is observed. Moreover, the singlet at 5.76 ppm is directly bonded to a

carbon at 76.7 ppm (from the HSQC experiment), confirming that Cj in compound 3 is bond to a hydroxyl group. The protons of the propionic chain (Hb and Hc) are also low field shifted with respect to their ketoprofen analogues.

Compound 1 is present in the 24 h sample in a very low amount. However, it was possible to characterize it completely (see Table III.1). It presents two coupled doublets at 7.72 ppm and at 6.89 ppm corresponding to Hl/Hp and Hm/Ho, with a coupling constant of 8.8 Hz (figure III.3), and their directly coupled carbons resonate at 133.7 ppm and 116.0 ppm respectively. The HMBC experiment presents a correlation between Hl/Hp and Hm/Ho and a carbon at 163.4 ppm (Cn), which confirms a hydroxyl substitution in the Cn.

Finally, a partial characterization of compound 2 was achieved and made possible the identification of its structure. A proton presented as a doublet at 6.72 ppm (Hh) coupled to a less intense doublet at 7.15 ppm (Hi), with a coupling constant of 8.3 Hz was observed. The HSQC experiment shows that they are directly bonded to a carbon at 115.8 ppm (Ch) and at 128.9 ppm (Ci). Hh is correlated via HMBC to a carbon at 157.5 ppm (Cg), which could correspond to an aromatic carbon bonded to a hydroxyl group, and to a quaternary carbon at 136.7 ppm (Cf). In the HMBC experiment it is also observed a correlation between Hf and a quaternary car carbon at 145.4 ppm (Cd) and a weak correlation with the carbon at 157.7 ppm (Cg). It was not possible to get more NMR information about compound 2 due to its low concentration and probably by the overlapping of its peaks. However, considering the above NMR data and the MS results the structure of compound 2 was identified as a hydroxyl derivative of ketoprofen with the hydroxyl substitution in Cg.

III.3.4. Quantification of the metabolites of ketoprofen and proposed degradation pathway

The above identified metabolites were quantified at experimental times 0, 1, 24 h and 7 d, adding a specific amount of DSS as an internal standard in each NMR sample (see Section 2.3.2.ii). As shown in Table III.2, the major degradation metabolite was 3 (2-(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid) that appeared at low

concentrations after 1 h of incubation and reached the maximum value after 24 h. Metabolites 2 (2-(3-benzoyl-4-hydroxyphenyl)-propanoic acid) and 1 (2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid) were formed in minor proportion and solely appeared after 24 h, although probably higher concentrations could be achieved in the period from 1 to 24 h. After 7 d of incubation only small amounts of 3 (0.32 µmol, 0.08 mg) were observed in comparison with the initial dose of ketoprofen added (3.93 µmol, 1.0 mg). The mass balance of this experiment together with the fact that only very weak signals of compound 3 were observed at the aromatic region of the NMR spectrum at day 7, suggest ketoprofen mineralization by *T. versicolor* (figure III.4).

Table III.2: Quantification by NMR of ketoprofen and derivates 1-3 at experimental times 0, 1, 24 h and 7 d.

Compound	**µmol of compound**[a] (mg of compound)			
	t_0[b]	1 h	24 h	7 h
Ketoprofen	3.93 (1,00)	3.11 (0.79)	0.19 (0.05)	n.d. [c]
1	n.d.	n.d.	0.09 (0.02)	n.d.
2	n.d.	n.d.	0.08 (0.02)	n.d.
3	n.d.	0.23 (0.06)	2.06 (0.53)	0.32 (0.08)

[a] Measuments done considering the amount of DSS added to each sample (0.283 mg, 1.259 µmol) and the relative areas between DSS and the compound of interest.
[b] Feed solution with Ketoprofen before treatment.
[c] Not detected.

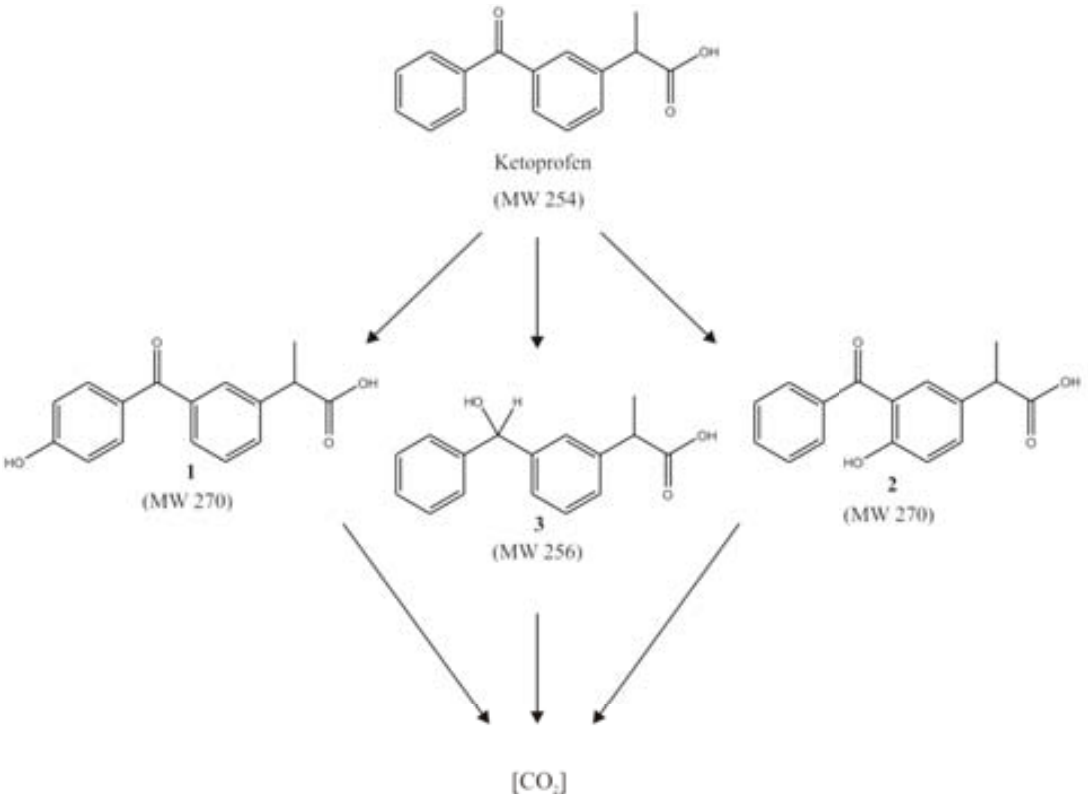

Figure III.4: Suggested degradation pathway of ketoprofen by *T. versicolor*.

Biotransformation of drugs by higher animals is usually carried out in two steps known as phase I and phase II reactions, which either modify the toxicity of drugs or change them to water-soluble forms that are readily excreted from the body. Phase I reactions typically involved oxidation by the cytochrome P450 monooxygenase, leading to a new intermediate that contained a reactive chemical group (hydroxyl). Following administration of ketoprofen to mammals, formation of compounds 1 and 2-[3-(3-hydroxybenzoyl)phenyl]-propanoic acid (non-identified in our study) by hydroxylation of the aromatic ring, and compound 3 by reduction of the ketone group, were previously reported as phase I degradation metabolites (Alkatheeri et al., 1999; Skordi et al., 2004). The fact that ketoprofen degradation was also inhibited after addition of cytochrome P450 inhibitor 1-aminobenzotriazole (Section 3.2) suggests the possible involvement of this intracellular system in the first step of ketoprofen degradation by *T. versicolor*.

Quintana et al. (2005) also proposed metabolite 3 as intermediate of ketoprofen mineralization from batch trials with activated sludge. In this case,

metabolite 3 underwent dioxygenation and formation of the respective catechol, which was subsequently ring-opened by oxidation. As far as we know, metabolite 2 is for the first time reported.

In the case of mammals, both ketoprofen and hydroxylated metabolites obtained in Phase I underwent extensive conjugation with *D*-glucuronic acid in Phase II reactions, and were excreted mainly in urine (Alkatheeri et al., 1999; Skordi et al., 2004). Phase II enzymes such as glucosides and xylosidases and the formation of the conjugated derivatives of some xenobiotics were previously reported in WRF (Bezalel et al., 1997; Reddy et al., 1997; Ichinose et al., 1999; Hundt et al., 2000). Nevertheless, conjugates of ketoprofen in *T. versicolor* metabolism were not observed, nor in their ESI–MS spectra neither by NMR (2D HMBC or 2D NOESY) experiments.

III.4. Conclusions

The feasibility of *T. versicolor* to degrade ketoprofen at 10 mg L^{-1} but also at the environmental relevant concentration of 40 μg L^{-1} was demonstrated. A high degradation rate of the parent compound and almost all the three identified metabolites was observed, suggesting possible mineralization of ketoprofen, which is of interest for bioremediation purposes. Cytochrome P450 system seems to catalyze the first step of ketoprofen degradation, similarly to the degradation pathway described for mammals, but ligninolytic enzyme system (laccase) did not play a role on ketoprofen degradation. It is noteworthy that one of the identified ketoprofen degradation metabolites, 2-(3-benzoyl-4-hydroxyphenyl)-propanoic acid, was reported for the first time in biological systems.

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Chapter IV:

Degradation of the drug sodium diclofenac by *Trametes versicolor* pellets and identification of some intermediates by NMR.

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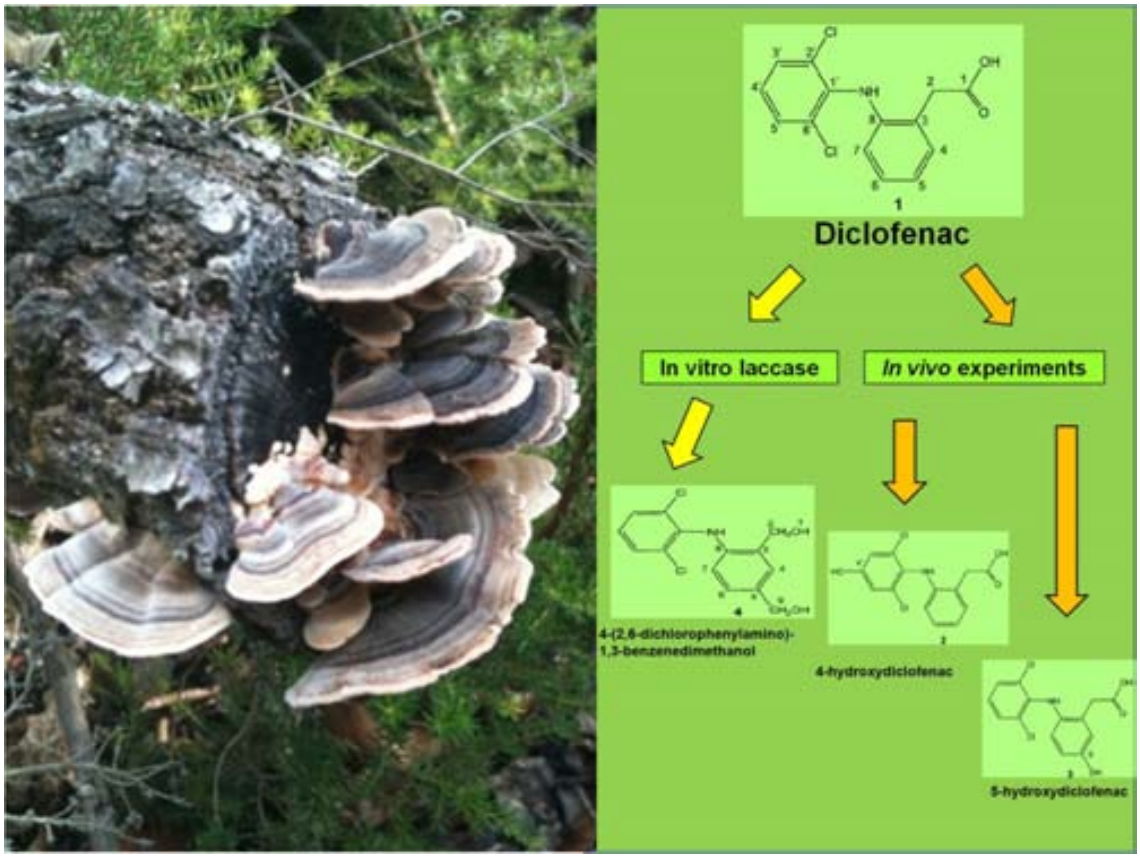

Abstract

Degradation of diclofenac sodium, a nonsterodial anti-inflammatory drug widely found in the aquatic environment, was assessed using the white-rot fungus *Trametes versicolor*. Almost complete diclofenac removal (≥ 94%) occurred the first hour with *T. versicolor* pellets when the drug was added at relatively high (10 mg L^{-1}) and environmentally relevant low (45 μg L^{-1}) concentrations in a defined liquid medium. *In vivo* and *in vitro* experiments using the cytochrome P450 inhibitor 1-aminobenzotriazole and purified laccase, respectively, suggested at least two different mechanisms employed by *T. versicolor* to initiate diclofenac degradation. Two hydroxylated metabolites, 4'-hydroxydiclofenac and 5-hydroxydiclofenac, were structurally elucidated by nuclear magnetic resonance as degradation intermediates in fungal cultures spiked with diclofenac. Both parent compound and intermediates disappeared after 24 h leading to a decrease in ecotoxicity calculated by the Microtox test. Laccase-catalysed transformation of diclofenac led to the formation of 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol, which was not detected in *in vivo* experiments probably due to the low laccase activity levels observed through the first hours of incubation.

Keywords: Diclofenac, degradation, laccase, *Trametes versicolor*, white-rot fungus, NMR

IV.1. Introduction

Diclofenac sodium is a nonsterodial anti-inflammatory drug widely prescribed as an anti-inflammatory and antipyretic analgesic. The globally consumed volume of diclofenac is estimated to be 940 tons per year (Zhang et al., 2008), with a defined daily dose of 100 mg. Due to its extensive use its presence was reported in surface waters, groundwaters and drinking waters in the range of ng L^{-1} to µg L^{-1} in the last years (Zhang et al., 2008).

The metabolism of diclofenac in humans and animals has been studied extensively. Diclofenac undergoes bioactivation by cytochrome P450 oxidation to hydroxylated derivatives including 4'-hydroxy (OH) and 5-OH metabolites. Minor metabolites include 3'-OH, 3'-OH-4'-methoxy and 4',5- dihydroxy diclofenac (Stierlin et al., 1979; Shen et al., 1999; Sparidans et al., 2008). Thus, after intake, unchanged diclofenac is excreted together with its hydroxylated metabolites, and all either not conjugated or conjugated (e.g. as glucuronides) with urine and faeces enter the wastewater treatment plants (WWTP) via wastewater (Stülten et al., 2008). Once in the WWTP, its removal efficiency is still very contradictory, ranging from 0% up to 80%, but mainly in the scope of 21-40% (Zhang et al., 2008). The operating conditions of WWTP such as anoxic-oxic ratios, acidic conditions and sunlight irradiation have been proposed as a possible explanation of the wide variety of diclofenac removal efficiency (Zhang et al., 2008).

Evidences of diclofenac transformation by microbial communities are scarce in the literature. It is known that biodegradation of diclofenac in activated sludge from WWTP is very limited. Quintana et al. (2005) found no degradation of diclofenac over 28 days neither when it was the sole source of carbon nor when an external carbon source was added to achieve co-metabolic degradation. Batch elimination tests using an activated sludge system and membrane bioreactors also revealed very poor degradability of diclofenac in comparison with other pharmaceuticals (Kimura et al., 2007). Gröning et al. (2007) showed that under aerobic conditions biofilms of river

sediment extensively transformed diclofenac to *p*-benzoquinone imine of 5-OH diclofenac, which was not further degraded, in an apparent co-metabolic process.

Initial demonstrations of the ability of white-rot fungi (WRF) to remove pharmaceuticals such as antibiotics (tetracycline and oxytetracycline), lipid regulator (clofibric acid), antiepileptic (carbamazepine), analgesic (ibuprofen) and estrogens (17beta-estradiol and 17alpha-ethynylestradiol) suggests the feasibility of this group of microorganisms for pharmaceuticals bioremediation purposes (Marco-Urrea et al., 2009a; Blánquez and Guieysse 2008; Wen et al., 2009). WRF, believed to be the most effective lignin-degrading microbes in nature, produce high redox potential peroxidases (lignin peroxidase, manganese peroxidase, and versatile peroxidase), laccase and the intracellular cytochrome P450 system, among others (Marco-Urrea et al., 2009a; Martínez et al., 2005). Ligninolytic enzymes are relatively non-specific and use free radical mechanisms that allowed them to catalyze the degradation of a wide variety of environmental pollutants (Pointing, 2001).

In this work the capability of *Trametes versicolor* to degrade diclofenac in a defined liquid medium was studied. An effort was done to identify the possible enzymes involved in diclofenac degradation by *in vitro* and *in vivo* inhibitory experiments. Since products of microbial or physico-chemical transformation of pharmaceuticals can show increased toxicity compared to the parent compound (Isidori et al., 2005), degradation intermediates were identified by nuclear magnetic resonance (NMR) experiments. Finally, the ecotoxicity of the fungal treated medium was considered by using the Microtox test.

IV.2. Materials and methods

IV.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 days.

Pellets of *T. versicolor* were produced by inoculating a 1 L Erlenmeyer flask containing 250 mL of malt extract medium with 1 mL of a mycelial suspension and shaking (135 rpm, r = 25 mm) at 25 ºC for 5 d (Marco-Urrea et al., 2008). Pellets formed by this process were washed with sterile deionised water.

Diclofenac sodium salt was obtained from Sigma-Aldrich. Purified laccase from *T. versicolor* was obtained from Fluka. The following chemicals were used in the analyses for identification and quantification of the metabolites: CH_3CN supergradient HPLC grade, CH_3CO_2H (96%, v/v) reagent grade (Scharlau Chemie), and CD_3OD (99.80% D) (CortecNet).

All other chemicals used were of the highest available purity and were purchased from Sigma–Aldrich.

IV.2.2. Experimental procedures

IV.2.2.1. In vivo degradation experiments

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing appropriate amounts of mycelial pellets in a total volume of 25 mL of a chemically defined. Defined medium contained per liter: 8 g glucose, 498 mg N as ammonium tartrate, 10 and 100 ml, respectively, of a micro and macronutrient solution, and 1.168 g of 2,2-dimethylsuccinate buffer (pH 4.5) (Marco-Urrea et al., 2008). Degradation experiments included uninoculated controls containing 25 mL of defined medium as well as heat-killed controls that consisted of autoclaved cultures performed under conditions identical to those of the experimental cultures. When required, controls containing 10 mM of sodium azide were included.

Diclofenac was added into the flasks (20 µL) to give the desired final concentration (approximately 10 mg L^{-1} except where specified) from a stock solution in ethanol. After diclofenac addition, flasks were incubated under shaken conditions (135 rpm) at 25 ºC. To obviate the possible influence of light on diclofenac stability, all the experiments were carried out in the dark.

In time course experiments, 1-mL sample was withdrawn at each time point, it was filtered through a Millex-GV (Millipore) 0.22 µm filter and subsequently analyzed by HPLC. Degradation at a specified interval was calculated by comparing diclofenac concentration in the uninoculated controls with that in the experimental flasks. All the degradation values were corrected for the sorption values determined in either heat-killed or sodium azide control flasks.

When diclofenac was added at low concentration (~45 µg L^{-1} we proceed as stated above but Erlenmeyer flasks contained a total volume of 50 mL of defined medium instead of 25 mL. Entire flasks were sacrificed at each experiment time and they were filtered through 0.45 µm glass fiber filter from Whatman. The target compound in the liquid medium was extracted in one step by solid phase extraction with Oasis HLB cartridges (60 mg adsorbent, Waters) as is described elsewhere (Radjenovic et al., 2007). Briefly, the cartridges were preconditioned sequentially with 5 mL of methanol and 5 mL of deionized water at the sample pH. After that, the sample was passed through the cartridge and it was dried under vacuum. Then, the adsorbed compounds were eluted with methanol (2 x 2 mL) and subsequently concentrated to dryness under a gentle nitrogen stream. The extracts were reconstituted with 0.5 mL 25:75 (v/v) methanol-water. Extraction efficiency of diclofenac, evaluated by recovery experiments, was 97.8%.

IV.2.2.2. Experiments with purified laccase and cytochrome P450 inhibitor

Laccase-mediated degradation experiments were performed in 250 mL Erlenmeyer flasks containing 20 mL of a purified laccase solution at a final enzyme activity of 2000 AU L^{-1} (pH 4.5). Controls containing water at pH 4.5 were included. In

both treatments, diclofenac was added at a final concentration of 40 mg L^{-1} and flasks were incubated on an orbital shaker (135 rpm) at 25 ºC. At designated times, 1-mL samples were taken and 100 µL of acetic acid was added to stop the reaction prior to high-performance liquid chromatography (HPLC) analysis as described in section 2.3.1.

For those microcosms that were tested with the cytochrome P450 inhibitor, 1-aminobenzotriazole was added to a final concentration of 5 mM in experiments performed as described in section 2.2.1. Heat-killed and inhibitor-free controls were included in duplicate.

IV.2.3. Analytical procedures

IV.2.3.1. Analysis of diclofenac

Analysis of diclofenac was performed using a Dionex 3000 Ultimate HPLC equipped with a UV detector at 230 nm. The column temperature was 30 ºC and a sample volume of 20 µL was injected from a Dionex autosampler. Chromatographic separation was achieved on a GraceSmart RP 18 column (250 mm × 4 mm, particle size 5 µm). The mobile phase consisted of 6.9 mmol L^{-1} acetic acid in water adjusted to pH 4 (by NaOH) with 35% v/v acetonitrile. It was delivered isocratically at 1 mL min^{-1} as was described elsewhere (Stafiej et al., 2007). The detection limit was > 0.4 µmol L^{-1} and it was determined as the diclofenac concentration that yielded a peak height equal to three times that of baseline noise.

IV.2.3.2. Identification of degradation products by NMR analysis

To identify the major metabolites of diclofenac by *T. versicolor* pellets, 80 mL of defined medium was added to 500-mL Erlenmeyer flasks and subsequently they were inoculated with 20 g of wet pellets of *T. versicolor*. Each flask contained diclofenac at a final concentration of 10 mg L^{-1} and they were incubated under shaking conditions (135 rpm) at 25 ºC. Uninoculated flasks were included as controls as well as flasks containing the fungus without diclofenac. At each time point (0 h, 1, 3, 6, 24 h and 7 days) one flask was sacrificed, filtered through a 0.45 µm glass fiber filter (Whatman)

and cooled immediately. Extraction and preconcentration was performed by solid-phase extraction as described in section 2.2.1 but the extracts were reconstituted in 0.6 mL of CD_3OD after evaporation to dryness under nitrogen.

To identify the metabolites formed in *in vitro* experiments with pure laccase, the experiment was performed as described in section 2.2.2 but a total volume of 100 mL was added. Controls containing water at pH 4.5 with diclofenac as well as flasks with laccase solution without diclofenac were included. At each time point (0 h, 45 min, 4.5 h, 24 h and 7 days) one flask was sacrificed, 5 mL of acetic acid was added to stop the reaction and extraction and preconcentration was performed as described above.

^{1}H (500.13 MHz) and ^{13}C (125.76 MHz) NMR spectra of the samples were recorded on an AVANCE 500 Bruker spectrometer equipped with a high-sensitivity cryogenically cooled triple-resonance TCI probehead. 2D NMR correlations were needed for the structural characterisation of the metabolites. By means of COSY (Correlated Spectroscopy), HSQC (Heteronuclear Single-Quantum Correlation), Edited HSQC and HMBC (Heteronuclear Multiple Bond Correlation) experiments, compounds were ^{1}H and ^{13}C fully characterised. The ^{13}C NMR spectroscopic peaks were assigned by HSQC and HMBC experiments. NMR analyses were performed in methanol-d4 at 25ºC. The proton and carbon chemical shifts were referenced to solvent, methanol-d4 (1H, 3.32 ppm; 13C, 47.9 ppm). The patterns of peaks were reported as singlet (s), doublet (d), triplet (t), or double doublet (dd).

IV.2.3.3. *Vibrio ficheri* luminescence reduction test (Microtox test)

A Microtox bioassay was used to perform toxicity tests. *V. fischeri* is a marine luminiscent bacterium that liberates energy in the form of visible light (maximum intensity at 490 nm). Toxicity data were based on a 15 min exposure of bacteria to a filtered solution (pH 7) at 25 ºC. Effluent toxicity was expressed in units of EC_{50}. The experimental sample tested was collected from time course degradation experiments

described in section 2.2.1 at 24 h. The results were compared with an uninoculated control. The initial diclofenac concentration was 10 mg L^{-1}.

IV.2.3.4. Other analyses

Laccase activity was assayed in 100 mM sodium phosphate buffer, pH 5, using 10 mM DMP as substrate and measuring the production of coerulignone (ε_{469}= 27,500 M^{-1} cm^{-1}, when referred to DMP concentration) (Martínez et al., 1996).

Mycelial dry weights were determined by vacuum filtering the cultures through preweighed glass filters (Whatman). The filters containing the mycelial mass were placed in glass dishes and dried at 100 ºC to constant weight.

IV.3. Results and discussion

Time-course degradation experiments of diclofenac, added at 10 mg L^{-1}, are shown in figure IV.1. As can be observed, almost complete diclofenac removal (94%) was obtained the first hour of incubation and after 4 h diclofenac was not detected in the liquid medium. This fast removal was not in accordance with previous reports regarding degradation of environmental pollutants by *T. versicolor* pellets in the same experimental conditions, in which a lag phase of several hours was observed (Marco-Urrea et al., 2008; Marco-Urrea et al., 2009b). Besides heat-killed controls, sodium azide-killed controls were also included in this experiment. Our results revealed that adsorption of diclofenac in heat-killed cultures (47%) was higher than that obtained in cultures containing sodium azide (10%) if compared with uninoculated controls.

Since pharmaceuticals are found in the aquatic environment at low concentrations (in the range of ng L^{-1} to µg L^{-1}), additional experiments were carried out at 45 µg L^{-1} to assess the capability of *T. versicolor* to degrade diclofenac at environmentally relevant concentrations. As can be observed in figure IV.2, removal of diclofenac appeared almost immediately after its addition into the medium, and it was not detected after 0.5 h. The sorption values obtained in heat-killed controls are considerably higher (80%) than those obtained at the higher concentration of 10 mg L^{-1}.

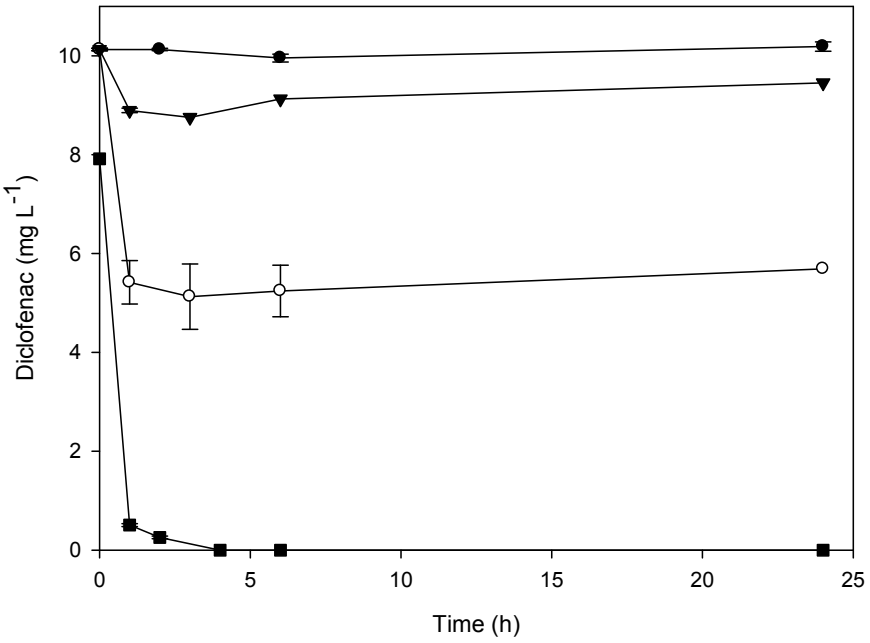

Figure. IV.1: Time course of diclofenac degradation by *T. versicolor* pellets. Symbols: uninoculated controls (●), experimental cultures (■), heat-killed (○) and sodium azide controls (▼). Diclofenac was added at a final concentration of 10 mg L^{-1} in the liquid medium. Values plotted are means ± standard deviations for duplicate cultures. The initial mycelial pellets dry weight added to each flask was 135.2 ± 4.3 mg.

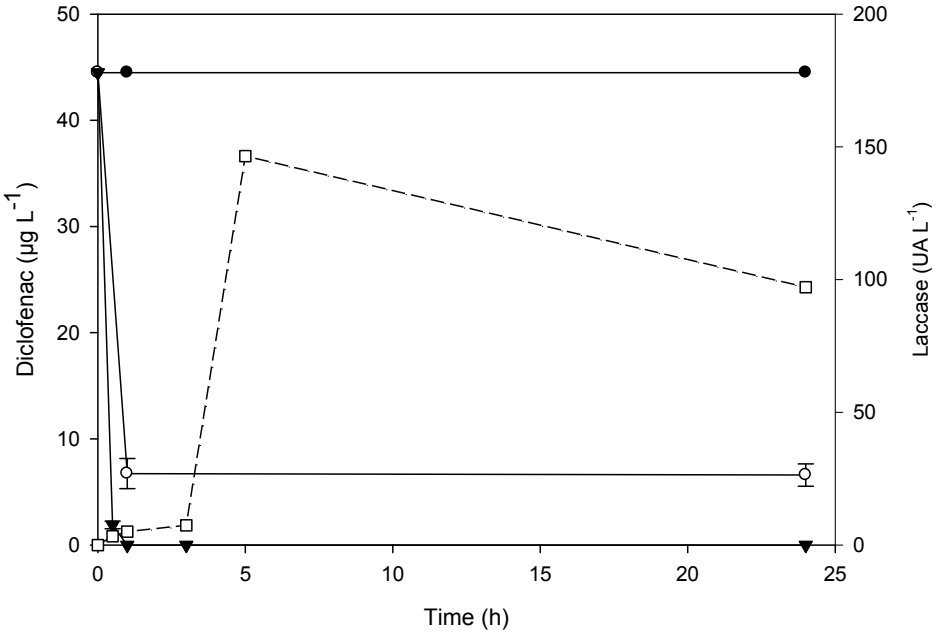

Figure IV.2: Time course of diclofenac degradation added in the range of µg L^{-1} by *T. versicolor* pellets. Symbols: Lacasse activity (□) and diclofenac concentration in uninoculated controls (●), experimental cultures (▼) and heat-killed controls (○). Diclofenac was added at a concentration of ~45 µg L^{-1}. Values plotted are means ± standard deviations for duplicate or triplicate cultures. The initial mycelial pellets dry weight added to each flask was 265.2 ± 6.2 mg.

It is well known that fungal cells exposed to heat treatments alter the physicochemical properties of the cell wall leading to a greater, equivalent to or less bioadsorptive capacities than that of living cells depending the pollutant (Arica et al., (2003). However, in contrast with experimental cultures, disappearance of diclofenac in heat-killed and sodium azide controls was noticeable lower and was not accompanied of degradation metabolites in the medium, corroborating that removal in both controls was solely due to sorption. As stated above, high levels of sorption can be ascribed to the heat treatment of fungus, but it is noteworthy that adsorption and transformation of xenobiotic in live cultures of white-rot fungi cannot be clearly distinguished since they can also degrade adsorbed pollutants by intracellular mechanisms (Blánquez et al., 2004).

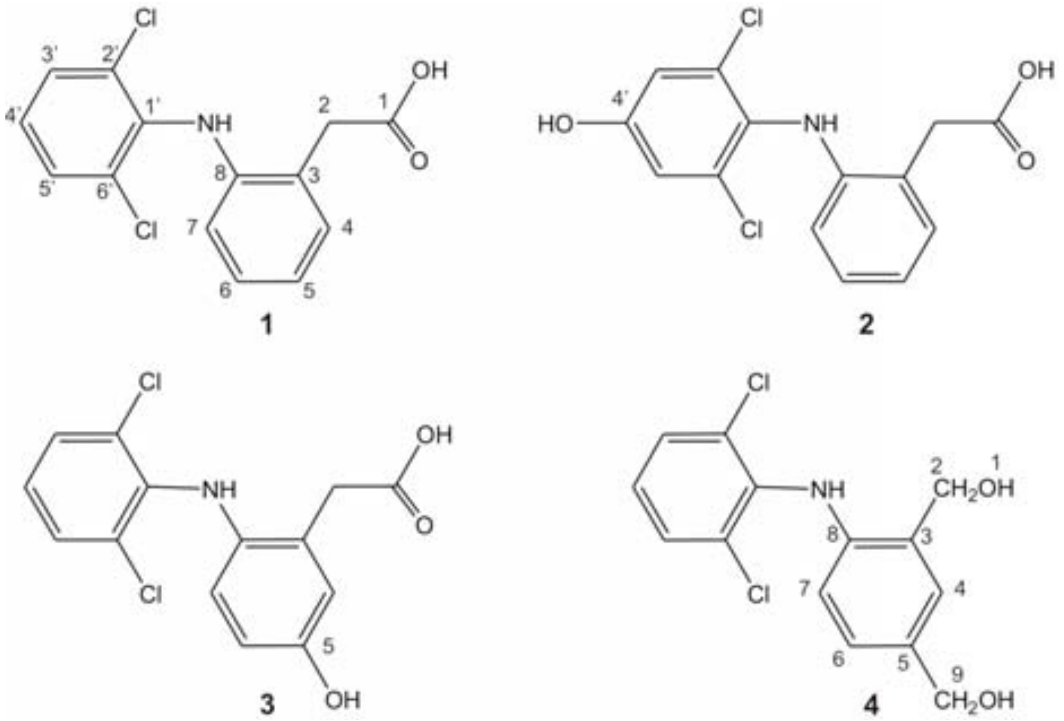

Figure IV.3: Structures of diclofenac and the degradation products identified by NMR.

NMR analyses allowed the identification of the degradation metabolites of diclofenac by *T. versicolor* pellets: 4'-OH-diclofenac (metabolite **2**) and 5-OH-diclofenac (metabolite **3**) (figure IV.3). *In vivo* samples at experimental times 0 h, 1 h, 3 h, 6 h, 24

h and 7 days were prepared for the NMR analysis (see section 2.3.2) and ^{1}H NMR spectra of each sample were recorded and compared. From sample at 0 h, which consists in an uninoculated sample, diclofenac (**1**) was ^{1}H and ^{13}C NMR completely characterized and from sample at 3 h the identification of metabolites **2** and **3** was achieved (figure IV.4). The performance of 2D NMR experiments, such as COSY, HSQC and HMBC, was needed for the full ^{1}H and ^{13}C NMR assignment of **1**, **2** and **3** (Table 1). All ^{13}C NMR assignments were determined by the HMBC and HSQC experiments. ^{1}H and ^{13}C NMR assignments of compounds **1**, **2** and **3** are consistent with those described previously **(Stierlin et al., 1979; Kenny et al., 2004; Osorio-Lozada et al., 2008)**.

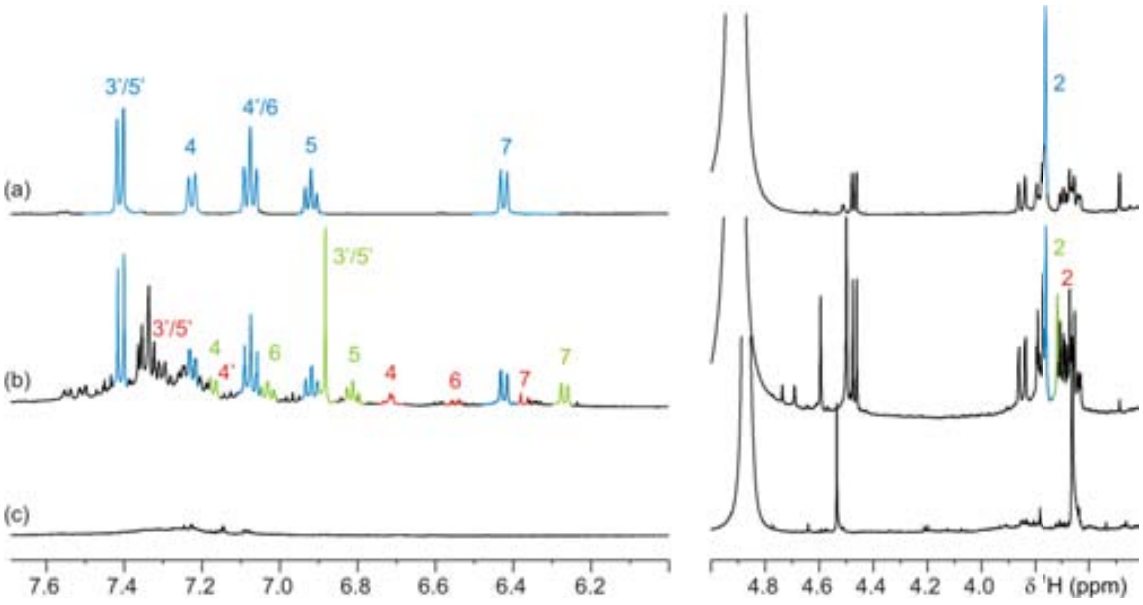

Figure IV.4. ^{1}H NMR spectra of the NMR samples corresponding to the *in vivo* experiments at 0 h, consisting in the uninoculated sample (a), at 3 h of experiment (b) and at 24 h of experiment (c) with *T. versicolor*. Peaks corresponding to diclofenac **1** are blue colored, those corresponding to metabolite **2** are green colored and those of **3** are red highlighted. All samples were dissolved in CD_3OD and the spectra were acquired at 25 ºC and at a magnetic field of 500 MHz.

These metabolites were previously described in biological system during diclofenac degradation, mostly when fungi and bacteria were used to synthesize hydroxylated diclofenac, which is of interest in the pharmaceutical industry. Webster et al. (1998) described the production of 4'-OH diclofenac and small amounts of 3'-OH and 5-OH diclofenac using filamentous fungi. More recently, conversion of diclofenac

to 4'-OH diclofenac using an extracellular peroxygenase of the basidiomycete *Agrocybe aegerita* was reported (Kinne et al., 2009). Also, metabolites **2** and **3** but also 4',5-OH-diclofenac were produced by the bacterium *Actinoplanes* sp in hollow fiber cartridges (Osorio-Lozada et al., 2008). Metabolites **2** and **3** were also identified as metabolites of diclofenac in humans (Dorado et al., 2008). In the environment, diclofenac degradation by indigenous microflora of river sediments leads to the p-benzoquinone imine of 5-hydroxy diclofenac as major metabolite (Gröning et al., 2007). However, our results showed that after 24 h of experiment no presence of diclofenac, neither metabolites **2** and **3** were observed in the liquid medium (see Table 2) suggesting either diclofenac mineralization or diclofenac transformation to non-detected metabolites.

Table IV.1: Description of ^{1}H and ^{13}C NMR chemical shifts (δ) and H,H-coupling constants ($^{3}J_{H,H}$ and $^{4}J_{H,H}$) of compounds **1**, **2**, **3** and **4**. Data were obtained from samples dissolved in CD_3OD and spectra were acquired at 25.0 ºC and at a magnetic field of 500 MHz. In brackets are indicated δ_H and δ_C values for metabolites **2** and **3** (with the same solvent conditions), described previously (Osorio-Lozada et al., 2008).

	1[a,b]		**2**		**3**		**4**	
	δ (^{1}H) and $^{3/4}J_{H,H}$	δ (^{13}C)	δ (^{1}H) and $^{3/4}J_{H,H}$	δ (^{13}C)	δ (^{1}H) and $^{3/4}J_{H,H}$	δ (^{13}C)	δ (^{1}H) and $^{3/4}J_{H,H}$	δ (^{13}C)
Atom	[ppm] and [Hz]	[ppm]	[ppm] and [Hz]	[ppm]	[ppm] and [Hz]	[ppm]	[ppm] and [Hz]	[ppm]
1	-	174.7	-	174,2 [174.4]	-	174.2 [174.4]	-	-
2	3.77 (s, 1H)	38.3	3.73 (s, 1H) [3.72]	37.8 [37.8]	3.72 (s, 1H) [3.71]	37.8 [37.8]	4.81 (s, 4 H)	63.1
3	-	125.4	-	122.7 [123.0]	-	127.9 [127.9]	-	132.7
4	7.24 (d, *J*=7.4, 1H)	130.3	7.18 (d, *J*=7.6, 1H) [7.18]	130.4 [130.0]	6.73 (d, *J*=2.8, 1H) [6.72]	116.8 [116.9]	7.49 (d, *J*=2.3, 1H)	126.5
5	6.93 (t, *J*=7.4, 1H)	121.4	6.82 (t, *J*=8.2, 1H) *J*=7.6, 1H) [6.82]	119.9 [119.8]	-	152.7 [152.6]	-	128.5
6	7.09 (t, *J*=8.0, 1H)	127.3	7.04 (dd, *J*=8.2 *J*=8.0, 1H) [7.03]	127.4 [127.4]	6.56 (dd, *J*=8.4 *J*=2.8, 1H) [6.56]	113.8 [113.8]	7.35 (dd, *J*=8.3 *J*=2.3, 1H)	125.7
7	6.43 (d, *J*=8.0, 1H)	117.2	6.28 (d, *J*=8.0, 1H) [6.28]	115.0 [114.9]	6.39 (d, *J*=8.4, 1H) [6.38]	120.5 [120.4]	6.40 (d, *J*=8.3, 1H)	115.1
8	-	143.3	-	144.2 [143.8]	-	134.8 [134.9]	-	132.7
9	-	-	-	-	-	-	4.81 (s, 4 H)	63.1
1'	-	137.7	-	132.1 [132.1]	-	138.9 [138.9]	-	137.1
2'	-	130.1	-	128.7 [128.9]	-	[c]	-	130.9
3'	7.42 (d, *J*=8.0, 1H)	128.5	6.89 (s, 1H) [6.89]	115.5 [115.3]	7.36 (d, *J*=8.0, 1H) [7.34]	128.6 [128.5]	7.47 (d, *J*=8.1, 1H)	128.6
4'	7.09 (t, *J*=8.0, 1H)	124.5	-	154.5 [154.7]	6.98 (t, *J*=8.0, 1H) [6.96]	122.8 [122.6]	7.15 (t, *J*=8.1, 1H)	125.2
5'	7.42 (d, *J*=8.0, 1H)	128.5	6.89 (s, 1H) [6.89]	115.5 [115.3]	7.36 (d, *J*=8.0, 1H) [7.34]	128.6 [128.5]	7.47 (d, *J*=8.1, 1H)	128.6
6'	-	130.1	-	128.7 [128.9]	-	[c]	-	130.9

[a] Experiments acquired in CD_3OD, solvent signal appears at δ_H 3,32 ppm and δ_C at 47,9 ppm.

[b] Values in brackets correspon to δ_H and δ_C described in reference (Osorio-Lozada et al., 2008).

[c] Not possible to detect due to overlap.

Table IV.2: Relative concentrations (%) of metabolites **2**, **3** and of diclofenac **1** at experimental times 0, 1, 3, 6, 24 h and 7 days. Data were obtained from samples dissolved in CD_3OD and spectra were acquired at 25 ºC and at a magnetic field of 500 MHz.

Exp.Time	**1**	**2**	**3**
t = 0 h	100[a]	n.d.[b]	n.d.
t = 1 h	94	n.d.	6
t = 3 h	55	37	8
t = 6 h	70	21	9
t = 24 h	n.d.	n.d.	n.d.
t = 7 d	n.d.	n.d.	n.d.

[a] The relative area (%) values were measured by the integration of those ^{1}H NMR signals with no overlapping and considering only the peaks of the identified metabolites and of the remaining diclofenac. Nevertheless, ^{1}H NMR spectra show that there could be other minor metabolites not identified.

[b] Not detected.

To test whether cytochrome P450 played a role on diclofenac degradation, the use of the cytochrome P450 inhibitor 1-aminobenzotriazole was used. As shown in figure IV.5, a fast disappearance of diclofenac from the medium (approximately 75%) occurred the first 15 min in both inhibitor and inhibitor-free flasks. It is interesting to compare this percentage of diclofenac removal with that obtained in sodium azide cultures (10%) at approximately the same concentration of diclofenac (figure IV.1). Besides oxidases inhibition, sodium azide is an inhibitor of ATPase activity of cells in humans and it is commonly used to discriminate between energy dependent transports of drugs across the cell membrane (virtually inhibited by sodium azide) and passive uptake, which is usually associated to physicochemical processes (Gabor and Wirth, 2008). On this basis, we suggest that the steep decrease on diclofenac concentration observed the first 15 min in cyt P450 inhibitor and inhibitor-free flasks (figure IV.5) may be due to an energy-dependent transporter of diclofenac into the cell, which would be inhibited in sodium azide flasks (figure IV.1). Once into the cell, diclofenac degradation would take place by cytochrome P450 system in inhibitor-free controls after a short plateau whereas it would be inhibited in cultures containing 1-aminobenzotriazole (figure IV.5). However, more research is needed in the future to corroborate this transport mechanism for diclofenac degradation by *T. versicolor*. A further evidence to strengthen the role of cytochrome P450 in diclofenac degradation

by *T. versicolor* pellets is the the fact that diclofenac is also converted to metabolites **2** and

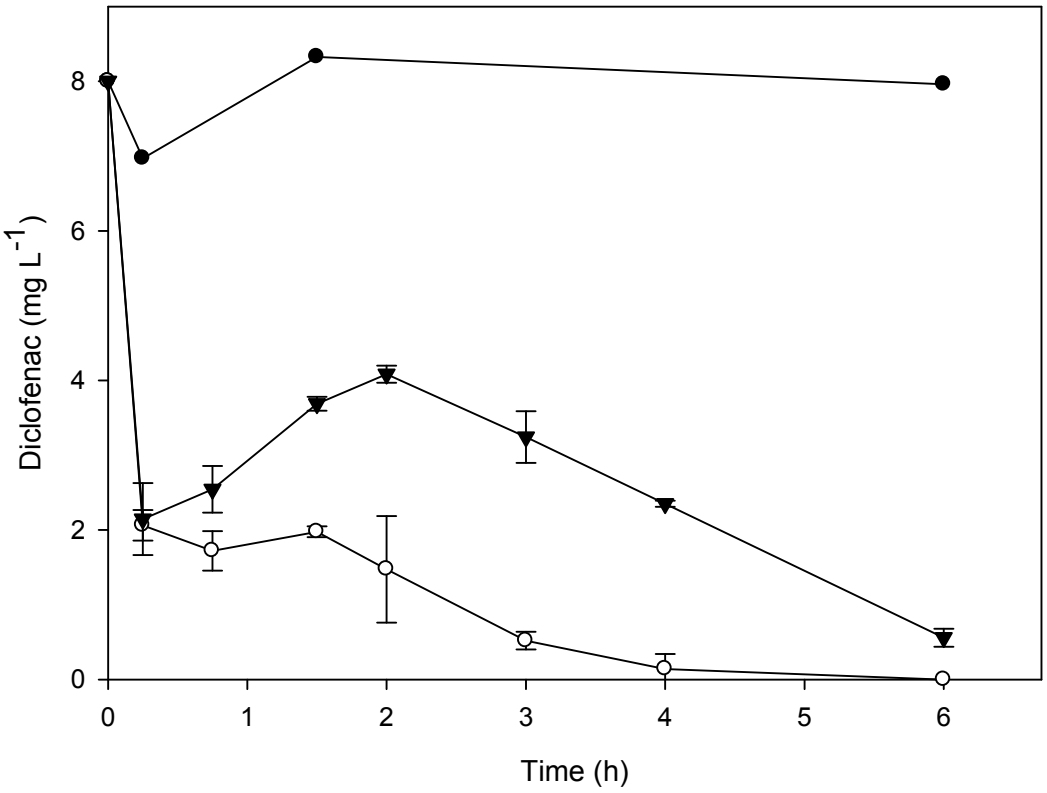

Figure IV.5: Influence of the cytochrome P450 inhibitor 1-aminobenzotriazole (5 mM) on the degradation of diclofenac. Symbols: uninoculated bottles (●), inhibitor-free controls (○), and cultures containing 1-aminobenzotriazole (▼).Values plotted are means ± standard deviations for duplicate cultures. The initial mycelial pellets dry weight added to each flask was 129.2 ± 7.5 mg.

The effect of purified laccase on diclofenac was also assessed, although most diclofenac was transformed before laccase activity reached a peak (see figure IV.2). Laccases generally catalyze the removal of a hydrogen atom from the hydroxyl groups of phenolic substrates and from aromatic amines using molecular oxygen as a primary electron acceptor (Martínez et al., 2005). Thus, diclofenac was not apparently an optimal substrate for laccase. However, results in figure IV.6 showed a steep decrease of diclofenac when purified laccase was added at 2000 AU L^{-1}. After 4.5 h more than 95% of the added diclofenac was degraded. The reaction mixture was initially colourless and through the experiment it adopted a brown colour possibly due to the accumulation of a diclofenac degradation product, that was identified as 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol (metabolite **4**) by NMR. The spectra

show the decreasing of diclofenac signals and the appearance of small new signals at 45 min (figure IV.7). When comparing these spectra with those of the *T. versicolor* samples, neither metabolites **2** nor **3** are observed.

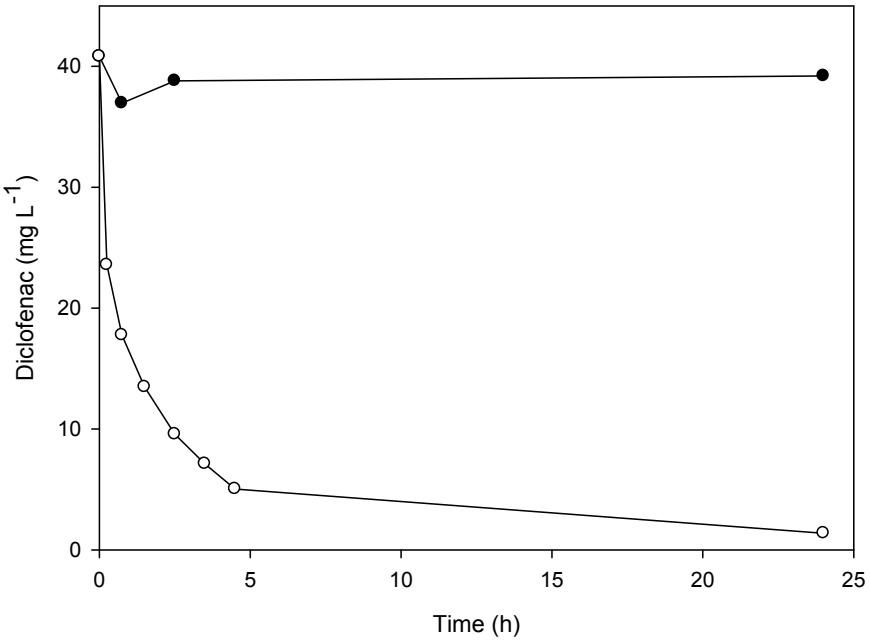

Figure IV.6: Time-course degradation of diclofenac by purified laccase. Symbols: Laccase-free controls (●) and flasks containing laccase at 2000 activity units (AU) per liter. In both cases diclofenac was added at a final concentration of ~40 mg L^{-1}, the medium was adjusted at pH 4.5 and was incubated under shaking conditions (135 rpm, 25 ºC).

The study in depth by NMR of sample at 24 h allowed the identification of the major degradation metabolite. Figure IV.7d shows the ^{1}H NMR spectrum of the sample at 24 h, where signals corresponding to metabolite **4** are indicated. For the identification of **4**, ^{1}H and ^{13}C NMR characterization of the molecule was needed and that was achieved by means of the 2D NMR experiments COSY, Edited HSQC and HMBC. Table 1 shows the ^{1}H and ^{13}C NMR assignments of compound **4**. The dichloride substituted ring (figure IV.3 and Table 1) has not been modified if compared with the diclofenac **1,** and it shows proton and carbon chemical shifts very similar to **1**. For the ring initially bonded to the acetylic chain, several changes have taken place. Considering the multiplicity and the coupling constants values of the aromatic protons, the ring presents a triple (*orto* and *para*) substitution.

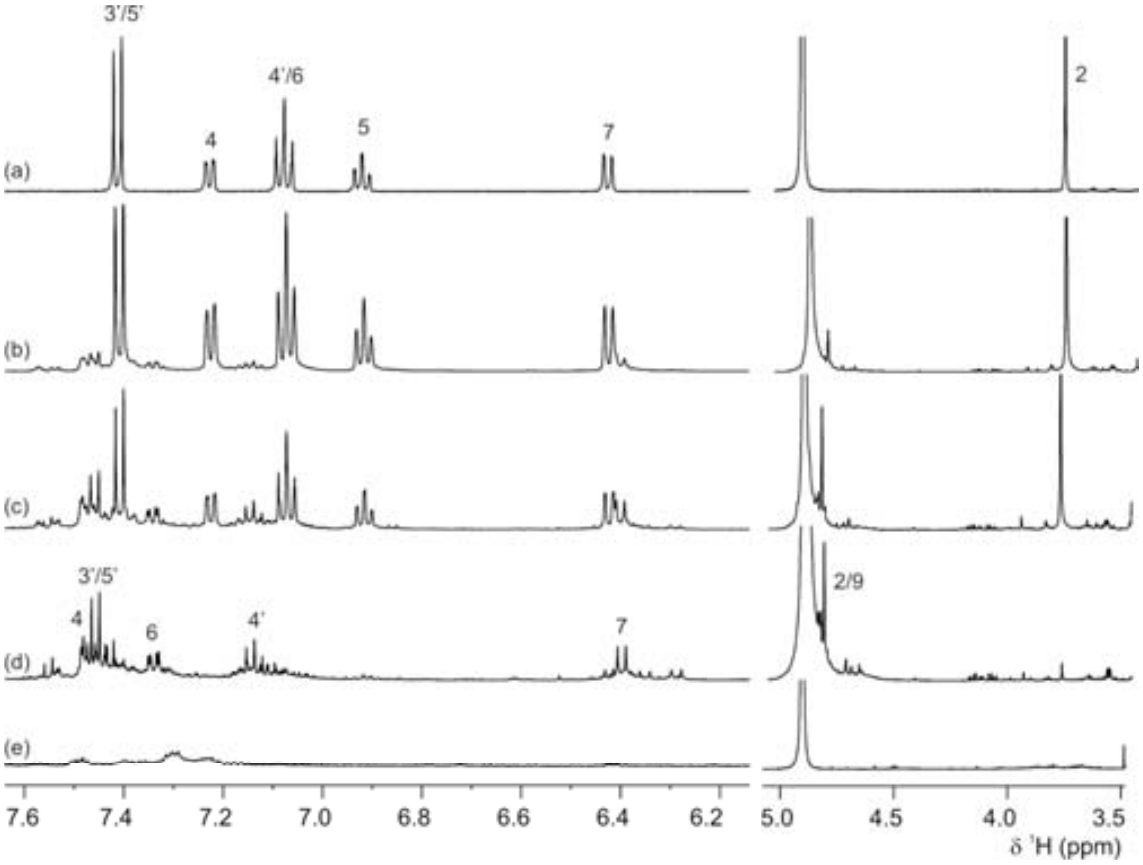

Figure IV.7: ^{1}H NMR spectra of the NMR samples corresponding to the *in vitro* experiments with laccase at 0 h, consisting in the uninoculated sample (a), at 45 min (b), 4.5 h (c), 24 h (d) and 7 days of experiment (e). Peaks corresponding to diclofenac **1** are indicated in the spectrum (a) and peaks corresponding to metabolite **4** are indicated in spectrum (d). All samples were dissolved in CD_3OD and the spectra were acquired at 25 ºC and at a magnetic field of 500 MHz.

By the edited HSQC experiment the ^{13}C values of C2, C4, C6 and C7 were determined and C2 was identified as a methylene (CH_2) group. The values of H2 (4.80 ppm) and C2 (64.2 ppm) correspond to a typical CH_2OH group. Via the HMBC correlation peaks, the substitution of the ring in positions 3, 5 and 8 was confirmed, the ^{13}C values of quaternary carbons, C3, C5, and C8 were determined and the presence of carboxylic or carbonylic carbons in the molecule was rejected. Finally, the substitution in position 5 was identified as a second CH_2OH group, resonating at H9 4.80 ppm, as H2. That was concluded because of the HMBC intense correlation peak observed between singlet at 4.80 ppm (H2/H9) with C3 and C5 and because of the ^{13}C values of C3 and C5. The integration of the singlet at 4.80 ppm (H4/H9) was not

possible because of a partial overlap of it with the HDO signal. In order to characterize the main biodegradation product of laccase more exhaustively, an analytical procedure involving the isolation of compound 4 and the comparison of its NMR results with those of the synthetic molecule will be needed (Caviglioli et al., 2002)

Thus, 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol (metabolite **4**) was identified by NMR as the major degradation metabolite of diclofenac by laccase and, as far as we know, it is described for the first time. In the case of the diclofenac degradation with *T. versicolor* pellets, neither metabolite **4** nor the colour developments were observed, indicating that laccase was not the primary enzyme in the diclofenac transformation by *T. versicolor*.

Finally, to evaluate the risk involved with the emission of the treated medium containing diclofenac by *T. versicolor* pellets, a standard bioassay was performed with the bacterium *V. fischeri* (Microtox test). Our results showed that only the uninoculated control containing diclofenac at 10 mg L^{-1} was sufficiently toxic to produce any effect, resulting in a 15 min EC_{50} of 34.0%. These results are in accordance with our previous observation of disappearance of both diclofenac and degradation metabolites after 24 h and suggest that diclofenac degradation by *T. versicolor* is an environmental friendly strategy.

IV.4. Conclusions

The use of *T. versicolor* pellets to degrade diclofenac led to an unusual fast degradation rate at concentrations in the range of µg L^{-1} to mg L^{-1} in a defined liquid medium. Both parent compound and degradation metabolites identified in fungal cultures (4'-OH and 5-OH diclofenac) disappeared after 24 h. This is in accordance with the decrease in ecotoxicity assessed by the Microtox bioassay. Cytochrome P450 system appeared to play a key role in the first step of diclofenac degradation according the results obtained with the cytochrome P450 inhibitor 1-aminobenzotriazole. Purified laccase can also catalyze the transformation of diclofenac to 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol but it did not appear to be the enzymatic system responsible of diclofenac degradation in *T. versicolor* pellets. Further

experiments scaling up the reactors *are needed to confirm* the feasibility of using *T. versicolor* to remove diclofenac from aquatic environments.

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Chapter V:

Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bio reactor and identification of intermediates.

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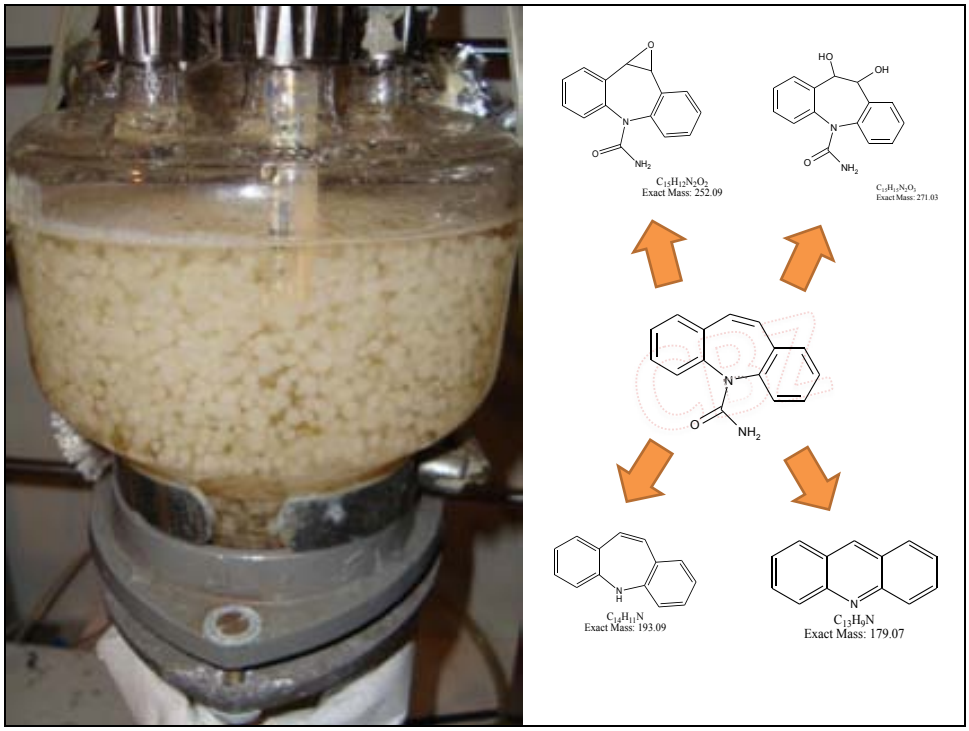

Abstract

The paper describes the aerobic degradation of carbamazepine (CBZ), an anti-epileptic drug widely found in aquatic environment, from Erlenmeyer flask to bioreactor by the white-rot fungus *Trametes versicolor*. In Erlemeyer flask, CBZ at approximately 9 mg L^{-1} was almost completely eliminated (94%) after 6 d, while at close environmentally relevant concentrations of 50 µg L^{-1}, 61 % of the contaminant was degraded in 7 d. Acridone, acridine, 10,11-dihydro-10,11-dihydroxy-CBZ, and 10, 11-epoxy-CBZ were identified as major metabolites, confirming the degradation of CBZ. The degradation process was then carried out in an air pulsed fluidized bioreactor operated in batch and continuous mode. Around 96% of CBZ was removed after 2 days on batch operation, and 10,11-dihydro-10,11-epoxycarbamazepine was found as unique metabolite. In bioreactor operated in continuous mode with a hydraulic retention time of 3 d, 54% of the inflow concentration (approx. 200 µg L^{-1}) was reduced at the steady state (25 d) with a CBZ degradation rate of 11.9 µg CBZ g^{-1} dry weight d^{-1}. No metabolite was detected in the culture broth. Acute toxicity tests (Microtox) indicated that the final culture broth in both batch and continuous mode operation were non toxic, with 15 min EC50 values of 24% and 77%, respectively.

Keywords: *Trametes versicolor*, pharmaceuticals, carbamazepine, bioreactor, continuous treatment.

V.1. Introduction

Carbamazepine (CBZ), 5H-dibenzazepine-5-carboxamide, an iminostilbene derivative with a tricyclic structure, is one of the most widely prescribed and very important drug for the treatment of epilepsy, trigeminal neuralgia and some psychiatric diseases (Fertig and Mattson, 2008). In 2002 the annual consumption in Spain was approximately 25 tons, which increased up to 32 tons in 2006 (De la Fuente et al., 2007), and its global consumption was estimated to be approximately 1000 tons per year (Zhang and Geiβen, 2010).

This antiepileptic is one of the most studied pharmaceuticals detected in the environment. It is hardly or no degraded during wastewater treatment and many studies have found it ubiquitous in various environmental matrices (soil, surface and ground water) (Clara et al., 2005, Joss et al., 2005, Zhang et al., 2008). Measured CBZ concentrations in wastewater effluents ranged from ng L^{-1} up to μg L^{-1} (Camacho-Muñoz et al., 2010; Gros et al., 2010; Jelic et al., 2011; Kasprzyk-Hordern et al., 2009). It was also detected in drinking water, though at low ng L^{-1} concentrations (Benotti et al., 2009).

Degradation of CBZ has become a topic of concern given the fact that it is recalcitrant to biological attack and it is neither removed during conventional biological wastewater (<10%) nor membrane bioreactor treatments (<20%) (Miao and Metcalfe, 2003; Clara et al., 2004; Joss et al., 2005; Radjenovic et al., 2007; Zhang et al., 2008). Physicochemical processes such as coagulation-flocculation and flotation also give low results concerning its elimination (20-35 %) (Carballa et al., 2004; Suárez et al., 2008). On the other hand, advanced oxidation processes (AOP) as ozonization (Ternes et al., 2002), UV/H_2O_2 induced photolytic degradation (Vongna et al., 2004), photocatalytic degradation with TiO_2 (Doll and Frimmel, 2005), or direct photolysis (Chiron et al., 2006), resulted in high percentages of CBZ degradation (>90%) (Esplugas et al., 2007) but the main limitation is the formation of undesirable and sometimes toxic by-products (Negrón-Encarnación and Arce, 2007).

To date, the only microorganisms able to degrade CBZ are white-rot fungi (Marco-Urrea et al., 2009; Hata et al., 2010; Zhang and Geiβen, 2010; Golan-Rozen et al., 2011). This group of microorganisms possesses a high capability to degrade a wide range of xenobiotics and recalcitrant pollutants due to their non-specific ligninolytic enzymatic system that includes manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) and laccase (Tanaka et al., 1999; Durán and Esposito, 2000). *In vitro* experiments using LiP from *Phanerochaete chrysosporium* showed limited CBZ degradation (<10%) (Zhang and Geiβen, 2010). Although CBZ is not a substrate for laccase, repeated treatments with this enzyme and a redox mediator 1-hydroxybenzotriazole (HBT) led to degradation values of 60% after 48 h (Hata et al., 2010; Marco-Urrea et al., 2009). MnP and VP produced by *Pleurotus ostreatus* have also been shown to oxidize CBZ to a significant level (98%) (Golan-Rozen et al., 2011). Besides ligninolytic enzymes, inhibition experiments indicated that the cytochrome P450 (CYT P450) enzyme system also play an important role in CBZ degradation (Marco-Urrea et al., 2009; Golan-Rozen et al., 2011). In addition, a novel strategy based on the induction of hydroxyl radicals in *Trametes versicolor* using the quinone redox cycling resulted in a high percentage of CBZ degraded (80%) in 6 h (Marco-Urrea et al., 2010a).

Aside CBZ, there is an increasing list of pharmaceuticals that are degraded by white-rot fungi, that make these organisms an interesting catalysts to be taken into account for pharmaceutical remediation processes (Blánquez and Guieysse, 2008; Rodríguez-Rodríguez et al., 2009; Marco-Urrea et al., 2009; Hata et al., 2010; Marco-Urrea et al., 2010b; Marco-Urrea et al., 2010c; Marco-Urrea et al., 2010d). However, studies applying fungus in lab-scale bioreactors are very scarce in the literature, and they commonly deal with endocrine disrupting compounds and dyes (Blánquez and Guieysse, 2008; Cabana et al., 2007; Pakshirajan et al., 2011). Due to the poor removal of CBZ in wastewater treatment plants we here evaluated the capability of *T. versicolor* to degrade this recalcitrant compound applying two different strategies of operation in bioreactor, i.e. batch and continuous. In addition, we identified transformation products formed in the time-course experiments. Finally, acute toxicity bioassay was

carried out to evaluate the risk involved with the emission of the treated aqueous medium in both systems.

V.2. Materials and methods

V.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 days.

Pellets production was done as previously described by Font et al. (2003). Pellets formed by this process were washed with sterile deionised water.

All the pharmaceutical standards were of high purity grade (>97%). CBZ, acridine, acridone and 10,11-dihydro-10,11-epoxycarbamazepine (CBZE) were purchased from Sigma-Aldrich (Barcelona, Spain). The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98% were provided by Merck (Darmstadt, Germany). Glucose, ammonium tartrate dibasic and 2,2-dimethylsuccinic acid 99 % were purchased from Sigma-Aldrich (Barcelona, Spain).

V.2.2. Experimental procedures

V.2.2.1. Degradation experiments in Erlenmeyer flasks

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing appropriate amounts of mycelial pellets (0.48 g dry weight) in a total volume of 50 mL of Kirk medium (pH 4.5) (Kirk et al., 1978). CBZ, from a stock solution in ethanol, was added into the flasks to give the desired final concentration (9 mg L^{-1} and 50 µg L^{-1}). After CBZ addition, flasks were incubated under constant shaking (135 rpm) at 25 ºC. To avoid the possible influence of light on CBZ stability, all the experiments were carried out in the dark. Each flask was sacrificed for analysis at 2-3 hours intervals over the first 8 h, then twice a day during next 4 days, and daily until the end of the experiment (15 days).

Degradation of CBZ in time-course experiments was evaluated by comparing its concentration in the heat-killed control flasks and in the experimental flasks. The heat-killed controls consisted of autoclaved cultures (121 ºC for 30 min) that were processed under the same conditions as the experimental cultures. The amount of adsorbed CBZ was determined using the heat-killed controls.

In time course degradation experiments, where CBZ was added at high concentrations (approx. 9 mg L^{-1}), the entire flask contents were collected at selected intervals during the experiment, and filtered through 0.45 μm glass fiber filter from Whatman (Barcelona, Spain). Subsequently, 1 mL-sample was withdrawn to be analyzed by high performance liquid chromatography with UV detection (HPLC-UV). Also, glucose and laccase production were measured. The remaining part of the samples was used for the identification of the transformation products.

When CBZ was added at low concentration (50 μg L^{-1}), we proceeded as previously explained, but the entire flask contents was preconcentrated by solid phase extraction (SPE) and afterwards analyzed by HPLC-UV. The experiment was performed in triplicate. The target compound in the liquid medium was extracted in one step by solid phase extraction with Oasis HLB cartridges (60 mg adsorbent, Waters, Barcelona, Spain) (Gros et al., 2006). Briefly, the cartridges were preconditioned sequentially with 5 mL of methanol and 5 mL of deionized water adjusted at sample pH. After that, the sample was passed through the cartridge and dried under vacuum. Then, the adsorbed compounds were eluted with methanol (2 x 2 mL) and subsequently concentrated to dryness under a gentle nitrogen stream. The extracts were reconstituted with 0.5 mL 25:75 (v/v) acetonitril-water. Extraction efficiency of carbamazepine, evaluated by recovery experiments, was 98.5%.

V.2.2.2. Degradation experiments in bioreactor

A glass fluidized bioreactor with a volume of 1500 mL was used for the degradation experiments (Blánquez et al., 2007) (Figure V.1). Fluidized conditions were maintained by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 second open, 5 seconds close) and the air flow was 12 L h^{-1}. Temperature was maintained stable at 25ºC and pH in the bioreactor was controlled at 4.5. Approximately 3.8 g dry weight pellets were inoculated. Glucose and nitrogen (as ammonium tartrate) were added at a rate of 0.879 g glucose g^{-1} dry weight pellets d^{-1} and 1.98 mg ammonium tartrate g^{-1} dry weight pellets d^{-1}, respectively.

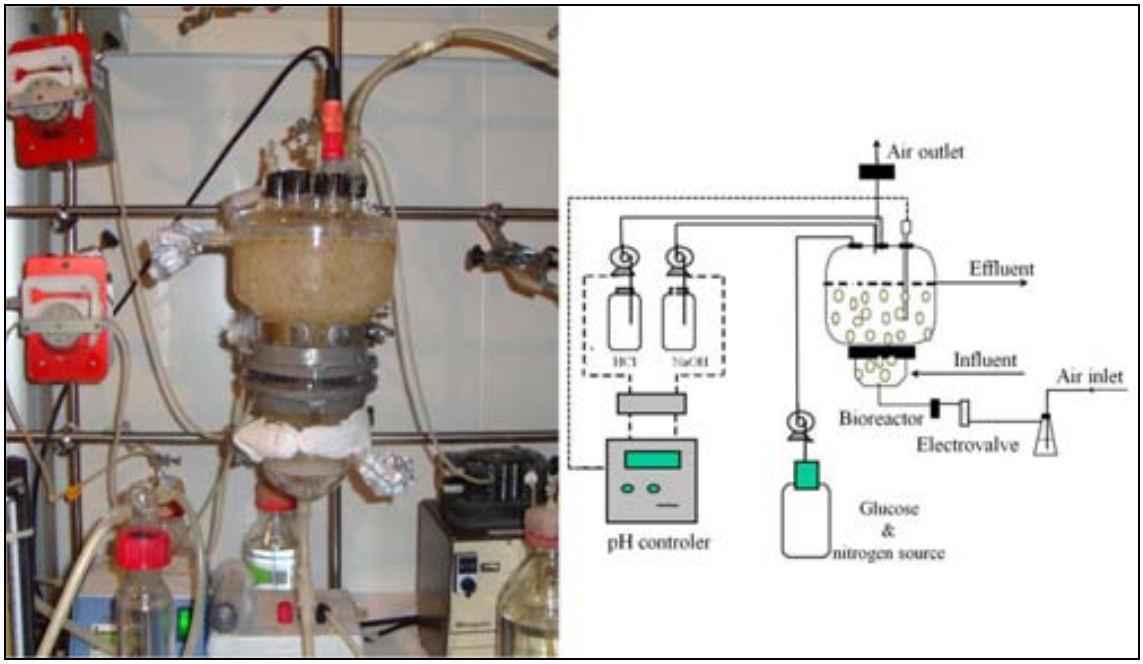

Figure V.1: Picture (left) and scheme (right) of the bioreactor.

The batch reactor medium contained 10 ml L^{-1} and 100 ml L^{-1} of micro and macronutrient solution, respectively, and 4 antifoam drops. The medium was sterilized at 121º C for 30 minutes. After it was sterilized, CBZ, from a stock solution in ethanol, was added into the medium to the final concentration of approx. 200 µg L^{-1}.

The start-up of the experiments in the bioreactor, when operated in continuous mode, was the same as in the batch reactor. Medium containing CBZ at 200 µg L^{-1} was fed into the reactor at a flow rate of 114 ml h^{-1} and cyclic temporized at 10 min on

every hour, to provide a hydraulic retention time (HRT) of 3 days. Glucose and ammonium tartrate was fed from a stock solution (75 g L^{-1} and 169 mg L^{-1} respectively) at a flow rate of 21.7 mL h^{-1} and also temporized (1.2 min on every hour). The biomass, in pellet form, was retained in the bioreactor throughout the experiment with no loss in the effluent and no extra addition of biomass was needed.

Samples from the liquid phase of both experiments (12.5 mL) were collected once a day until the end of the experiments and pre-concentrated as described in section 2.2.1.

V.2.3. Analytical procedures

V.2.3.1. Analysis of CBZ

Analysis of CBZ was performed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) equipped with a UV detector at 230 nm. The column temperature was 30 ºC and a sample volume of 20 µL was injected from a Dionex autosampler (Barcelona, Spain). Chromatographic separation was achieved on a GraceSmart RP 18 column (250 mm × 4 mm, particle size 5 µm). The mobile phase consisted of 6.9 mmol L^{-1} acetic acid in water adjusted to pH 4 (by NaOH) with 35% v/v acetonitrile. It was delivered isocratically at 1 mL min^{-1} as described elsewhere (Stafiej et al., 2007). The detection limit was 0.1 mg L^{-1}.

V.2.3.2. Identification and quantification of metabolites

i UPLC/ESI-QToF-MS analysis

Accurate mass measurements of CBZ and its biotransformation products formed in time-course degradation experiment (section 2.2.1.) were carried out in full-scan and product ion scan mode using a Micromass QqToF-system interfaced with a Waters ACQUITY UPLC system (Micromass, Manchester, UK). Samples from the biodegradation experiments were separated on a Waters ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 µm particle size) equipped with precolumn (5 × 2.1 mm) of the same packing material. The mobile phases were (A) formic acid 0.05% in water and (B) acetonitrile/methanol, 2/1. After 1 min isocratic conditions at 90 % A, the portion of A was linearly decreased to 5 % within 10 min. This condition was held for 2 min and

then the initial mobile phase composition was restored within 1 min and maintained for column regeneration for another 2 min. The flow rate was 300 µL min^{-1}. The injection volume was 10 µL. The MS analysis was performed with an electrospray ionization (ESI) interface in the positive ion mode applying a capillary voltage of +3500 V. The nebulizer gas flow was set to 600 L h^{-1} and the drying gas flow to 50 L h^{-1} with a temperature of 350 °C. The ToF analyzer operated at a resolution of 5000 (FWHM) and ESI mass spectra were recorded in 1-s intervals with automatic switching of the dual-sprayer every 10 s for infusion of the internal calibrant for a duration of 1 s. Tyrosine-valine-tyrosine served as internal lock mass with $[M+H]^+$= *m/z* 380.2185. All MS data acquisition and processing was done using the software package MassLynx V4.1.

ii HPLC/ESI-QqLIT-MS analysis (low concentration experiments)

The quantitative analysis of CBZ and its transformation products (for which their chemical standards were available) was performed using Symbiosis Pico™ (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). More information on the analytical methods is shown in the Supplementary Information (SI).

V.2.3.3. *Vibrio fishceri* luminescence reduction test (Microtox test)

Microtox system was used for toxicity assessment. *V. fischeri* is a marine luminescent bacterium that liberates energy in the form of visible light (maximum intensity at 490 nm). Toxicity data were based on a 15 min exposure of bacteria to a filtered solution (pH 7) at 25 ºC. Effluent toxicity was expressed in units of EC50. The experimental samples tested were collected from time-course degradation experiments in both batch and continuous bioreactor.

V.2.3.4. Other analyses

Laccase activity was assayed in 100 mM sodium phosphate buffer, at pH 5, using 10 mM of 2,6-dimethoxyphenol (DMP) as substrate and measuring the production of coerulignone as described elsewhere (Martinez et al., 1996). The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

For determining mycelial dry weight, the cultures were vacuum filtered over preweighed glass-fiber filters (Whatman, Barcelona, Spain). The filters containing the mycelial mass were dried at 100 ºC to constant weight.

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instrument and Co. (Yellow Springs, OH, USA)

V.3. Results and discussion

V.3.1. Degradation of CBZ by *T. versicolor* in Erlenmeyer flasks

Time-course degradation experiments performed in Erlenmeyer flasks showed that CBZ added at 9 mg L^{-1} was almost completely degraded (94 %) by *T. versicolor* after 6 d of incubation (Figure V.2). In a previous report, the percentage of degradation of CBZ by this fungus was considerably lower (57%) even for a longer incubation period (7 d) (Marco-Urrea et al., 2009). This was explained by the depletion of oxygen that may have occurred in the sealed microcosms used in the study. The experiments performed at mg L^{-1} concentration range simplified the analytical procedure, but CBZ is typically found at much lower concentration in municipal wastewaters. Therefore, additional experiments were carried out at a concentration of approx. 50 µg L^{-1} in order to assess the capability of *T. versicolor* to degrade the contaminant at close environmentally relevant concentrations. As can be observed in figure V.3, 61 % of CBZ was degraded within 7 d. In all the experiments, only 17% of CBZ was removed due to adsorption in the biomass as observed from the difference in CBZ concentration between the heat-killed controls and the uninoculated ones.

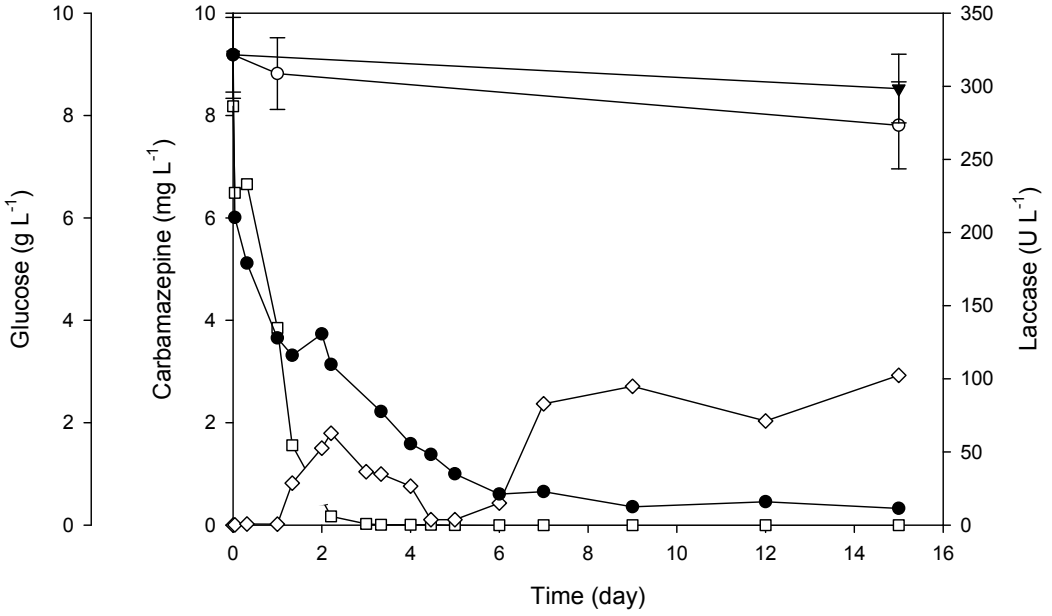

Figure V.2. Time course of carbamazepine degradation added at 9 mg L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Symbols: uninoculated controls (▼), experimental cultures (●), heat-killed (○), glucose (□) and laccase activity (◊).

Previous experiments showed that purified laccase did not significantly degrade CBZ, although the addition of a redox mediator HBT facilitated and improved the degradation (60% after 48 h) (Marco-Urrea et al., 2009; Hata et al., 2010). It is known that white-rot fungi produce lignin-related phenols or unsaturated fatty acids in the mycelium hyphae that can act as natural mediators expanding the oxidative potential of laccase for degradation of xenobiotics (Cañas and Camarero, 2010). Thus, the role of laccase on CBZ degradation cannot be underestimated. As shown in figures V.2 and V.3, after 7 d of experiments, extracellular laccase activity was approximately 100 U L^{-1} and 200 U L^{-1} in the experiments at high (mg L^{-1}) and low (µg L^{-1}) concentrations of CBZ, respectively. However, since a steep decrease of CBZ was observed during the first hours of the experiment, while laccase was still not detected (figure V.2 and V.3), no conclusive correlation between extracellular laccase activity and degradation of CBZ could be drawn.

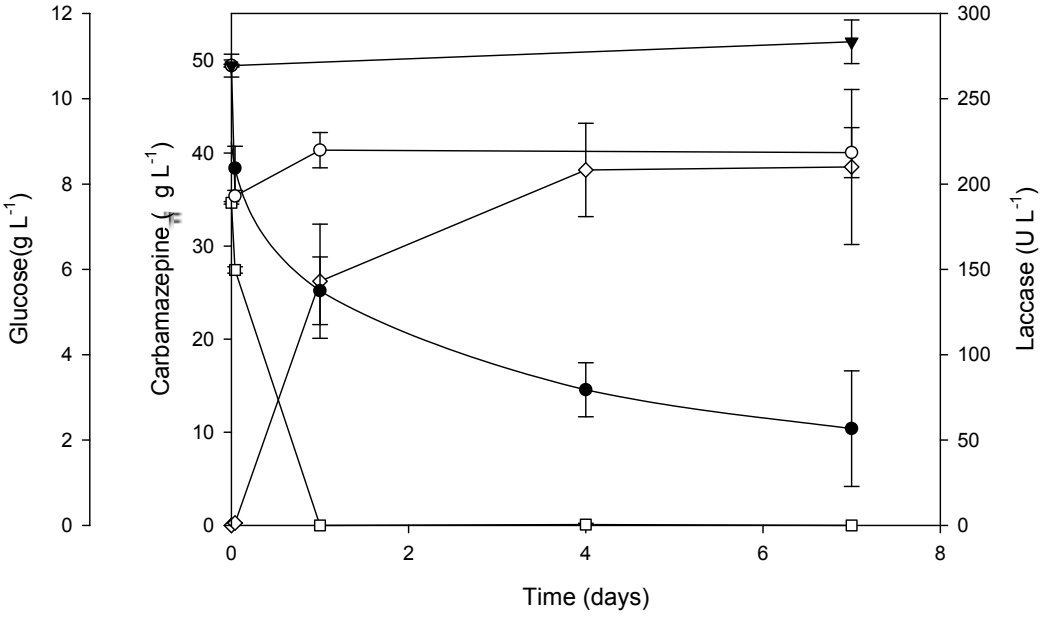

Figure V.3. Time course of carbamazepine degradation added at 50 µg L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Symbols: uninoculated controls (▼), experimental cultures (●), heat-killed (○), glucose (□) and laccase activity (◊).

In addition to laccase, other ligninolytic enzymes have been assumed to be involved in CBZ degradation by white-rot fungi. The addition of crude LiP from *P. chrysosporium* to CBZ resulted in degradation percentage below 10% (Zhang and Geiβen, 2010), but *T. versicolor* does not produce this enzyme. Crude enzyme MnP from *Bjerkandera* sp. strain BOS55 did not have any significant effect on CBZ oxidation (Marco-Urrea et al., 2009). However, Golan-Rozen et al., (2011) reported high degradation rates of CBZ (up to 99%) in glucose peptone (GP) medium with Mn^{2+} and suggested the involvement of MnP on CBZ removal since this medium expressed genes encoding MnP in *P. ostreatus*. In absence of Mn^{2+}, the degradation of CBZ by *P. ostreatus* was attributed to the activity of another enzyme VP, on the basis of the high enzymatic activity of this enzyme (Golan-Rozen et al., 2011). Another *enzymatic mechanism* involved in degradation of CBZ by *white-rot fungi is the CYT P450 system.* Its capability of degrading CBZ in liquid medium was demonstrated when the degradation rates were compared in the presence of CYT P450 inhibitors in *T. versicolor* and *P. ostreatus* and without them (Marco-Urrea et al., 2009; Golan-Rozen et al., 2011). In our study, rather negligible levels of laccase and MnP (data not shown)

were detected during the first hours in the time-course degradation experiments, which could indicate that this intracellular system was involved in CBZ oxidation at the beginning of the incubation period (Figure V.2 and V.3). The fact that ligninolytic enzymes and the CYT P450 system influence CBZ degradation indicated the applicability of whole cells of white-rot fungi in the removal of this pharmaceutical in aqueous media.

V.3.2. Identification of the transformation products of CBZ

In order to identify the molecular ions of the transformation products of CBZ, to propose empirical formulas and to elucidate their chemical structures, first full scan MS data were collected on a QqToF-MS instrument followed by acquisition of the product ion spectra of the tentatively assigned metabolites. Table 1 shows the results of these high-resolution measurements along with the relative mass errors for the proposed elemental compositions. As observed, four major transformation products from CBZ were identified when exposed to *T. versicolor*: CBZE, 10,11-dihydro-10,11-dihydroxycarbamazepine (CBZD), acridine, and acridone.

As regards the parent compound CBZ (6.95 min, in figure V.4), it formed a protonated molecule at *m/z* 237 under (+) ESI conditions with a minor contribution of the sodium adduct at m/z 259. Upon collision-induced dissociation of the protonated CBZ molecule, fragment ions were detected at *m/z* 220 and *m/z* 194 corresponding to the neutral loss of NH_3 and HNCO (43 Da), respectively (figure V.5). In search of transformation products in the samples from the biodegradation experiments, full-scan chromatograms were recorded over a mass range from *m/z* 70 to 800. This allowed discerning the emergence of five major peaks, four of which were attributed to CBZ-related metabolites having molecular ions of *m/z* 180, 196, 253 and 271 (figure V.5).

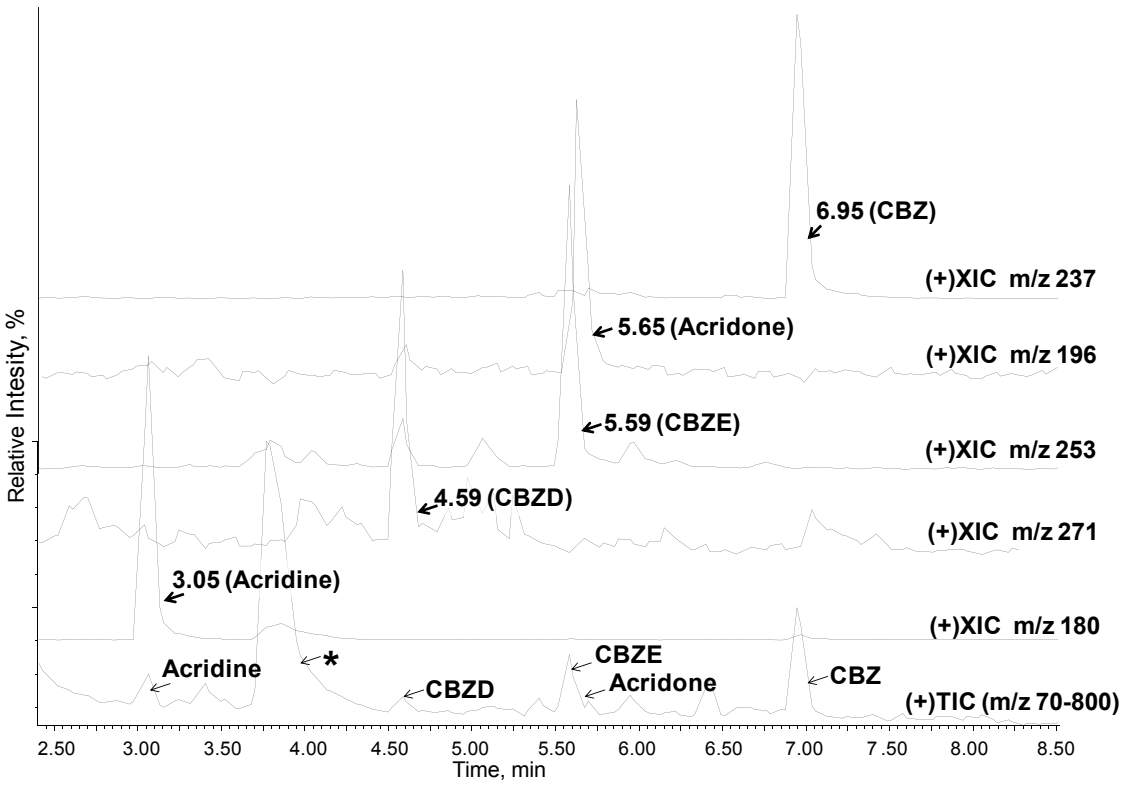

Figure V.4: UPLC- ESI-QqToF-MS chromatograms of a sample taken on the third day of the incubation of CBZ by *T. versicolor*: (+)XIC of m/z 237 (CBZ), (+)XIC of m/z 196 (acridone), (+)XIC of m/z 253 (CBZE), (+)XIC of m/z 271 (CBZD)), (+)XIC of m/z 180 (acridine) and (+)TIC (m/z 70-500).

The mass spectrum of the most intense peak in turn (3.55 min, in figure V.4) was characterized by a number of peak clusters across the entire mass range (data not shown). At least three series of peaks with repeating and alternating m/z units could be discerned in the full-scan spectrum while their product ion spectra revealed a similar fragmentation pattern with the characteristics of oligomeric products. As the signal at 3.55 min was also observed in the chromatographic analysis of the test medium from a parallel study, dealing with the degradation of another pharmaceutical compound by *T. versicolor*, no further attempts were made to elucidate the structure of these apparently endogenous fungal metabolites.

Table 1. Accurate mass measurements of the biodegradation products of CBZ as determined by UPLC–(+)ESI-QqToF-MS. Data for (pseudo)-molecular ions correspond to acquisitions in full-scan mode, those of fragment ions to product ion spectra of the protonated molecules.

Retention time [min]	Compound	Ion	Measured mass [m/z]	Elemental composition	Calculated mass [m/z]	Relative error [ppm]	Double-bond equivalents
3.05	Acridine	$[M+H]^+$	180.0806	$C_{13}H_{10}N$	180.0813	-3.9	9.5
		152	152.0581	$C_{11}H_6N$	152.0500	53.2	9.5
		128	128.0518	C_9H_6N	128.0500	14.1	7.5
4.59	CBZD	$[M+Na]^+$	293.0909	$C_{15}H_{14}N_2O_3Na$	293.0902	2.4	9.5
		$[M+H]^+$	271.1096	$C_{15}H_{15}N_2O_3$	271.1083	4.8	9.5
		253	253.0986	$C_{15}H_{13}N_2O_2$	253.0993	3.6	10.5
		236	236.0700	$C_{15}H_{10}NO_2$	236.0712	-5.1	11.5
		210	210.0947	$C_{14}H_{12}NO$	210.0909	8.3	9.5
5.59	CBZE	$[M+H]^+$	253.0985	$C_{15}H_{13}N_2O_2$	253.0977	3.2	10.5
		236	236.0711	$C_{15}H_{10}NO_2$	236.0712	-0.4	11.5
		210	210.0918	$C_{14}H_{12}NO$	210.0919	-0.5	9.5
		180	180.0825	$C_{13}H_{10}N$	180.0813	6.7	9.5
5.65	Acridone	$[M+H]^+$	196.0763	$C_{13}H_{10}NO$	196.0762	0.5	9.5
		167	167.0743	$C_{12}H_9N$	167.0735	4.8	9.0
6.95	CBZ	$[M+H]^+$	237.1027	$C_{15}H_{13}N_2O$	237.1028	-0.4	10.5
		194	194.0975	$C_{14}H_{12}N$	194.0970	2.6	9.5

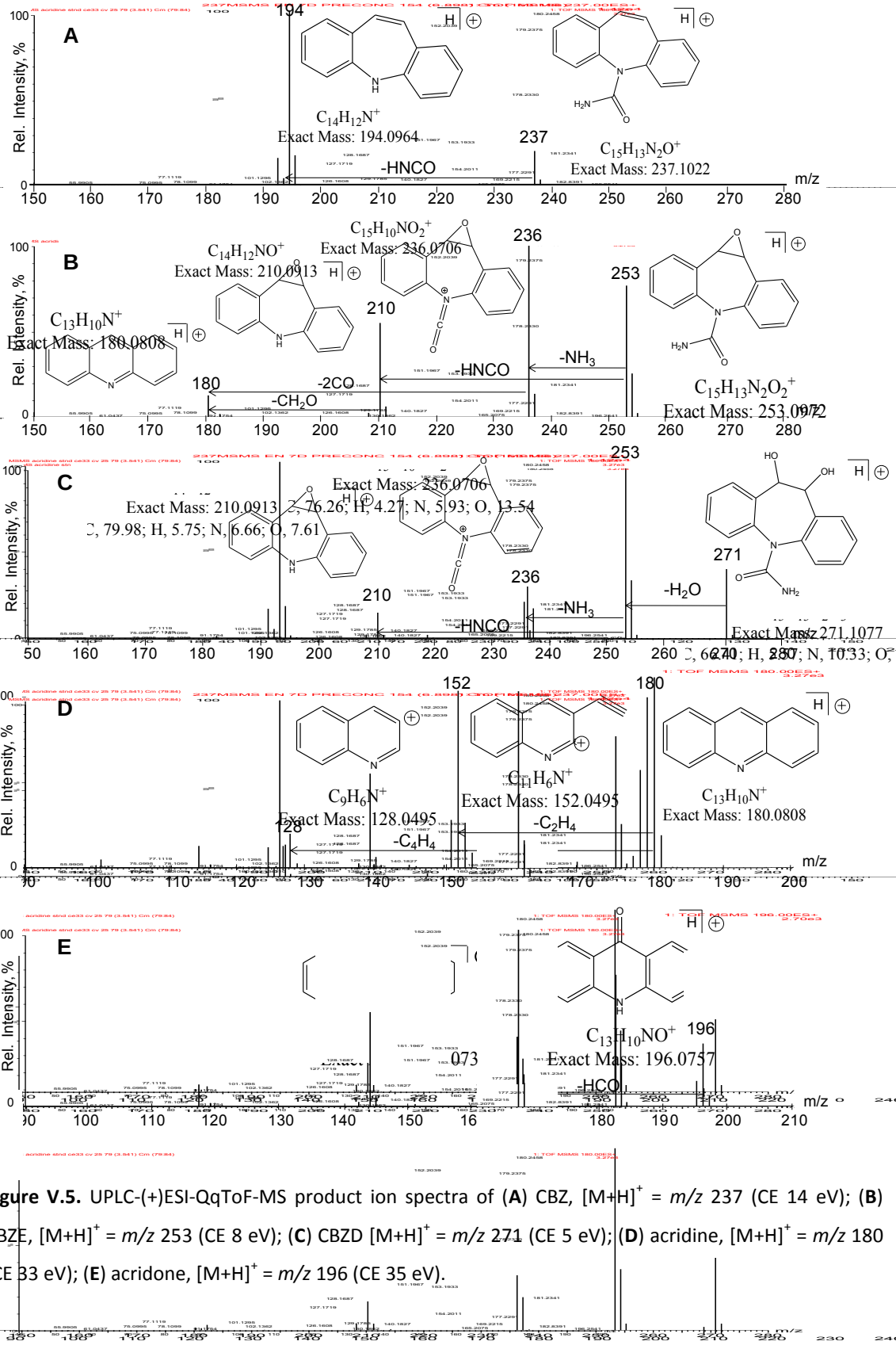

Figure V.5. UPLC-(+)ESI-QqToF-MS product ion spectra of (**A**) CBZ, $[M+H]^+$ = *m/z* 237 (CE 14 eV); (**B**) CBZE, $[M+H]^+$ = *m/z* 253 (CE 8 eV); (**C**) CBZD $[M+H]^+$ = *m/z* 271 (CE 5 eV); (**D**) acridine, $[M+H]^+$ = *m/z* 180 (CE 33 eV); (**E**) acridone, $[M+H]^+$ = *m/z* 196 (CE 35 eV).

With respect to the two degradates with ion masses lower than that of the parent compound, the molecular ion of the product at *m/z* 196 showed an accurate mass of *m/z* 196.0767 suggesting the elimination of C_2H_3N from CBZ. In the product ion spectrum (figure V.5), the only detected fragment ion was at *m/z* 167 while the characteristic loss of 43 Da observed for CBZ was absent. This fragment ion was attributed to the loss of $HCO^{\cdot}$ upon formation of a radical cation as indicated by the integer double-bond equivalent (DBE). The structure of this compound was proposed to correspond to acridone, which was corroborated by the match in retention time and mass spectral fragmentation with an authentic standard. The accurate mass data obtained for the molecular ion of the breakdown product at *m/z* 180 suggested an elemental composition of $C_{13}H_{10}N$ (-3.9 ppm). Fragmenting the $[M+H]^+$ ion with a collision energy of 33 eV yielded signals at *m/z* 152 ($-C_2H_4$) and 128 ($-C_4H_4$) yet the precursor ion proved fairly stable. At more energetic conditions in the collision cell the dissociation process led to complex spectra of many peak clusters with ions of similar abundance. Based on the likely molecular formula and the fragmentation scheme, the metabolite at *m/z* 180 was proposed to correspond to the aromatic nitrogen heterocycle acridine. Analysis of a commercially available standard of acridine under identical UPLC-MS conditions corroborated the metabolite identity.

The extracted ion chromatogram of *m/z* 253, i.e. the ion mass of possible monooxygenation products, displayed a dominant peak at a retention time of 5.59 min (figure V.4). Accurate mass measurements on the QToF-MS instrument were in line with the postulated incorporation of an oxygen atom into the CBZ structure ($C_{15}H_{13}N_2O_2$ for the $[M+H]^+$). The product ion spectrum of *m/z* 253 resembled the CBZ spectrum in that protonated molecule underwent loss of ammonia (17 Da) or HNCO (43 Da) to produce the ions at *m/z* 236 and 210, respectively. The ion *m/z* 180 was rationalized to originate from *m/z* 210 by loss of formaldehyde (-0.3 ppm) resulting in a stabilized fragment ion with the structure of the protonated acridine (cf. figure V.5). Hydroxylation of the phenyl ring was ruled out because standard solutions of 2-hydroxy and 3-hydroxy-CBZ gave distinct mass spectra (data not shown). Epoxidation of the double bond in the central 7-membered ring was therefore proposed, which

was further supported by the (+)ESI-MS^2 data of CBZE reported in Miao and Metcalfe (2003) where the same set of fragment ions was described. As far as the transformation product at 4.95 min is concerned, the full-scan mass spectrum showed beside the molecular ion $[M+H]^+$ at *m/z* 271 an abundant sodiated molecule at m/z 293 indicating the ease for coordinating the metal cation. The proposed elemental composition was $C_{15}H_{15}N_2O_3$ for m/z 271.1096 with a mass error of 4.8 ppm (Table 1). Generation of the product ion spectrum of the $[M+H]^+$ resulted in three fragment ions at m/z 253, 236 and 210 corresponding to dehydration followed by the loss of ammonia or HNCO (figure V.5), respectively. This fragmentation pathway was consistent with CBZD, presumably the hydrolysis product of the aforementioned epoxide in the degradation pathway of *T.versicolor*. The fragmentation pattern of m/z 271 in the present study matched with the authentic standard described elsewhere (Miao and Mecalf, 2003). The observation of the intense sodium adduct in the full-scan mass spectrum of the present study reflected the capacity of the analytes to complex the cation via the hydroxyl groups of the vicinal diol.

Figure V.6 depicts the decay of CBZ (added approx. at 9 mg L^{-1}) and the evolution of its transformation products during the experiment in Erlenmeyer flasks. About 94% of the initially present amount of CBZ was eliminated already after 6 d, and no further decrease of the concentration was observed. Three of four products, CBZE, acridone and acridine, were formed in the first few hours of the experiment, with different rates of production; whereas CBZD emerged after one day. After 2 d, all degradation products concentration remained constant along the time except acridine, which began to be removed from day 9.

Figure V.6. Plot of degradation of CBZ and evolution of its metabolites in Erlenmeyer flask experiment using *T. versicolor* (CBZ added at 9 mg L^{-1}).

CBZE was reported to be the major subproduct of the transformation of CBZ by the fungi *Cunninghamella elegans* and *Umbelopsis ramanniana* (Kang et al., 2008). Hata et al. (2010) also reported the formation of CBZE in the presence of laccase. Golan-Rozen et al. (2011) identified CBZE as major metabolite of CBZ degradation with whole cells of *P. ostreatus* and reported minor amounts of CBZD and 2- or 3-hydroxycarbamazepine. It is worth mentioning that the first step of the oxidative breakdown of CBZ in the CYT P450-mediated metabolism in humans is the oxidation to CBZE (Sillanpaa, 1996) and consecutively to CBZD and other hydroxylated compounds (Lertratanangkoon et al., 1982). The CYT P450 system has been shown to have a major role on CBZ degradation in white-rot fungi (see section 3.1) and the identification of the metabolites in this and similar studies (Golan-Rozen et al., 2011) might serve as an additional proof of its involvement. Nevertheless, in the present study we cannot demonstrate that CBZD was formed by oxidation of CBZE. As for acridine, it was detected in the study of Hata et al. (2010) when the redox mediator HBT was added to the medium. Acridine was also found as major photodegradation intermediate (Chiron et al., 2006), and it was detected as a transformation product of CBZ exposed to a UV/H_2O_2 treatment (Vogna et al., 2004).

V.3.3. Degradation of CBZ in bioreactor and evaluation of the toxicity of the culture broth.

The high degradability showed by white-rot fungi is in contrast with the negligible levels of CBZ elimination showed in conventional biological wastewater treatment systems. Therefore, the next step to assess the use of this technology is the application of white-rot fungi in bioreactors. First, a batch bioreactor was used for the degradation of CBZ charged approximately at 200 μg L^{-1}. As observed in figure V.7A, almost complete removal of CBZ (95.6%) was obtained within 48h. The higher degradation efficiency obtained here, in comparison with Erlenmeyer flasks, can be explained by the continuous addition of glucose, pH control and air pulses supplies that allow the fungus to thrive and obtain better degradation yields. Regarding operational parameters, glucose was continuously consumed throughout the experiment, which indicates that *T. versicolor* was active until the end. Low activity values of laccase were detected during the process (up to 18.97 U L^{-1} at day 5) and the pH was maintained at 4.5. CBZE was found as unique metabolite in batch bioreactor treatment at a concentration of 127 μg L^{-1}, when CBZ was almost completely degraded within two days (figure V.7A). After this point, CBZE tended to drop but at 5 d began to accumulate in the broth up to a concentration of 80 μg L^{-1}. The fact that no other metabolites were detected is attributed to the low concentration of the parent compound (CBZ) in this experiment that produced the minor metabolites at concentrations below the detection limit.

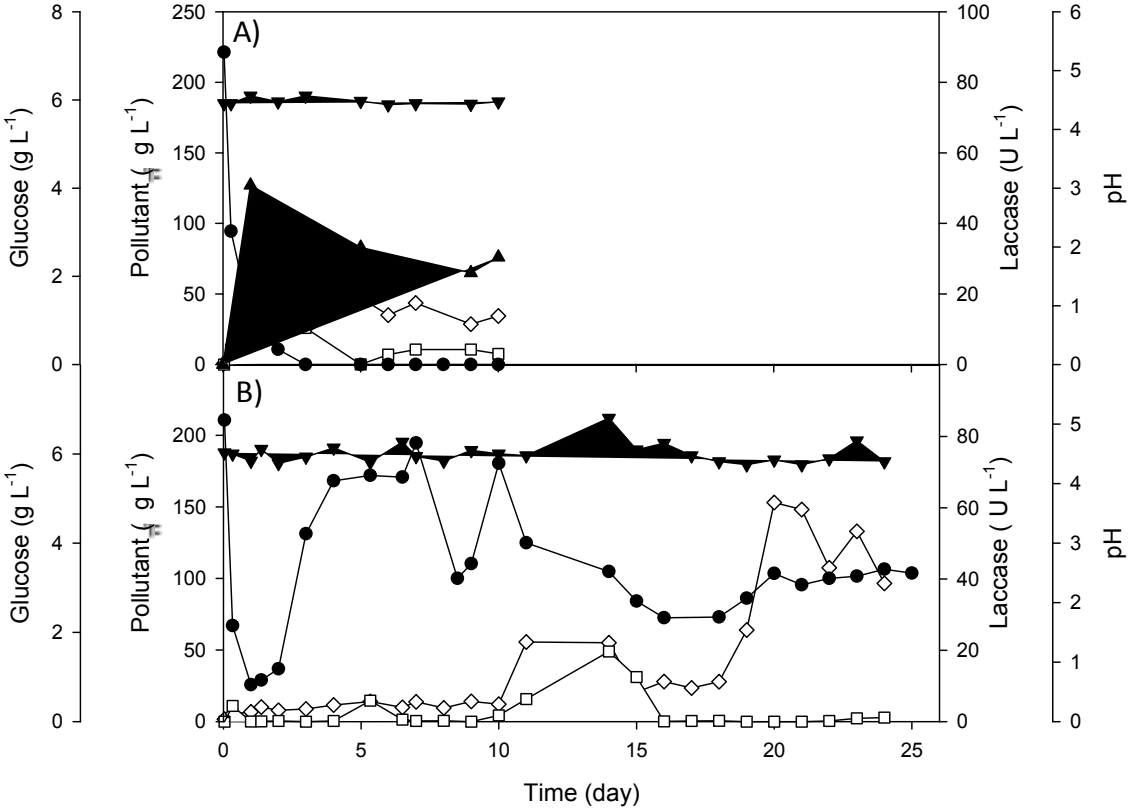

Figure V.7. Concentration profiles of CBZ and its CBZE metabolite in degradation study using *T. versicolor* pellets in batch (A) and continuous (B) bioreactor treatment. In addition, analysis of glucose, laccase production and pH are included. Symbols: CBZ (●), CBZE (▲), glucose (□), laccase activity (◊) and pH (▼).

The next step in the experiments was to operate the bioreactor in continuous mode (figure V.7B). Hydraulic retention time of 3 d was used with the aim to degrade CBZ but to avoid, as less as possible, the appearance of its transformation products. After 20 d of the experiment, when the steady state was reached, CBZ concentration in the outflow decreased to 54% of its inflow concentration, where the CBZ degradation rate was 11.9 µg CBZ g^{-1} dry weight pellets d^{-1}. Regarding the continuous addition of glucose, no accumulation was observed in the steady state, indicating that the fungus was active and therefore was not necessary to re-inoculate biomass at least during the first 25 d. pH was mantained at 4.5. Samples taken on the 3rd, 10th and 25th days were analyzed to identify transformation products but no metabolites were detected, probably due to their low concentration, as stated above.

Finally, a standard bacterial bioassay (Microtox) was performed for assessing the toxicity of the treated aqueous medium. The control containing CBZ at 200 g L^{-1} was analysed by Microtox test and showed a 15 min EC50 of 95%. At the end of the batch bioreactor treatment (10 d), the measured acute toxicity, expressed as 15 min EC50, was 24.0 %. In continuous mode, a 15 min EC50 of 77 % was determined in the steady state (23 d). These results showed low toxicity in both bioreactor treatments. However, in both cases (bath and continuous modes) the results of the acute toxicity were below the EC50 of the control indicating that transformation products of CBZ may be more toxic than the parent compound.

V.4. Conclusion

The results of this study indicated that T. versicolor is capable of degrading CBZ in aqueous medium in an air pulsed fluid- ized bioreactor operated in batch and continuous mode. Acridone, acridine, CBZE and CBZD were identified as the major transformation products of CBZ degradation. In batch reactor, CBZ concentration decreased by 96% within 2 days. More than a half (54%) of CBZ fed to the bioreactor operated in continuous mode (HRT 3 d) was removed when the process reached the steady state. Acute toxicity test showed that the final culture broths in both batch and continuous mode operation were non toxic. Therefore, the applied treatment might be a good strategy for the degradation of CBZ. Never- theless, further experiments are planned to study the degra- dation of CBZ by T. versicolor in bioreactor fed with real domestic wastewater, and then evaluate the possibility of full scale application of the process.

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Chapter VI:

Continuous treatment of clofibric acid by *Trametes versicolor* in a fluidized bed bioreactor: identification of transformation products and toxicity assessment.

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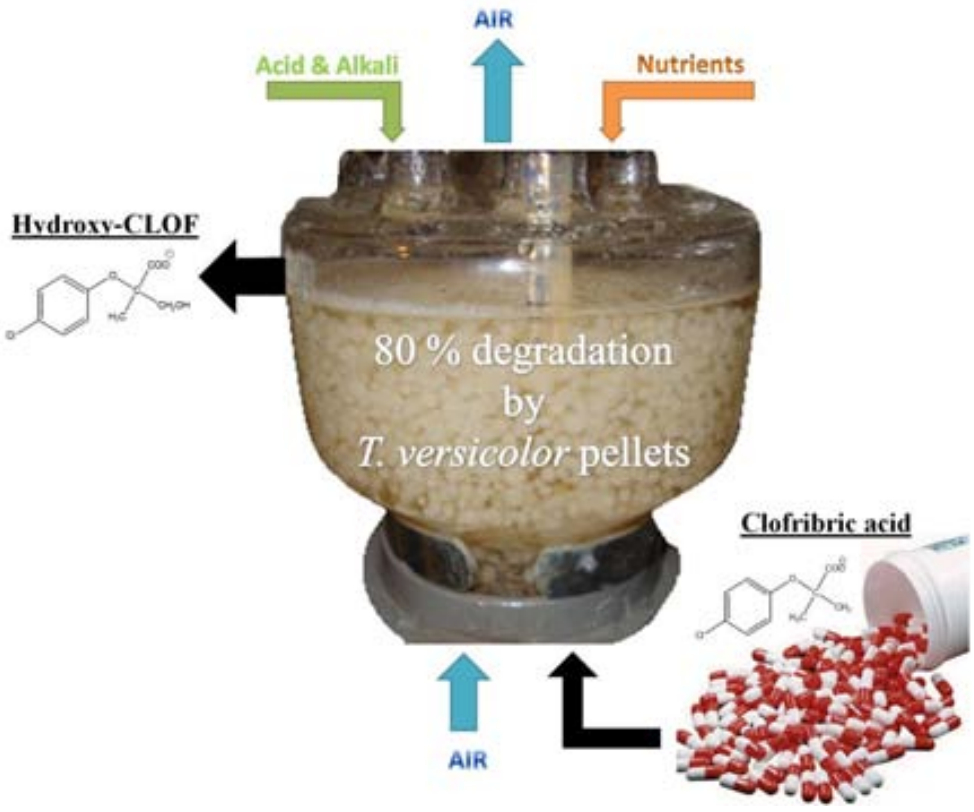

Abstract

The aerobic degradation of the blood lipid regulator clofibric acid (CLOF) was studied in a continuous bioreactor treatment using the white-rot fungus *Trametes versicolor*. Experiments in Erlenmeyer flasks with the compound at 30 µg L^{-1} showed that CLOF can be completely degraded at near environmentally relevant concentration after 4 d. The degradation process was scaled-up in an air-pulsed fluidized bioreactor operated in continuous mode with a hydraulic retention time of 4 d. The results show that 80 % of the fed concentration (160 µg L^{-1}) was reduced at the steady state (from day 12 to the end). Here, CLOF removal rate was 12.5 µg g^{-1} dry weight biomass d^{-1}. The 2-(4-chlorophenoxy)-2-(hydroxymethyl)propanoic acid (hydroxy-CLOF) was identified as major metabolite, confirming the degradation of CLOF, but its concentration remained constant in the medium. In addition, in a batch bioreactor treatment the undegradability of hydroxy-CLOF was demonstrated. Finally, acute toxicity tests (Microtox) performed with the bacterium *Vibrio fischeri* showed that the final culture broth in both batch (15 min EC_{50} of 55 %) and continuous (11 %) experiments were more toxic than the beginning (61 %).

Keywords: Continuous treatment; bioreactor; *Trametes versicolor*; pharmaceuticals; clofibric acid.

VI.1. Introduction

Clofibric acid (CLOF) is the main pharmacologically active metabolite of the lipid lowering drugs clofibrate, etofibrate and etophyllinclofibrate. Numerous studies have demonstrated the occurrence of pharmaceuticals, including clofibric acid, in surface, ground water and even drinking water (Kasprzyk-Hordern et al., 2008; Kim et al., 2009). The main point of collection and subsequent release of these micropollutants into the environment are wastewater treatment plants (WWTP) (Verlicchi et al., 2012), where they arrive via domestic and hospital sewages or through industrial discharges (Ternes et al., 2004). Although present at low concentrations in the environment, ranging from ng L^{-1} to μg L^{-1} (Heberer et al., 2002), the pharmaceutically active compounds can produce adverse effects on aquatic and terrestrial organisms, especially on the former since they are exposed to long-term continuous influx of wastewater effluents (Cleuvers, 2004; Nentwig et al., 2004; Santos et al., 2010; Schnell et al., 2009). The studies of effluent waters and river sediment show that wastewater treatment achieves only partial removal of organic pollutants. Regarding the removal of CLOF, Petrović et al. (2009) reported a removal efficiency of only 28 % in conventional treatment technologies. Salgado et al. (2012) studied the biotransformation of CLOF in aerobic sequential batch reactors with mixed microbial cultures, achieving 51 % of CLOF degradation. They observed that the heterotrophic populations were more likely to be responsible for the CLOF degradation. They identified three main metabolites: α-hydroxyisobutyric acid, lactic acid and 4-chlorophenol, the latter known to exhibit higher toxicity than the parent compound.

The degradation of CLOF using alternative technologies becomes an attractive topic in research. Advanced oxidation processes have been shown to be effective technologies for the removal of this compound. Doll and Frimmel (2004) achieved a removal of 90 % of CLOF by means of photocatalysis under UV in aqueous TiO_2 suspensions. In another study, it was demonstrated that the photoelectro-fenton method with Fe^{2+} and UVA light as catalysts was able to mineralize more than 96 % of CLOF in aqueous medium (pH 3.0) (Sirés et al., 2007). Using phytoremediation by

Typha spp., CLOF was removed up to 80 % after 21 days of exposure, with over 50 % being removed just within the first 24–48 h (Dordio et al., 2009).

Besides phytoremediation, another bioremediation strategy for removing CLOF is the use of white-rot fungi. In fact, the use of these microorganisms has shown to be a possible solution for the recalcitrance of many organic contaminants, as for example polycyclic aromatic hydrocarbons and other pharmaceuticals in different matrices (Semrany et al., 2012). Thus, the fungus *Trametes versicolor* degraded CLOF up to 97 % after 7 days of treatment in liquid medium at high concentrations (10 mg L^{-1}) (Marco-Urrea et al., 2009). In addition, a higher removal rate was achieved (80 % after 6 h) by inducing hydroxyl radical production via quinone redox cycling in this fungus (Marco-Urrea et al., 2010).

Most of the works published about the degradation of pharmaceuticals by fungi were performed in Erlenmeyer scale, while only few have examined the degradation in bioreactors (Rodarte-Morales et al., 2012a and b; Jelić et al., 2012), which is the next step in the process scaled-up to pilot plants. As an example, Rodarte-Morales et al. 2012b carried out experiments for degradation of pharmaceuticals by fungi in stirred tank and fixed-bed reactors operated in continuous mode. They observed complete removal of three anti-inflammatory drugs (diclofenac, naproxen and ibuprofen) and partial removal (50-60 %) for psychiatric drugs (carbamazepine and diazepam) by *Phanerochaete chrysosporium d*uring a treatment of more than 50 d. Jelić et al. 2012 demonstrated the continuous biodegradation of carbamazepine in a fluidized bed bioreactor with *T. versicolor* pellets, achieving 54 % of removal in the outflow when the steady state was reached (25 d), although it was accompanied by an increase in the toxicity. Therefore, it is not only important to study the primary degradation of the target compound, but also to identify the transformation products and assess the toxicity of the treated effluent, which eventually allowed us to evaluate the suitability of the treatment.

In this study, we implement the CLOF degradation process by *T. versicolor* in an air pulsed fluidized bioreactor operated in continuous mode. Furthermore, we also aim to identify major transformation products and to assess the effluent toxicity.

VI.2. Materials and methods

VI.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on 2 % malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 days.

Pellet production was done as previously described by Font et al. (2003). Pellets obtained by this process were washed with sterile deionized water.

CLOF (>97 % of purity) was purchased from Sigma-Aldrich (Barcelona, Spain). The solvents, HPLC-grade methanol, acetonitrile, water (Lichrosolv), and formic acid (98 %) were provided by Merck (Darmstadt, Germany). Glucose, ammonium tartrate dibasic, 2,2-dimethylsuccinic acid (99 %) and antifoam 204 (mixture of organic polyether dispersions) were purchased from Sigma-Aldrich (Barcelona, Spain).

VI.2.2. Experimental procedures

VI.2.2.1. Degradation experiments in Erlenmeyer flask

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing appropriate amounts of mycelial pellets (0.201 g dry weight) in a total volume of 50 mL of defined medium: 8 g L^{-1} of glucose, 3.3 g L^{-1} of ammonium tartrate, 1.168 g L^{-1} of 2,2-dimethylsuccinate buffer, 10 and 100 mL, respectively, of a micro and macronutrient solution from Kirk medium (Kirk et al., 1978) and adjusted to pH 4.5. This medium induces the production of laccase and it was used previously for CLOF and other PhACs degradation by *T. versicolor* (Marco-Urrea et al. 2009). CLOF, from a stock solution (5000 mg L^{-1}) in ethanol, was added into the flasks to give the desired final concentration (30 µg L^{-1}). After CLOF addition, the flasks were incubated under orbital shaking (135 rpm) at 25 ºC. To avoid the possible influence of light on CLOF stability, all the experiments were carried out in the dark. Experiments were performed in triplicate and under sterile conditions (all the containers and the medium was autoclaved at 121ºC for 30 min).

The whole content of the flasks was sacrificed on day 1, 2, 4 and 7 and each flask was filtered through 0.45 µm glass fiber filter GF/A from (Whatman, Spain). Subsequently, the filtrated residue was preconcentrated by solid-phase extraction (SPE) and the extract was analyzed by high performance liquid chromatography (HPLC). The target compound in the broth was concentrated in one step by SPE with Oasis HLB cartridges (60 mg adsorbent, Waters, Barcelona, Spain) as described elsewhere (Gros et al., 2006). Briefly, the cartridges were preconditioned sequentially with 5 mL of methanol and 5 mL of deionized water adjusted at sample pH. After that, the sample was passed through the cartridge and the cartridge was dried under vacuum. The adsorbed compounds were eluted with methanol (2 x 2 mL) and subsequently concentrated to dryness under a gentle nitrogen stream. The dried extracts were reconstituted with 0.5 mL (25:75, v/v) acetonitrile-water. Extraction efficiency of CLOF, evaluated by recovery experiments, was 99.4 % ± 0.47. Also, glucose and laccase production were measured.

Degradation of CLOF in time-course degradation experiments was assessed by comparing its concentration in the heat-killed control flasks with that in the experimental flasks. Heat-killed controls consisted of autoclaved cultures (121 ºC for 30 min) which were set-up under identical conditions to those of the experimental cultures. The amount of adsorbed CLOF was determined from the difference in CLOF concentration between uninoculated and heat-killed control.

VI.2.2.2. Degradation experiments in bioreactor

A glass fluidized bed bioreactor with a working volume of 1500 mL (Blánquez et al., 2007) was used for the CLOF continuous and batch treatment. Fluidized conditions were maintained by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 second open, 5 seconds close) and the air flow was 12 L h^{-1}.

The medium loaded in both continuous and batch bioreactor treatment contained 10 mL L^{-1} and 100 mL L^{-1} of micro and macronutrient solution, respectively, and 4 antifoam drops L^{-1} (Kirk et al., 1978). The medium and the reactor were sterilized

at 120º C for 30 min. After sterilization, CLOF was added into the medium to reach a final concentration of 160 µg L^{-1}, from a stock solution (1000 mg L^{-1}) in ethanol. Glucose and ammonium tartrate were fed continuously in both treatments from their stock solution (300 g L^{-1} and 169 mg L^{-1}, respectively) at a flow rate of 0.43 mL h^{-1} to ensure an uptake rate of 0.879 g g^{-1} dry weight pellets d^{-1} and 1.98 mg g^{-1} dry weight pellets d^{-1}, respectively. The temperature was maintained at 25ºC and the pH in the bioreactor was controlled at 4.5. Approximately 3.8 g dry weight pellets were inoculated.

In continuous bioreactor treatment, the flow rate (15.62 mL h^{-1}) of the influent (containing medium with CLOF) was adjusted to provide a hydraulic retention time (HRT) of 4 d. The biomass, in pellet form, was retained in the bioreactor throughout the experiment with no loss in the effluent, thus not requiring any extra addition.

Samples from the liquid phase in both treatments, were collected once a day until the end of the experiment, and pre-concentrated as explained in section 2.2.1.

The CLOF removal rate in batch (Erlenmeyer flasks) and continuous treatments was calculated following equations 1 and 2, respectively. R_{CLOF} is the CLOF removal rate in batch (b) or continuous (c) treatment (µg CLOF removed g^{-1} d.w. of biomass d^{-1}). M is the mass (µg) of CLOF at the beginning (i) and at the end (f) of the experiment. m is the mass flow (µg d^{-1}) of CLOF in the influent (i) and in the effluent (e) at the steady state. B is the inoculated fungal biomass (g d.w.) and t corresponds to degradation time (d).

$$R_{CLOF,b} = \frac{M_i - M_f}{B \cdot t} \qquad (1)$$

$$R_{CLOF,c} = \frac{m_i - m_e}{B} \qquad (2)$$

VI.2.3. Analytical procedures

VI.2.3.1. Analysis of CLOF

Analysis of CLOF was performed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) equipped with a UV detector at 230 nm. The column temperature was 30 ºC and a sample volume of 20 µL was injected from a Dionex autosampler (Barcelona, Spain). Chromatographic separation was achieved on a GraceSmart RP 18 column (250 × 4 mm, particle size 5 µm). The mobile phase consisted of 6.9 mmol L^{-1} acetic acid in water adjusted to pH 4 (by NaOH) with 35 % acetonitrile v/v. It was delivered isocratically at 1 mL min^{-1} as described elsewhere (Stafiej et al., 2007). The method quantification limit was 4 µg L^{-1}.

VI.2.3.2. Identification and quantification of metabolites

Accurate mass measurements of CLOF and its transformation product formed in bioreactor treatments were carried out in full-scan and product ion scan mode using a Micromass QqToF-system interfaced with a Waters ACQUITY UPLC system (Micromass, Manchester, UK).

Samples from the degradation experiments in bioreactor were separated on a Waters ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 µm particle size) equipped with a precolumn (5 × 2.1 mm) of the same packing material. The mobile phases were (A) water, and (B) acetonitrile/methanol (50/50, v/v). After 1 min isocratic conditions at 95 % A, the portion of A was linearly decreased to 5 % within 6 min. This condition was held for 1 min and then the initial mobile phase composition was restored within 1 min and maintained for column regeneration for another 2 min. The flow rate was 400 µL min^{-1}. The injection volume was 5 µL.

The MS analysis was performed with an electrospray ionization (ESI) interface in the negative ion mode applying a capillary voltage of -2800 V and the cone voltages 10 and 40 V. The nebulizer gas flow was set to 500 L h^{-1} at a temperature of 300°C. The drying gas flow was 50 L h^{-1}, and the source temperature 120°C. For MS experiments, the instrument operated in the wide pass quadrupole mode with ToF data collected

between *m/z* 70 and 400. The ToF analyzer was operated at a resolution of 5000 (FWHM) and ESI mass spectra were recorded in 1-s intervals with automatic switching of the dual-sprayer every 10 s for infusion of the internal calibrant for a duration of 1 s. Val-Tyr-Val served as internal lock mass with $[M-H]^-$= *m/z* 378.2029. All MS data acquisition and processing was done using the software package MassLynx V4.1.

The quantitative analysis of CLOF, and the semi-quantitative determination of its transformation product was performed using Symbiosis Pico™ (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcap-ped column (125mm x 2.0 mm, particle size 5 µm) preceded by a C18 guard column (4 mm x 4 mm, particle size 5 mm), both supplied by Merck (Darmstadt, Germany).

The mobile phases were (A) acetonitrile/methanol (50/50, v/v) and (B) HPLC grade water. The gradient was as follows: isocratic for 5 min at 5 % eluent A, linear increase to 95 % A within 10 min, hold for 2 min, return to initial conditions in 2 min, equilibration for 3 min. The flow rate was 300 µL min^{-1} and the injection volume was 20 µL.

For the analysis, the Turbo Ion Spray source was operated in the negative ion mode using the following settings for the ion source and mass spectrometer: curtain gas 30 psi, spraying gas 50 psi, drying gas 50 psi, drying gas temperature of 700 °C and ion spray voltage of -4500 V.

The transitions for multiple reaction monitoring, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were as follows: *m/z* 213→127 (DP -50V, CE -26 eV, CXP -1) and *m/z* 213→85 (DP -50V, CE -14 eV, CXP -5 V).

VI.2.3.3. *Vibrio fischeri* luminescence test (Microtox® test)

A Microtox bioassay was used to perform toxicity test. This method is based on the percent decrease in the amount of light emitted by the bioluminescent bacterium *V. fischeri* upon contact with a filtered sample at pH 7. The effective concentration,

EC_{50}, was measured after 15 min. Effluent toxicity was expressed in percentages of EC_{50}. The experimental sample tested was collected from both batch and continuous treatments.

VI.2.3.4. Other analyses

Laccase activity was assayed using a modified version of the method for the determination of manganese peroxidase (MnP) as described elsewhere (Kaal et al., 1993). The reaction mixture used consisted in 200 µL of 250 mM sodium malonate at pH 4.5, 50 µL of 20 mM 2,6-dimethoxiphenol (DMP) and 600 µL of sample. DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV-vis spectrophotometer at 30ºC. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

Mycelial pellets dry weight was determined after vacuum-filtering the cultures through pre-weighed glass-fiber filters (Whatman GF/A, Barcelona, Spain). The filters containing the mycelial pellets were placed on glass plates and dried at 100 ºC to constant weight.

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instrument and Co. (Yellow Springs, OH, USA).

VI.3. Results and discussion

VI.3.1. CLOF degradation at low concentrations

Degradation of CLOF by *T. versicolor* pellets has been investigated previously in our group, obtaining 97 % CLOF degradation after 7 d when the contaminant was at 10 mg L^{-1} Marco-Urrea et al., 2009). However, pharmaceutical residues are detected in the environment at concentrations ranging from ng L^{-1} to µg L^{-1} (Heberer et al., 2002). Therefore, it is important to ensure that the degradation is also possible under these conditions, testing that the low concentration of the target compound triggers the enzymes required for its removal. Therefore, an additional series of experiments were carried out at a lower concentration of 30 µg L^{-1} CLOF. As observed in Figure VI.1, 33 %

of the initial CLOF concentration was removed after 1 h, reaching almost complete removal within 4 d. The average CLOF removal rate was 1.79 μg g^{-1} d.w. of biomass d^{-1}. In the experiment, less than 17 % of CLOF was removed due to adsorption in the biomass as observed from the difference in CLOF concentration between the heat-killed control and the uninoculated ones.

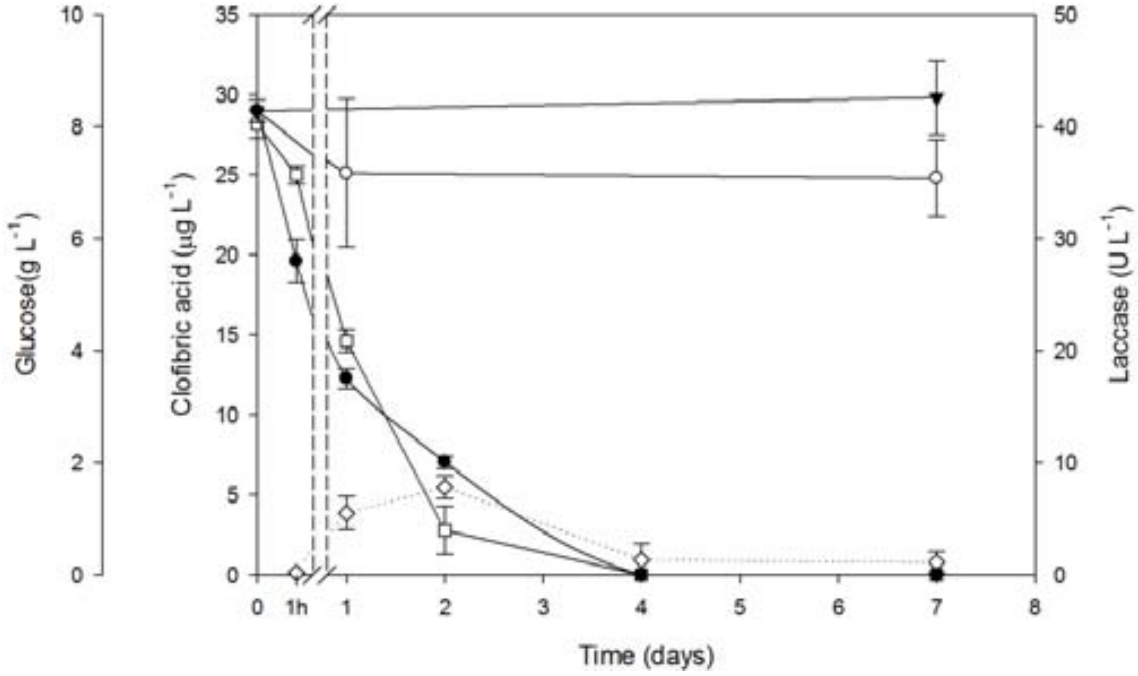

Figure VI. 1: Time course of CLOF degradation added at 30 μg L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Symbols: uninoculated controls (▼), experimental cultures (●), heat-killed (○), glucose (□) and laccase activity (◊). The experiment was performed in triplicate.

On the other hand, extracellular laccase activity did not exceed 7.9 U L^{-1}, although previous reports demonstrated that this enzyme was not involved in the degradation of CLOF (Marco-Urrea et al., 2009; Tran et al., 2010). However, it was used as an indicator of fungus activity. Other ligninolytic enzyme such as MnP, also excreted by *T. versicolor*, did not show significant oxidation of CLOF (Marco-Urrea et al., 2009). On the contrary, experiments using the cytochrome P450 inhibitors 1-aminobenzotriazole and piperonyl butoxide produced a reduction in the removal of CLOF, thus indicating that this intracellular enzymatic system plays a major role in the first step of CLOF oxidation by *T. versicolor* (Marco-Urrea et al., 2009).

VI.3.2. Continuous treatment

A fluidized bioreactor operated in continuous mode was used to performed experiments for the degradation of CLOF (Figure VI.2A). The biomass in pellet form was maintained fluidized by air pulses. The air pulses favored the formation of compact spherical pellets (Rodarte-Morales, et al., 2012a) and the homogenous distribution of the pellets within the liquid phase of the bioreactor volume. The hydraulic retention time was 4 d, since in previous Erlenmeyer flask experiment (section 3.1) CLOF was completely removed before that time.

Figure VI.2A depicts the CLOF concentration profile in the continuous bioreactor treatment. After 12 d of experiment, when the steady state was considered to be reached, the CLOF concentration was reduced to 80 % compared to the inflow concentration, with a removal rate of 12.5 μg g^{-1} d.w. biomass d^{-1}. This high rate of removal was maintained up to 24 d without re-inoculation of biomass. The high removal rate obtained in this treatment in comparison with Erlenmeyer flasks (1.79 μg g^{-1} d.w. biomass d^{-1}) can be explained by the continuous addition of glucose and nitrogen, the air pulses supply and the controlled pH that allow the fungus to give better degradation rates. Moreover, the continuous addition of CLOF may allow the adaptation of the fungus at these conditions, leading to higher degradation rates.

Regarding the operational parameters, no accumulation of glucose was observed, confirming that the fungus was active throughout the experiment. The pH was maintained at 4.5. Although laccase appeared not to be involved in the degradation of CLOF (Marco-Urrea et al., 2009), its production increased over the experiment (going up to 300 U L^{-1} at the end of the experiment) indicating that the fungus was still active.

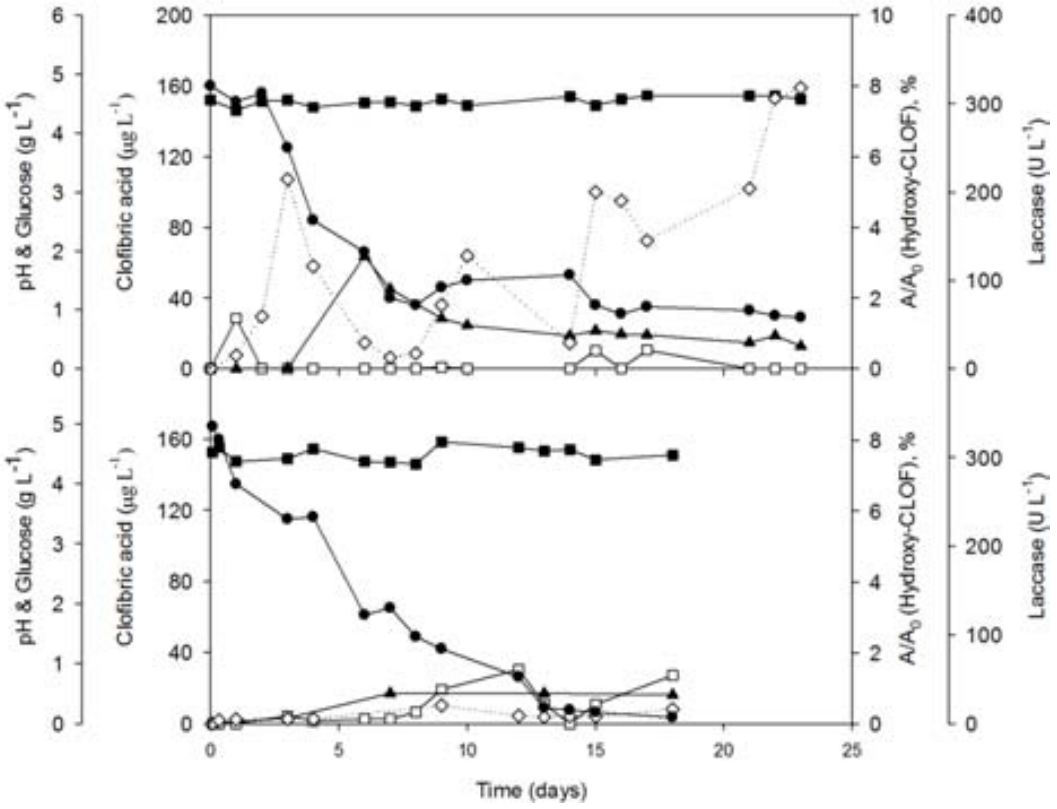

Figure VI.2: Time course of CLOF degradation and metabolite formation using *T. versicolor* pellets in continuous (A) and batch (B) bioreactor treatments. The HRT for the continuous treatment was 4 d. Analysis of glucose, laccase activity and pH are included. Symbols: CLOF (●), Hydroxy-CLOF formation expressed as relative area (A/A_0) where A is the metabolite and A_0 is CLOF at time zero (▲), glucose (□), laccase activity (◊) and pH (■).

VI.3.3. Identification of transformation products

Besides studying the degradation of the target compound, it is also important to assess the transformation products formed because they can be more toxic than the parent compound.

The negative ion mass spectra of the deprotonated species of CLOF and the transformation product, which was generated by *T. versicolor* in the liquid medium, were recorded using a QqToF-MS instrument. Table 1 shows the data from the full scan and product ion scan experiments used to elucidate the chemical structure of the transformation product.

Table VI.1: Accurate mass measurements of the biodegradation products of CLOF as determined by UPLC–(-)ESI-QqToF-MS.

Full-scan							
Compound	Ion	Elemental composition	Meas. Mass	Calc. Mass	Error (mDa)	Error (ppm)	DBE
CLOF	$[M-H]^-$	C10H10O3Cl	213.0326	213.0318	0.8	3.8	5.5
Hydroxy-CLOF	$[M-H]^-$	C10H10O4Cl	229.0270	229.0268	0.2	0.9	5.5
Product Ion Scan							
CLOF (m/z 213)	$[M-H]^-$	C10H10O3Cl	213.0309	213.0318	-0.9	-4.2	5.5
	127	C6H4OCl	126.9955	126.9951	0.4	3.1	4.5
	85	C4H5O2	85.0318	85.0290	2.8	33	2.5
Hydroxy-CLOF (m/z 229)	$[M-H]^-$	C10H10O4Cl	229.0270	229.0268	0.2	0.9	5.5
	127	C6H4OCl	126.9956	126.9951	0.5	3.9	4.5
	101	C4H5O3	101.0260	101.0239	2.1	21	2.5

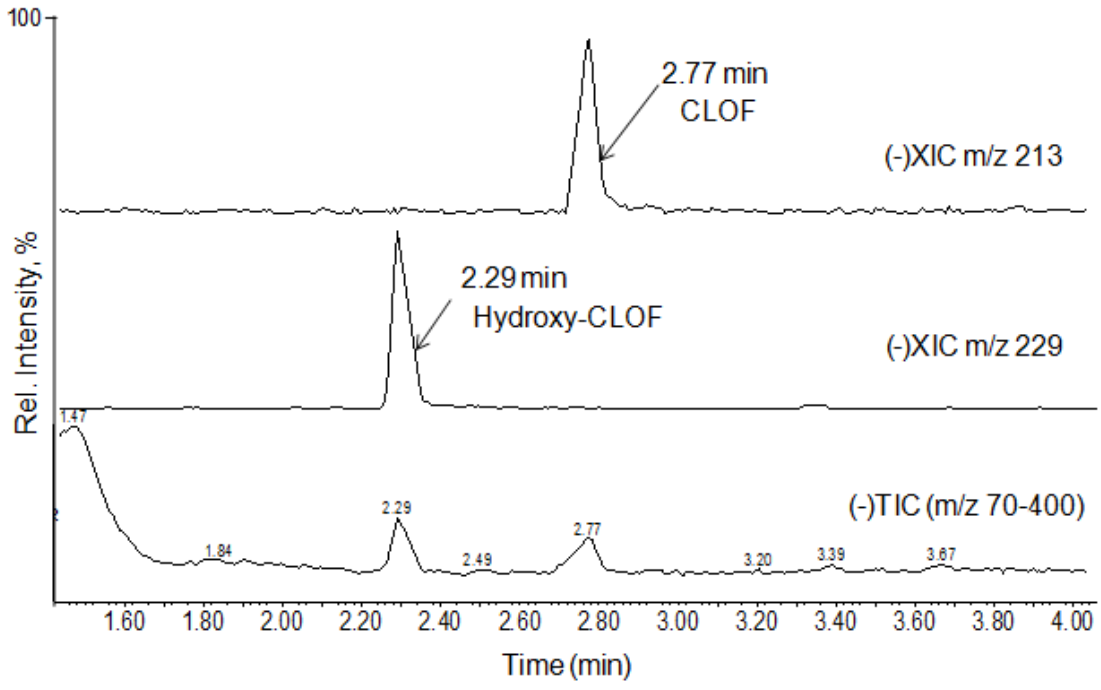

Figure VI.3: UPLC-(-)ESI-QqToF-MS chromatograms of a sample taken on the fourth day of the incubation of CLOF by *Trametes versicolor*: (-)XIC of *m/z* 213 (CLOF), and (-)XIC of *m/z* 229 (Hydroxy-CLOF)

As regards the mass spectrum of the parent compound CLOF (2.77 min, in figure VI.3), it formed a deprotonated molecule at *m/z* 213 under (-)ESI conditions. Upon collision-induced dissociation, two major fragments were observed: m/z 127 and m/z 85. The base peak at m/z 126.9955 corresponded to 4-chlorophenolate (mass error of +0.4 mDa) whereas the ion at *m/z* 85.0318 was attributed to deprotonated methacrylic acid (+2.8 mDa). Therefore both fragment ions originated from the same cleavage of the ether linkage on the aliphatic side in the CLOF molecule with charge retention in either moiety.

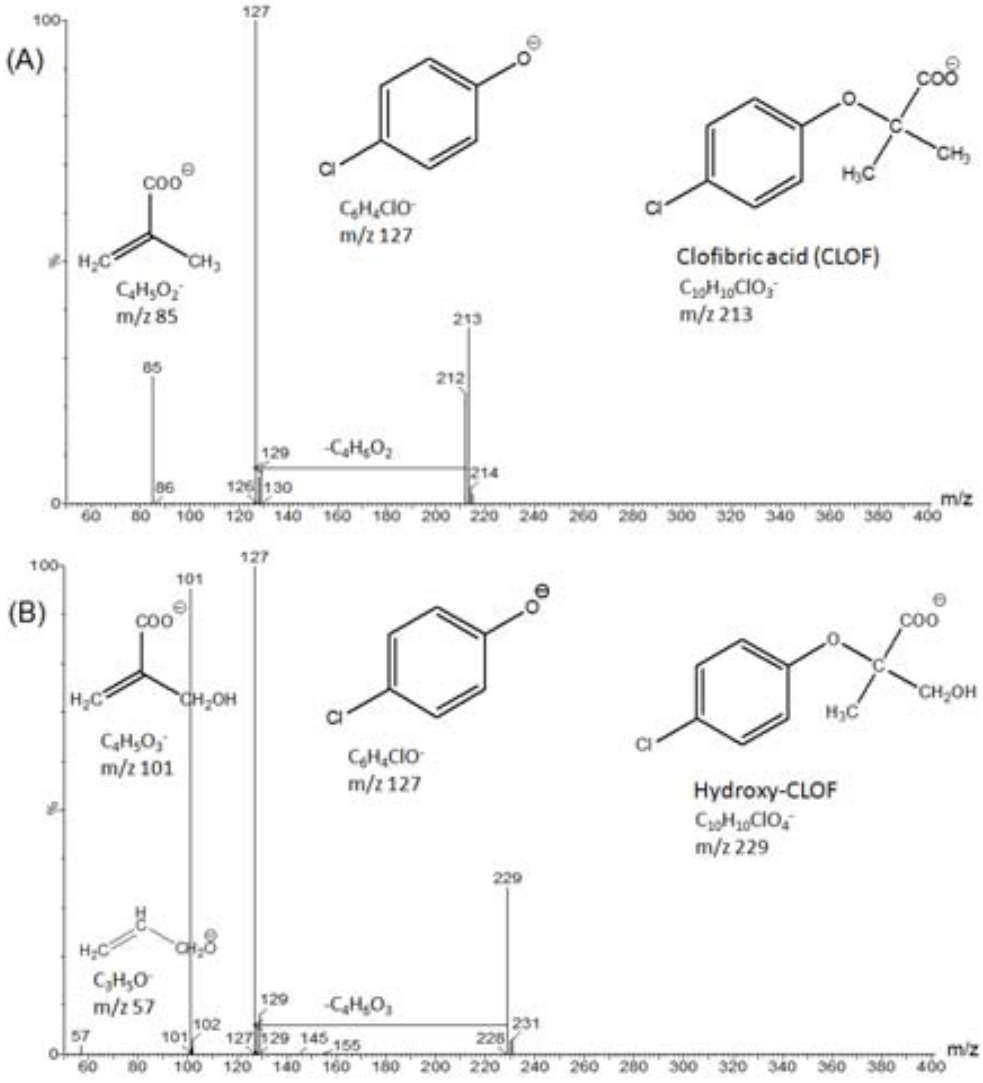

Figure VI.4: UPLC-(-)ESI-QqToF-MS product ion spectra of (**A**) CLOF, $[M-H]^-$ = *m/z* 213 (CE 9 eV); and (**B**) Hydroxy-CLOF, $[M-H]^-$ = *m/z* 229 (CE 8 eV)

Full-scan chromatograms recorded over a mass range from *m/z* 70 to 400 allowed discerning the emergence of two major peaks, where only one was attributed to CLOF-related metabolite having molecular ion of *m/z* 229 (Figure VI.4). The extracted ion chromatogram of *m/z* 229 displayed a dominant peak at a retention time of 2.29 min (Figure VI.3). The accurate mass of the deprotonated molecule was *m/z* 229.0268 with an isotopic peak cluster characteristic of a mono-chlorinated compound. The accurate mass measurement (+0.9 mg L^{-1}) indicated monooxygenation of CLOF to produce at metabolite with an elemental composition of $C_{10}H_{10}O_4Cl$ for the $[M-H]^-$ ion. Examination of its (-)ESI-MS/MS spectrum revealed the presence of the 4-chlorophenolate at mz/ 127 while the fragment ion at *m/z* 85 in the CLOF spectrum was shifted by 16 Da to *m/z* 101. The accurate mass data obtained for the fragment ion gave an elemental composition of $C_4H_5O_3$ (2.1 mDa). The only plausible structure of *m/z* 101, corresponded to the anion of 2-hydroxymethacrylic acid which provided evidence for mono-hydroxylation of a methyl group in the CLOF molecule as the metabolic route. This metabolite, 2-(4-chlorophenoxy)-2-(hydroxymethyl)propanoic acid, is henceforth referred to as *hydroxy-CLOF* (figure VI.4). To the author's knowledge, the side-chain hydroxylation of CLOF during biodegradation experiments was not reported previously.

Figure VI.2A depicts the decay of CLOF and the evolution of its transformation products in the continuous reactor treatment. As shown in the section 3.2, about 80 % of the inlet amount of CLOF was eliminated in the outflow when the steady state was reached (12 d). Hydroxy-CLOF began to be detected after 3 d, and after 6 d of the experiment it reached a maximum concentration that was equal to approx. 4 % of the initial CLOF concentration (A_0). Then, the concentration of the hydroxy-CLOF began to decrease until the steady state was reached (approx. 0.9 % of A_0), which was maintained constant until the end of the experiment. The identification of hydroxy-CLOF gives another proof for the involvement of cytochrome P450 in the degradation of CLOF, since the typical metabolites after the intake drugs in mammalian are hydroxylated products (Ortiz de Montellano, 2008). On the other hand the degradation by heterotrophic bacteria produced three metabolites, including α-hydroxyisobutyric

acid, lactic acid and 4-chlorophenol, not detected in this study, which implies the CLOF breakdown with the release of the methylpropanoic acid moiety (Salgado et al., 2012).

VI.3.4. Batch treatment

In order to observe whether a longer treatment with the fungus could degrade hydroxy-CLOF, experiments were carried out in a fluidized bioreactor operated in batch mode (Figure VI.2B). Glucose, supplied continuously, was not accumulated throughout the experiment and the pH was well controlled at 4.5. Almost complete removal of 160 µg L^{-1} CLOF (95 %) was observed after 13 d of the experiment. Regarding hydroxy-CLOF, it was detected after 3 days of the experiment as the only transformation product. It reached a maximum concentration (approx. 1 % of the A_0) on day 7, after which it remained constant until the end of the experiment. To sum up, the results showed that hydroxy-CLOF was not degraded by *T. versicolor* in the batch bioreactor treatment.

VI.3.5. Toxicity assessment of the bioreactor treatments

Finally, to evaluate the risk associated with exposure to the final broth in batch and the effluent of the continuous treatment, a bioassay was performed with the bacterium *V. fischeri* (Microtox test), which has been widely used test for assessing the toxicity of complex industrial effluents for more than two decades (DOGC, 1993). The lack of an accepted standard toxicity test for pure individual compounds at low concentrations and long-term exposure, as in the case of pharmaceutical concentration in wastewaters, have led to the use of Microtox test due to its reliability and easy-to-use technology. This test provides useful information about the toxicity of the treated broth in comparison with the blanks.

The influent containing 160 µg L^{-1} of CLOF shows a 15 min EC_{50} of 61 %, which means the influent is not toxic according to Microtox test. At the end of the treatment in batch bioreactor (day 18), a 15 min EC_{50} of 55 % was obtained. In the continuous reactor, a 15 min EC_{50} of 11 % was determined at day 21, when the steady state was

reached. These results show that hydroxy-CLOF and/or maybe any others transformation products of CLOF, which were not able to be detected as for example 4-chlorophenol identified in the degradation of heterotrophic bacteria (Salgado et al., 2012), are slightly more toxic than its parent compound. The treatment of contaminants by fungi is of interest because they are able to degrade a wide range of toxic compounds, but in some cases the transformation products are more toxic, like in the case of this study.

However, it should be noted that the toxicity in the present study just concerns the microorganism *V. fischeri*. Other authors (Parshikov et al., 2005) reported that hydroxylated derivatives increase the water solubility of the target compound, and therefore they could be more easily biodegradable by bacteria. Hence, the results of toxicity only should be taken as an indicator, because it cannot be generalized to other biological species.

VI.4. Conclusion

The results of this study showed that CLOF can be continuously degraded by *T. versicolor* in an air pulsed fluidized bed bioreactor. In the experiments performed in Erlenmeyer flasks containing CLOF at 30 µg L^{-1}, it was completely removed after 4 d, demonstrating that the target compound can be degraded at low concentration. The continuous bioreactor treatment, operated at 4 d of HRT, has allowed us to achieve eliminations of 80 % in the outflow when the process reached the steady state (12 d) and remained constant for a long period of time (24 d). In addition, the concentration of the major transformation product hydroxy-CLOF remained constant during the steady state. In addition, the recalcitrance of the hydroxy-CLOF by the fungus in a batch bioreactor treatment was demonstrated. Finally, standard toxicity bioassays with the bacterium *V. fischeri* were performed, showing that the treated effluent is more toxic than the initial feed, probably due to the presence of hydroxy-CLOF. Despite the low concentrations of the toxic contaminant, which are not harmful to health, this accumulation could cause environmental problems. Further experiments

are conducted in our lab to study the treatment of real wastewater containing CLOF and other pharmaceuticals.

VI.5. References

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Chapter VII:

Biodegradation of pharmaceuticals by fungi and metabolites identification- A review

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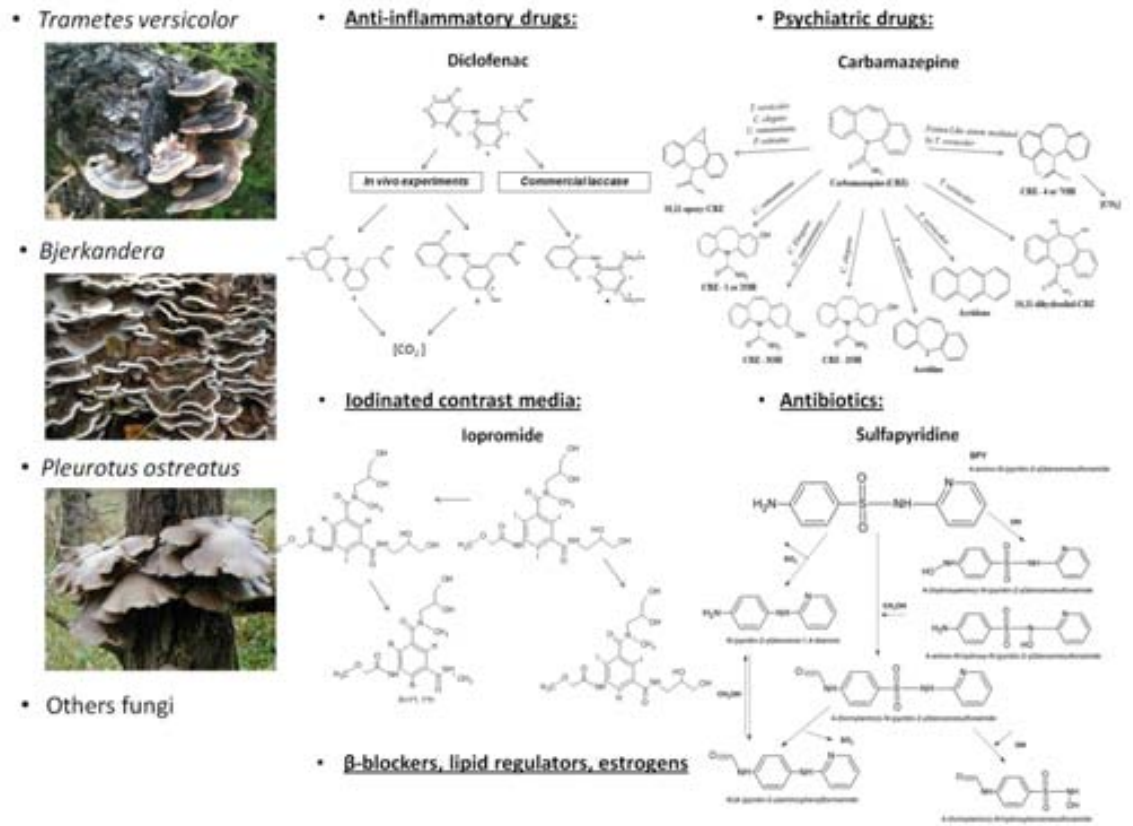

Abstract

Pharmaceutical compounds comprise a widely employed group of therapeutic agents now considered as emerging micropollutants. This chapter summarizes the state of the art in the degradation of pharmaceuticals by fungi in liquid matrices (with emphasis in white rot fungi), including the use of both whole cells and fungal enzymes. The identification of the metabolites produced as well as the proposed degradation pathways available for some drugs is discussed. The information is organized according to the activity of the pharmaceutical compounds, grouped in: anti-inflammatory/analgesic drugs, psychiatric drugs, lipid regulators, antibiotics and other antimicrobial agents, β-blockers, estrogens and iodinated contrast media. Considering the interest in potential application of fungal treatments in future real scale bioremediation of effluents, the ecotoxicology of the process is included when available.

Keywords: Transformation products, pharmaceuticals, degradation, toxicity, white rot fungi.

VII.1. Introduction

The most important removal pathways of organic compounds during wastewater treatment are biotransformation/biodegradation, adsorption to the sludge and stripping by aeration (volatilization). Also, abiotic removal from the aqueous phase by hydrolytic degradation and/or isomerisation/epimerisation can occur. In most of the studies, abiotic (adsorption) and biotic degradation (transformation by microorganisms) processes cannot be distinguished, and the term “removal” is usually indistinctly employed to refer to these phenomena. Moreover, without stoichiometric accounting for the transformation products (TPs) of biodegradation one cannot conclude whether the compound was completely destroyed (mineralized) or only structurally altered. Therefore, during the biological treatment of wastewater, microbial enzymatic activity may lead to the formation of TPs that preserved or even increased persistency and/or activity. Considering the potential persistency and toxicity of TPs generated during drinking and wastewater treatment, their identification and quantification, as well as elucidation of main reaction mechanisms is necessary for safe application of such processes. These products may have preserved the mode of action of the parent compound, or even be biologically more active, thus the disappearance of the parent drug does not necessarily equate to its detoxification. Quantitative evaluation of all the TPs formed, as well as their rate constants would afford kinetic and mechanistic data for the evaluation of efficiency in removing pharmaceuticals from real waters. More importantly, for a complete risk assessment study on the TPs of pharmaceuticals formed during drinking and wastewater treatment, determination of their ecotoxicity is fundamental and a prerequisite for a comprehensive protection of the environment.

However, studies dealing with identification of biodegradation products of drugs in wastewater treatment plants (WWTPs) are very scarce, possibly due to the complexity of screening and structural elucidation studies in environmental matrices such as wastewater and sludge.

Biotechnological approaches have emerged as promising tools for the elimination of micropollutants including pharmaceuticals. Some of them rely in the use of specific microorganisms with marked degrading ability, especially bacteria and less frequently fungi. The broad degrading ability of fungi and their enzymes, and their implication in the transformation and mineralization of several organic pollutants makes them potential agents for bioremediation processes, as it was reviewed elsewhere (Rodríguez-Rodríguez et al., 2012a). The present chapter summarizes the work done to date on the fungal-mediated degradation of pharmaceuticals and the identification of their TPs in liquid media. For the reasons above described, most of the research found in the literature focuses on the degradation in liquid media, since the easy control of parameters such as addition of nutrients, pH and the contaminant concentration facilitates the study of the degradation and the identification of TPs. On the other hand, many works presented in the next sections are referred to as degradation regardless whether the target compound is mineralized (completely removed) or transformed to another compound. As mineralization is hardly reported, in the discussion the degradation described refers to transformation, unless explicit mineralization is indicated.

The information is organized in two tables, comprising the degradation of pharmaceuticals by whole fungal cells (Table VII.1) or by fungal enzymes (Table VII.2); the discussion is grouped according to the therapeutic function of the pharmaceuticals in anti-inflammatory/analgesic drugs, psychiatric drugs, lipid regulators, antibiotics and other antimicrobial agents, β-blockers, estrogens and iodinated contrast media. Many of the studies were performed as preliminary approaches in order to determine the feasibility of applying fungal treatments to the biodegradation of pharmaceuticals from real liquid matrices. Therefore, when data is available, the toxicological characteristics of the residual effluent are discussed to assess the suitability of the treatment proposed.

VII.2. Pharmaceuticals

VII.2.1. Anti-inflammatory drugs

Non-steroidal anti-inflammatory agents are used extensively as non-prescription drugs, and residues of these compounds have been detected ubiquitously in WWTPs effluents at the μg L^{-1} levels, and they also frequently occur at the ng L^{-1} level in the aquatic environment (Hernandez-Raquet, 2012). As a result, many researchers have focused on the degradation of these pharmaceuticals, which are one of the most studied groups of therapeutic agents in terms of fungal transformation.

One of the most widely used anti-inflammatory drugs is ibuprofen. Marco-Urrea et al. (2009) performed a degradation screening using four white-rot fungi (WRF) (*Trametes versicolor, Ganoderma lucidum, Irpex lacteus* and *Phanerochaete chrysosporium)* for the removal of 10 mg L^{-1} of ibuprofen after 7 d of incubation in serum bottles and observed that the contaminant was degraded by all of the fungi. However, they noticed that *T. versicolor* was able to degrade the contaminant in only 1 h, while more time was needed to achieve the complete depletion with the other WRF. 1-hydroxy ibuprofen and 2-hydroxy ibuprofen were identified as TPs during the early stages of the degradation by *T. versicolor,* which were subsequently degraded to 1,2-dihydroxy ibuprofen (Figure VII.1). In addition, Microtox bioassay experiments performed at different incubation times , revealed an increase in the toxicity at the final time point (7 d), when only 1,2-hydroxy ibuprofen was detected (8.3 Equitox m^{-3} vs 0.05 Equitox m^{-3} in the controls with ibuprofen). This finding remarks the importance of the identification of metabolites in any treatment, because they may be more toxic than the parental compound. Rodarte-Morales et al. (2011a) confirmed the total elimination of ibuprofen by *P. chrysosporium* before 4 d, which agrees with the results by Marco-Urrea aforementioned. In addition, they reported complete degradation of this contaminant by *Bjerkandera* sp. R1 and *Bjerkandera adusta* before 4 d and 7 d respectively. However, no identification of metabolites or toxicological analysis was performed in order to evaluate the feasibility of the treatment.

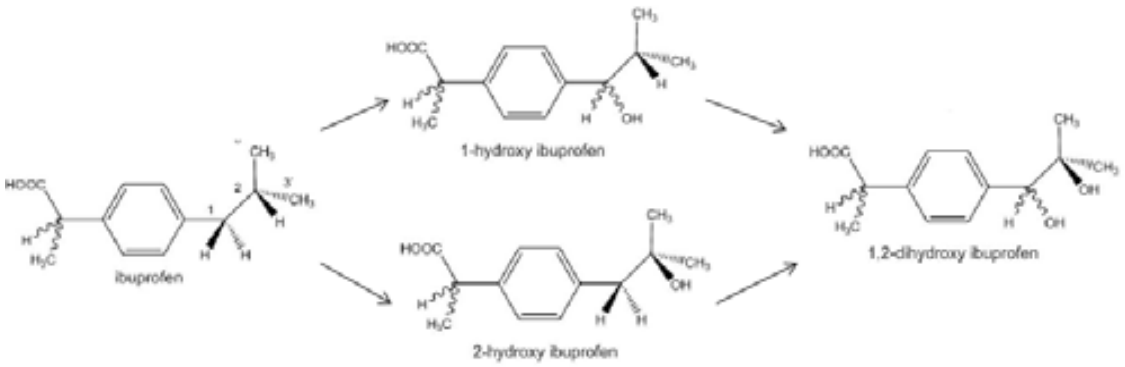

Figure VII.1 Suggested degradation pathway of ibuprofen by *T. versicolor* (Adapted from Marco Urrea et al. 2009).

Since it is known that WRF produce highly oxidative enzymes, several studies have also focused on the determination of which of these enzymes are involved in the degradation of contaminants. Marco-Urrea et al. (2009) reported that laccase (LAC) from *T. versicolor* and manganese peroxidase (MnP) from *Bjerkandera* sp. are not involved in the degradation of ibuprofen, according to the negligible levels (<10%) of degradation after 24 h. They also tested the degradation of ibuprofen by LAC adding different mediators, such as 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 1-hydroxybenzotriazol (HBT), violuric acid (VA) and 3,5-dimethoxy-4-hydroxyacetophenol (DMHAP), but their addition failed to oxidize ibuprofen as well. Tran et al. (2010) confirmed those results reporting low degradation values (35%) of ibuprofen (10 μg L^{-1}) when mediators were added. Marco-Urrea et al. (2009) also published that the addition of cytochrome P450 inhibitors did not affect in the degradation of that contaminant, thus concluding that an alternate enzyme system different from LACs, MnPs and cytochrome P450 monoxygenases is involved in the first step of ibuprofen degradation. Rodarte-Morales et al., (2011) carried out experiments for the biotransformation of three pharmaceutical active compounds (diclofenac, naproxen and ibuprofen) by *P. chrysosporium* in a fed batch stirred reactor under air and oxygen supply. They observed the complete removal of the contaminant before 24 h after the addition of ibuprofen pulses every 2 d and during 30 d. To sum up, ibuprofen was degraded for all the WRF so far studied. However, a more toxic transformation product was identified in the unique case where subproducts were

elucidated. This raises the question whether the treatment by fungi would be a good strategy for the removal of this drug.

The capability of diclofenac degradation by *T. versicolor* was demonstrated by Marco-Urrea et al. (2010a). In flask-scale experiments, they observed that almost complete diclofenac removal (>94%) occurred within the first hour with fungal pellets when the drug was added both at relatively high (10 mg L^{-1}) and environmentally relevant low (45 µg L^{-1}) concentrations in a defined liquid medium. In that treatment, 4'-hydroxydiclofenac and 5-hydroxydiclofenac were found as TPs (Figure VII.2), which in 24 h disappeared leading to a decrease in ecotoxicity calculated by the Microtox test, thus suggesting the complete mineralization of the drug. In addition, the authors carried out experiments to determine the possible role of some enzymes in the degradation of diclofenac.

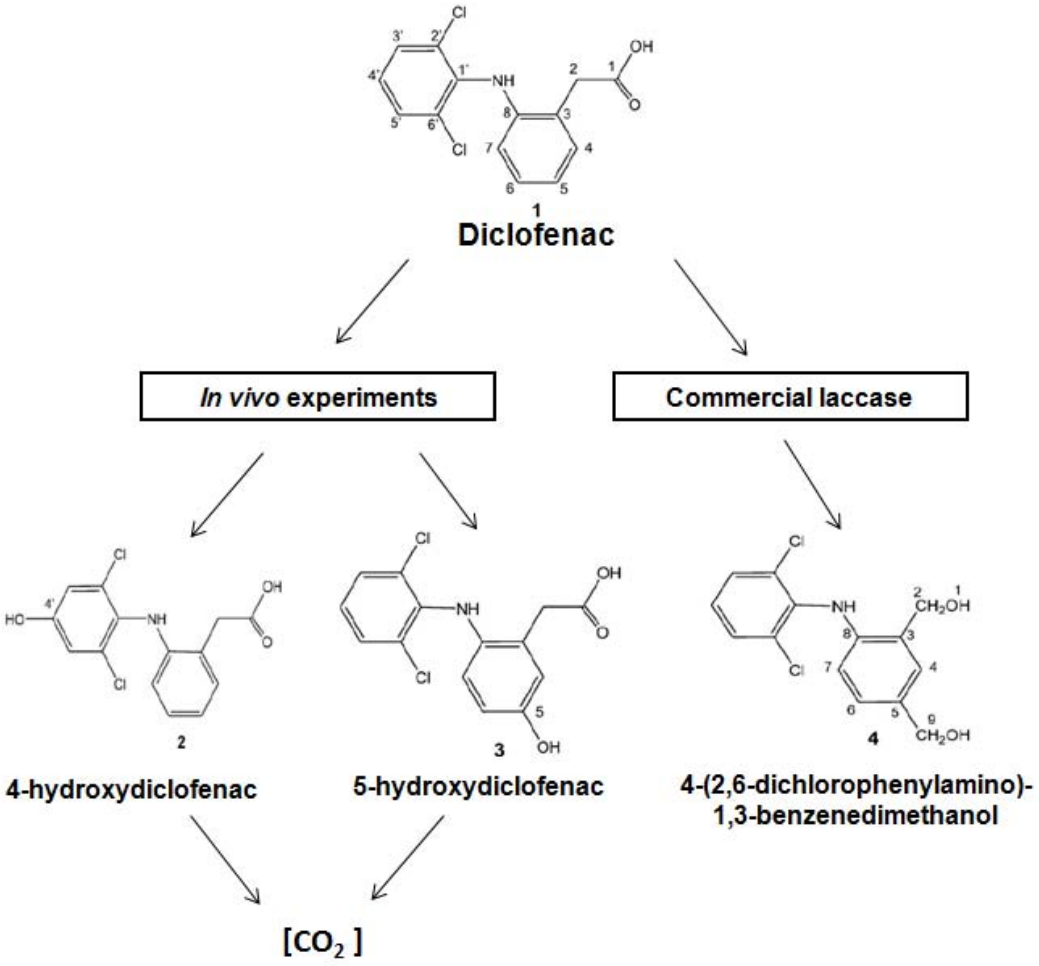

Figure VII.2 Suggested degradation pathway of diclofenac by *T. versicolor* and its enzyme LAC (Adapted from Marco-Urrea et al. 2010a).

They concluded that the cytochrome P450 system plays a key role in the first step of diclofenac degradation, because the addition of inhibitors of this enzyme resulted in a marked inhibition of diclofenac removal. Purified LAC catalyzes the transformation of diclofenac (>95% degradation) to 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol but it was not the only enzyme responsible for diclofenac degradation in *T. versicolor* pellets, as different subproducts were detected when the whole fungus was employed. Tran et al. (2010) confirmed the degradation of diclofenac by purified LAC, reaching more than 90% in 30 min. Likewise, Lloret et al. (2010) reported complete degradation of diclofenac by purified LAC from *Myceliophthora thermophila* using mediators, and 83% removal without them. Other enzymes such as VP from *B. adusta* (Eibes et al., 2011) and lignin peroxidase (LiP) from *P. chrysosporim* (Zhang and Geiβen et al., 2010) have also shown the ability to remove this micropollutant, but none of those reports refers to the identification of the TPs. Hata el al. (2010a) reported the degradation of diclofenac with other WRF, in particular *Phanerochaete sordida.* The target compound, in a concentration of 30 mg L^{-1}, completely disappeared after 4 d, indicating a slower degradation rate than *T .versicolor* (totally removed after 1 h). *P. sordida* also produced the hydroxylated metabolites found in the degradation by *T. versicolor*, but in addition 4,5-dihydroxydriclofenac was identified as a metabolite. The ability of *Bjerkandera* sp. R1*, B. adusta* and *P. chrysosporium* to degrade diclofenac was studied by Rodarte-Morales et al. (2011a). Their results revealed a complete degradation with the three strains after 7 d, except by *Bjerkandera*. sp. R1, which accomplished the elimination in 4 d. More recently research was focused on the scale up of the degradation process by *P. chrysosporium* in a fed batch stirred reactor under air and oxygen supply (Rodarte-Morales et al., 2011b). In that work, diclofenac was added into the reactor as pulses every 2 d during 30 d, resulting in complete removal, but metabolites were not analyzed. All these studies have demonstrated that diclofenac can be completely removed by WRF. The degradation of diclofenac by whole fungal cells produced hydroxylated subproducts, which at the end of the treatment had disappeared leading to a decrease in toxicity, suggesting diclofenac mineralization. In addition, researchers observed that many enzymatic systems are involved in the first step of the diclofenac

transformation, which could also participate in different steps of its possible mineralization.

Naproxen, is an arylpropionic acid, widely used to relieve mild to moderate pain and in the treatment of osteo- and rheumatoid arthritis. Rodarte-Morales et al. (2011a) reported complete removal of naproxen (1 mg L^{-1}) in 7 d by *Bjerkandera* sp. R1 and *B. adusta,* and in 4 d by *P. chrysosporium*. On the other hand, Marco-Urrea et al. (2010b) performed degradation experiments with *T. versicolor* and the contaminant at 10 mg L^{-1} and 55 µg L^{-1}. In both cases naproxen was completely degraded in a few hours (approx. 6 h), with a higher removal rate for *T. versicolor*. 2-(6-hydroxynaphtalen-2-yl)propanoic acid and 1-(6-methoxynaphthalen-2-yl)ethanone were identified as the main metabolites of naproxen, but after 6 h they disappeared, resulting in a final non-toxic medium, as determined by Microtox. On the other hand, Marco-Urrea et al. (2010b) and Tran et al. (2010) reported the possible implication of cytochrome P450 in the degradation, and the negligible elimination by purified LAC from *T. versicolor,* which was complete (95% after 30 min) when a mediator was added. The same behavior was observed by Lloret et al. (2010) when LAC from *M. thermophila* was in contact with the contaminant. Similarly Eibes et al. (2011) reported an 80% degradation of naproxen with versatile peroxidase (VP) from *B. adusta* after 7 h.

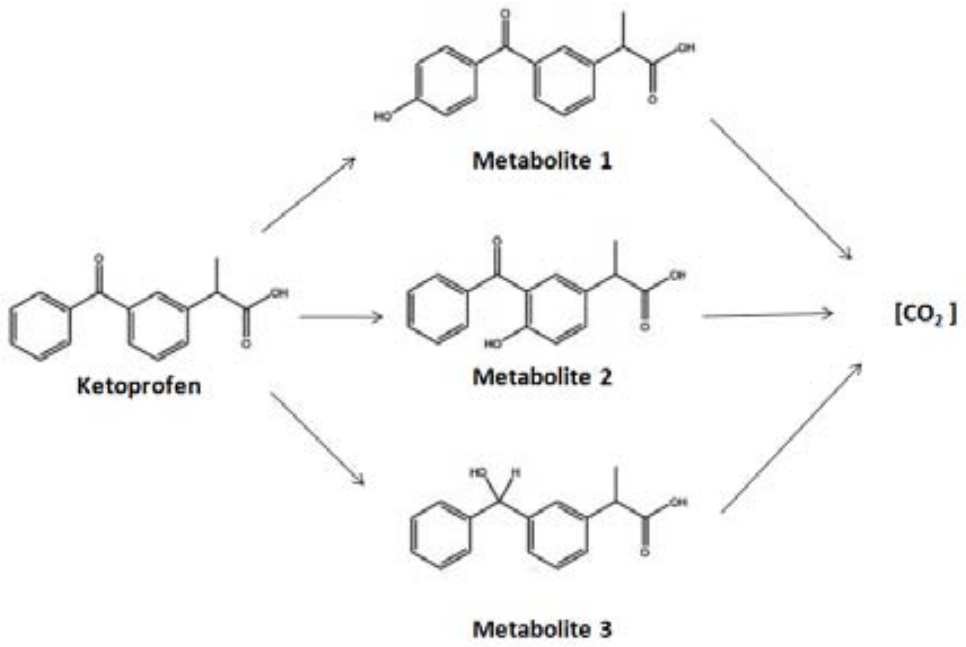

Figure VII.3: Suggested degradation pathway of ketoprofen by *T. versicolor* (Adapted from Marco-Urrea et al. 2010c).

Ketoprofen, is a therapeutic agent with analgesic and antipyretic effects, generally prescribed for toothaches that result in the inflammation of the gums. The fungal degradation of that drug has only been studied with *T. versicolor* by Marco-Urrea el at. (2010c), who reported the removal of ketoprofen to non-detectable levels in 24 h when it was added at 10 mg L^{-1}, whereas at low concentration of 40 µg L^{-1} it was almost completely removed after 5 h. During time-course degradation experiments, the metabolites 2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid (1), 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic (2) acid and 2-(3-benzyl-4-hydroxyphenyl)-propanoic acid were identified (3) (Figure VII.3). However, none of these intermediates was detected at the end of the experiment (7 d) except small amounts of 2-(3-benzyl-4-hydroxyphenyl)-propanoic acid (0.08 mg), which was lower in comparison with the amount detected at 24 h (0.53 mg), suggesting a possible mineralization of ketoprofen. The Microtox test showed minimal toxicity, with 15 min EC_{50} higher than 90%. Regarding the enzymatic degradation, LAC from *T. versicolor* is not involved in the first step of the degradation, as the addition of the LAC-mediator system failed to oxidize ketoprofen. In contrast, the cytochrome P450 inhibitor 1-aminobenzotriazole produced a reduction in the ketoprofen degradation rate, suggesting that the first

oxidation step of the ketoprofen is cytochrome P450-mediated (Tran et al., 2010; Marco-Urrea et al., 2010c).

Mefenamic is a non-steroidal anti-inflammatory drug used to treat pain, including menstrual pain. Hata et al. (2010a) treated that drug with *P. sordida*, and obtained a 90% reduction in mefenamic acid concentration (inicial concentration 24 mg L^{-1}) after 6 d. The system produced four metabolites, identified as 3'-hydroxymethylmefenamic acid, 3'-hydroxymethyl-5-hydroxymefenamic acid, 3'-hydroxmethyl-6'-hydroxymefenamic acid and 3'-carboxymefenamic acid. Moreover, the authors confirmed that the fungus almost completely removed the acute lethal toxicity of mefenamic towards the freshwater crustacean *Thamnocephalus platyurus* after 6 d of treatment, suggesting that the metabolites are less toxic than the parental compound.

Other anti-inflammatory drugs whose degradation by fungi has been studied include fenoprofen, indomethacin and propyphenazone. Tran et al. (2010) evaluated the degradability of these pharmaceuticals (10 µg L^{-1} of each) by cultures of *T. versicolor*. After 48 h of incubation, they observed complete degradation of fenoprofen and indomethacin and approximately 75% for propyphenazone. In addition, enzymatic assays with LAC from *T. versicolor* resulted in almost complete degradation for fenoprofen (>90% after 3 h) and indomethacin (>90% after 30 min), but negligible levels of degradation (25% after 3 h) were achieved in the case of propyphenazone. However, no analysis of the TPs neither toxicity assays were performed.

To summarize, the anti-inflammatory drugs studied are degraded at high rates by fungi (about some hours). Studies carried out with inhibitors of intracellular enzyme system (cytochrome p450) and purified extracellular enzymes like LAC, LiP, MnP and VP revealed that diverse enzymatic systems can act simultaneously to degrade anti-inflammatory drugs when whole fungal cells are used. In general, the first step in the degradation of these drugs involves the production of hydroxylated metabolites. However, in some cases such as ibuprofen, the TPs cannot be subsequently degraded, producing an increase in the toxicity, but in other cases (ketoprofen, diclofenac) the

hydroxylated products are degraded leading to a decrease in toxicity, suggesting the mineralization of the compound.

VII.2.2. Psychiatric drugs

Carbamazepine is one of the most widely prescribed drugs for the treatment of epilepsy, trigeminal neuralgia and some psychiatric diseases. The degradation of carbamazepine has concerned the scientific community during the last years due to its barely or non-degradability in conventional WWTPs (Hernandez-Raquet, 2012). In a degradation screening with four WRF (*T. versicolor, G. lucidum, I. lacteus* and *P. chrysosporium*), Marco-Urrea et al. (2009) reported the elimination of this pollutant at 10 mg L^{-1} after 7 d only with *T. versicolor* and *G. lucidum*, achieving removals of 58% and 47% respectively. The application of enzymatic degradation using MnP and comercial LAC-mediator system failed to improve the degradation, suggesting that the extracellular ligninolytic enzyme system did not play a role in the first step of carbamazepine degradation (Tran et al., 2010; Hata et al., 2010b). However, Hata et al. (2010b) increased the extent of carbamazepine degradation by pure LAC performing repeated treatments with the addition of LAC-mediator pulses every 8 h, achieving a removal of 60% after 48 h. During this process they found 10,11-dihydro-10,11-epoxyCBZ and 9(10H)-acridone as degradation products at the end of the experiment (48 h). On the other hand, experiments using the cytochrome P450 inhibitors 1-aminobenzotriazole and piperonyl butoxide produced a reduction in the removal, thus indicating a possible role of this enzymatic complex in the oxidation of carbamazepine by *T. versicolor* and *Pleurotus ostreatus* (Tran et al., 2010; Golan-Rozen et al., 2011). Jelić et al. (2011) confirmed the degradation of this psyquiatric drug by *T. versicolor* pellets obtaining a removal of 94% after 6 d when the contaminant was added at 9 mg L^{-1}, while at close environmentally relevant concentrations of 50 µg L^{-1}, 61% of the contaminant was degraded in 7 d. In experiments at 9 mg L^{-1} the compounds 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, acridone and acridine were found as TPs (Figure VII.4).

Kang et al. (2008) reported 10,11-dihydro-10,11-epoxycarbamazepine as the major byproduct in the transformation of carbamazepine by *Umbelopsis ramanniana* and *Cunninghamella elegans,* but also detected two hydroxylated byproducts (2-and 3-hydroxy carbamazepine). Other experiments with *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* were carried out by Rodarte-Morales et al. (2011a). Complete degradation was achieved by all the fungi after 14 d. Nevertheless, a slow removal was observed during the first week of assay (<33%), which agrees with previous works from Marco-Urrea et al. (2009) with *P. chrysosporium*. A study by Golan-Rozen et al. (2011) revealed 99% removal of carbamazepine by *P. ostreatus* with subsequent transformation to 10,11-epoxycarbamazepine. In addition, the use of inhibitors of MnP resulted in lower elimination levels (10% to 30%), which suggests MnP participation in the transformation process. The same authors also suggested that the carbamazepine removal might be partially attributed to the activity of VP, contrary to studies with VP from *B. adusta*, which have shown undetectable removal yields (Eibes et al., 2011). Low removal yields (<10%) were also obtained with LiP from *P. chrysosporium* (Zhang and Geiβen, 2010).

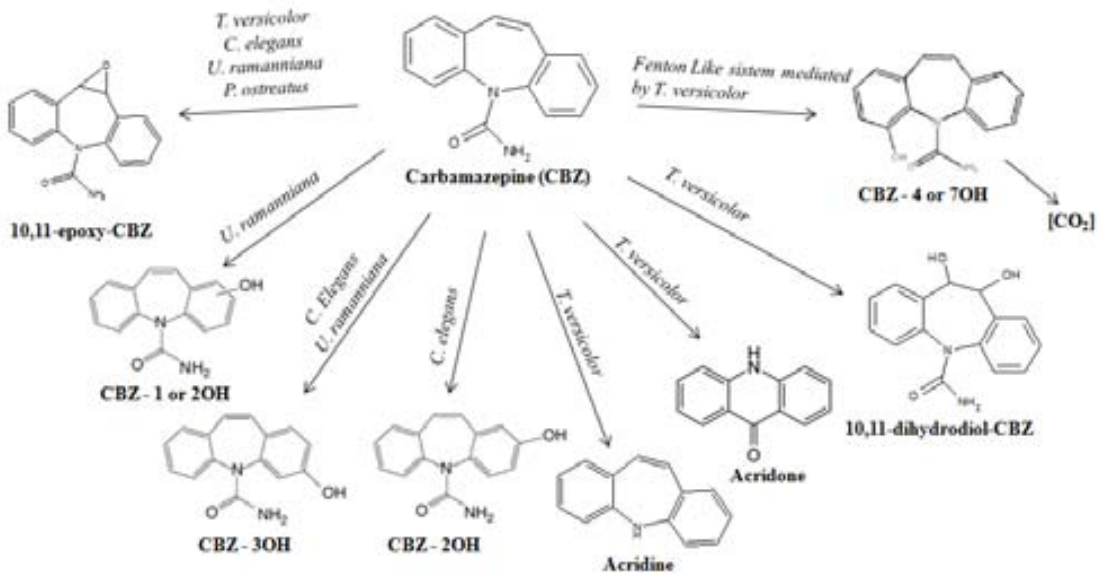

Figure VII.4 TPs in carbamazepine degradation by different fungi.

A biological Fenton-like system mediated by *T. versicolor* was used as a new treatment to degrade carbamazepine (Marco-Urrea et al., 2010d). That treatment consisted in producing extracellular oxidizing species responsible for the degradation

of the pollutants, through a quinone redox cycling mechanism catalyzed by an intracellular quinone reductase and any of the ligninolytic enzymes of *T. versicolor*, after addition of the lignin-derived quinone 2,6-dimethoxy-1,4-benzoquinone and Fe^{3+}-oxalate in the medium. In that treatment, 80% of the contaminant was degraded. In addition, researchers found two hydroxylated isomers of carbamazepine, named 4- and 7- hydroxycarbamazepine, which completely disappeared at the end of the incubation period, regarded by the authors as a possible mineralization indicator.

To summarize, carbamazepine can be completely degraded by some fungi like *T. versicolor, P. ostreatus*, *U. ramanniana* and *C. elegans.* The intracellular enzyme system cytochrome P450 seems to play a key role in the first step of the process. It is worth mentioning that the first step of the oxidative breakdown of carbamazepine in the cytochrome P450-mediated metabolism in humans is the oxidation to 10,11-epoxycarbamazepine (Sillanpaa, 1996) and consecutively to 10,11-dihydro-10,11-dihydroxycarbamazepine and other hydroxylated compounds (Lertratanangkoon and Horning, 1982). The identification of these metabolites in the studies above mentioned might serve as an additional proof of the involvement of cytochrome P450 in the degradation of carbamazepine. Experiments with MnP and VP indicated that the same enzymes in different fungi can or not participate in the degradation of carbamazepine. Finally, the reports indicate the accumulation of TPs in the medium, however no analyses of toxicity were carried out and subsequently, the suitability of the treatment is in doubt.

On the other hand, the continuous treatment of carbamazepine has been described by Jelić et al. (2011) in an air-pulsed fluidized bed bioreactor (FBR) with *T. versicolor* pellets. The system achieved a reduction of 54% in the outflow respect to the inflow concentration (approx. 200 μg L^{-1}) at the steady state (day 25) with a hydraulic retention time (HRT) of 3 d, corresponding to a degradation rate of 11.9 μg g^{-1} dry weight fungal pellets d^{-1}. Byproducts were not found during the process, probably due to the low concentration of the contaminant. Parallel experiments in a batch operated bioreactor resulted in high degradation (96%) after 2 d with the release of 10,11-dihydro-10,11-epoxycarbamazepine as the major transformation product. The authors reported low toxicity values (Microtox test) in both bioreactor

treatments. However, in both continuous and batch operation the values of toxicity were higher than those in the control (containing only carmabazepine and medium) indicating that TPs may be more toxic than the parent compound.

Citalopram and fluoxetine are antidepressant drugs widely used in human medicine and very persistent in WWTPs. However, there are few studies about the degradation of these pharmaceuticals by fungi and none of them identifies TPs. Rodarte-Morales et al. (2011a) observed the complete degradation of citalopram by the WRF strains *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* after 14 d. Nevertheless, the degradation rate was higher with *B. adusta,* as 58% of the citalopram was degraded after 4 d, while the other fungi removed less than 10% after 4 d. Regarding degradation of fluoxetine, the authors reported partial degradation by all the fungi tested, showing removal values ranging from 23 to 46% after 14 d. Eibes et al. (2011) studied the degradation of these two pharmaceuticals by VP from *B. adusta* with poor success: elimination of 18% and 10% for citalopram and fluoxacin, respectively.

The degradation of diazepam, a tranquilizer drug, has also been assessed with *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* (Rodarte-Morales et al., 2011a), however none of the WRF was able to completely degrade this compound, obtaining degradation yields between 39% and 57% after 14 d of incubation.

All the psychiatric drugs are degraded by WRF in long periods (days) in comparison with previous anti-inflammatory drugs described (some hours). The major metabolite from carbamazepine was consistently 10,11-epoxycarbamazepine; however other TPs, especially hydroxylated compounds, were identified during the fungal treatments. In the only study where toxicity was assessed, the TPs of carbamazepine seemed to be more toxic than the predecessor.

VII.2.3. Lipid regulators

Clofibric acid is the main pharmacologically active metabolite of the lipid lowering drugs clofibrate, etofibrate and etophyllinclofibrate. This compound shows low removal efficiency, approx. 28%, in conventional wastewater treatment technologies (Petrović et al., 2009) and its ubiquitous presence has been demonstrated in the environment (Jia et al., 2009). The first evidences for clofibric degradation by fungi were described by Marco-Urrea et al. (2009) and Tran et al. (2010). Their results showed that clofibric acid (10 mg L^{-1}) was almost totally degraded by *T. versicolor* after 7 d. Other WRF (*G. lucidum, I. lacteus* and *P. chrysosporium*) failed to degrade the contaminant. The failure of MnP and a LAC-mediator system to degrade clofibric acid led to rule out the participation of these extracellular fungal enzymes in the first step of clofibric acid degradation. In contrast, experiments with active fungal cultures and cytochrome P450 inhibitors suggested a key role of this enzymatic complex in the oxidation of clofibric acid by *T. versicolor.* This fungus was also applied in the treatment of clofibric acid by the induction of oxidizing agents via quinone redox cycling (Marco-Urrea et al., 2010d). This approach resulted in the elimination of more than 80% of the drug after 6 h when added at an initial concentration of 10 mg L^{-1}. The authors found a clofibric acid hydroxylated derivative as the main TP of the degradation process, which totally disappeared by the end of the experiment, thus suggesting the possible mineralization of the drug. Anyhow, toxicity was not assessed to evaluate the global applicability of the treatment.

Continuous bioreactor treatment was developed for the removal of clofibric acid by *T.versicolor* (data not yet published). That bioreactor was operated in continuous mode with a HRT of 4 d and a clofibric acid concentration in the inflow close to real concentrations (160 µg L^{-1}). The reactor achieved an 80% reduction in the concentration in the outflow at the steady state, indicating a clofibric acid degradation rate of 16.5 µg g^{-1} dry weight fungal pellets d^{-1}. During the treatment only 2-(4-chlorophenozy)-2-(hydroxymethyl)propanoic acid was detected as a major metabolite, confirming the degradation of the contaminant. However, that metabolite was not degraded by the fungus. In addition, results in the standard toxicity bioassay (Microtox

test) indicated that the treated effluent was more toxic than the initial inflow, suggesting the production of a more toxic metabolite, which casts doubts on the suitability of the treatment.

Gemfibrozil also belongs to the group of drugs known as fibrates, employed to lower lipid levels. Tran et al. (2010) studied gemfibrozil degradation by *T. versicolor* active cultures and its LAC. The researchers obtained a removal of 75% after 7 d when the whole fungus was inoculated. In addition, less than 30% was degraded in experiments with crude and commercial LAC, indicating that these extracellular enzymes are not involved in the first step of gemfibrozil degradation and suggesting that the oxidation of the target compound is done by intracellular enzymes.

Summarizing, intracellular enzyme citochrome P450 seems to play an important role in the oxidation of lipid regulators while extracellular ligninolyitc enzyme is not involved in their degradation. In addition, for clofibric acid hydroxylation products of higher toxicity were obtained after the treatment with fungal cells and also in fungal-mediated Fenton-like process, which in the former case was accumulated leading to an increase in the toxicity, but disappeared in the latter, suggesting mineralization.

VII.2.4. Antibiotics

VII.2.4.1.Sulfonamides

Among antibiotics, the group of the sulfonamides is, comparatively, one of the most studied in terms of fungal degradation. Enzymatic-mediated transformation has been demonstrated with LAC and VP. Schwarz and co-authors (Schwarz et al., 2010) reported transformations of 10%, 75% and 96% for sulfanilamide, sulfadimethoxine and sulfapyridine, respectively after 15 d with commercial LAC from *T. versicolor* (48000 U L^{-1}). Aniline was confirmed as a breakdown product of sulfapyridine, while the SO_2 extrusion products 4-(2-imino-1-pyridyl)aniline and 4-(6-imino-2,4-dimethoxypyrimidin-1-yl)aniline were determined for sulfapyridine and

sulfadimethoxine, respectively. An additional metabolite with elemental composition $C_{12}H_{11}N_3O$ was assigned to sulfapyridine transformation, however, only tentative structures were proposed: 4-(2,3-diaminophenyl)imino-cyclohexa-2,5-dien-1-one and *N*-(3-pyridyl)pyridine-3-sulfonamide. Degradation was also achieved in shorter periods (hours) in lower LAC activity systems (50-350 U L^{-1}) in the case of sulfamethazine (García-Galán et al., 2011), sulfapyridine and sulfathiazole (Rodríguez-Rodríguez et al., 2012b), though it took place in the presence of LAC-mediators. The desulfonated was a common metabolite obtained from the three sulfonamides. In addition, desamino-sulfamethazine and hydroxy-sulfamethazine were identified for sulfamethazine, while a formyl intermediate obtained after the loss of the pyrimidine/thiazole group was recognized from the transformation of sulfapyridine and sulfathiazole. Similarly, the use of VP from *B. adusta* (200 U L^{-1}) resulted in 80% transformation of sulfamethoxazole in 7 h (Eibes et al., 2011). Intermediate metabolites included 3-amino-5-methylisoxazole and a possible dimerization product of sulfamethoxazole, while anions such as nitrate, nitrite and sulfate were detected as well.

Degradation of sulfonamides has also involved the use of whole cell fungal systems. García-Galán et al. (2011) studied the degradation of sulfamethazine by *T. versicolor* pellets in liquid medium, obtaining almost complete removal after 20 h. Metabolites from *in vivo* experiments included the formylated (*N*-(4,6-dimethylpyrimidin-2-yl)-4-(formylamino)benzenesulfonamide) and desulfonated (*N*-(4,6-dimethylpyrimidin-2-yl)benzene-1,4-diamine) intermediates, the latter being also identified from enzymatic degradation. Similar work with *T. versicolor* by Rodríguez-Rodríguez et al. (2012b) led to the elucidation of several degradation intermediates of sulfapyridine and sulfathiazole. From the former sulfonamide, the recognized metabolites included the desulfonated, formyl and hydroxyl intermediates, formyl-desulfonated sulfapyridine and a formyl-hydroxylated metabolite produced after the loss of the pyridine ring (Figure VII.5). As in the case of sulfamethazine, the desulfonated was the only intermediate found both in cell-mediated and enzymatic degradation. For sulfathiazole the formyl intermediate was found, as well as a more transformed metabolite result of the hydroxylation of the desaminated and desulfonated parental compound. In those studies the possible role of the cytochrome

P450 was also demonstrated in the transformation of sulfamethazine and sulfathiazole, but it was unclear in the case of sulfapyridine. In addition, the simultaneous removal of the three latter sulfonamides was achieved under continuous operation in a 1.5 L FBR containing *T. versicolor* pellets. Elimination was >94% for each sulfonamide at a HRT of 72 h (Rodríguez-Rodríguez et al., 2012b).

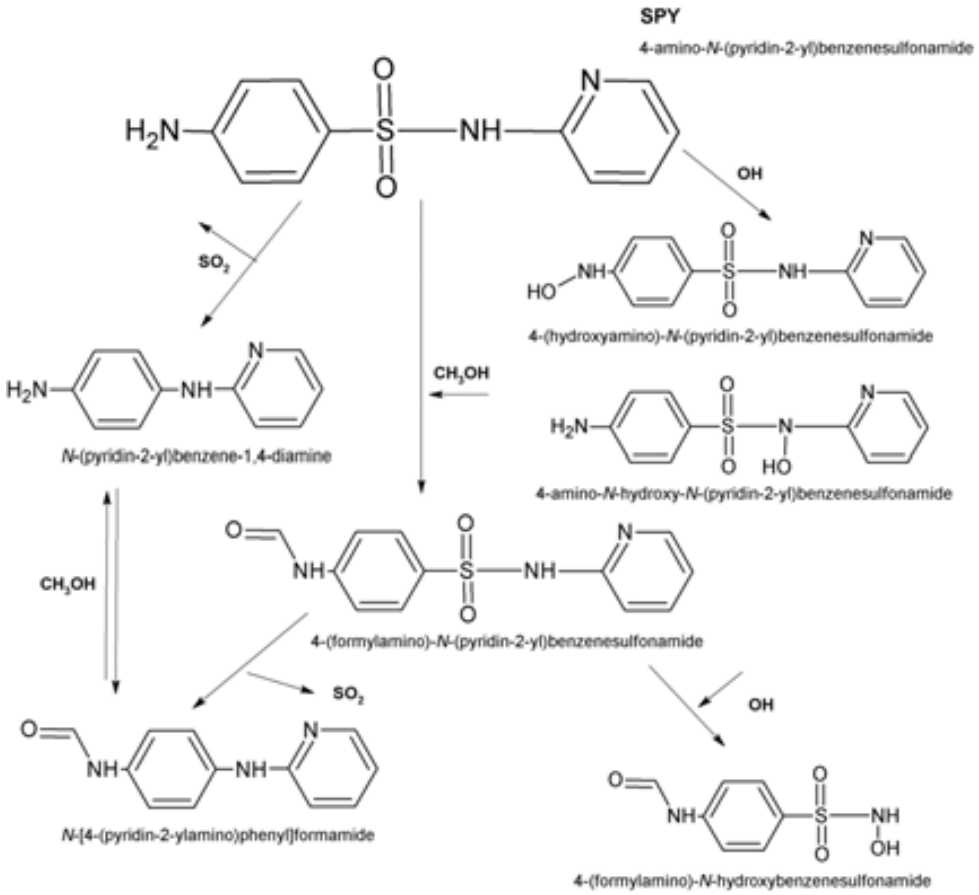

Figure VII.5: TPs of sulfapyridine after degradation by *T. versicolor* (Adapted from Rodríguez-Rodríguez et al. 2012b).

The complete degradation of sulfamethoxazole was also reported within 14 d with *P. crhysosporium*, *Bjerkandera* sp. R1 and *B. adusta* (Rodarte-Morales et al., 2011a), although, contrary to the reports of enzymatic transformation, metabolites were not identified. Partial removal (from 30% to 55%) of sulfamethoxazole from activated-sludge-mixed liquor and the effluent of a WWTP was demonstrated at bench scale within 5 d with *P. chrysosporium* propagules entrapped in a granular bioplastic formulation (Accinelli et al., 2010). This approach was also successful in the partial

elimination of other kinds of antibiotics, e.g. ciprofloxacin (see below) and the macrolide erythromycin.

Both enzymatic and whole cell mediated transformation of sulfonamides has been described, usually with high removal efficiencies and relatively short treatments. Although different metabolites have been elucidated, no clear pathways have been defined; however, the desulfonated metabolites have been widely identified (with LAC and fungal cells) along with products of hydroxylation, formylation and deamination reactions, and combinations of them.

VII.2.4.2.Tetracyclines

Fungal degradation of tetracyclines has been only described by enzymatic means but not with fungal cells. Wen et al. (2009) described the use of crude lignin peroxidase from *P. chrysosporium* (40 U L^{-1}) which produced a 95% removal of tetracycline and oxytetracycline in 5 min; the degradation was pH-dependant and was enhanced by increasing concentrations of veratryl alcohol and H_2O_2. Similarly, Suda et al. (2012) reported the complete elimination of tetracycline and chlortetracycline in 15 min, and doxycycline and oxytetracycline in 1 h by LAC from *T. versicolor* in the presence of HBT. Transformation resulted in the complete loss of inhibitory effect towards *Escherichia coli*, *Bacillus subtilis* and the green algae *Pseudokirchneriella subcapitata*. Nonetheless, the identification of TPs of these therapeutic drugs by fungal enzymes has not been yet reported.

VII.2.4.3.Quinolones

Transformation of quinolones by fungi, especially fluoroquinolones, has received some attention. In this respect the most widely studied antibiotic is ciprofloxacin. Wetzstein et al. (1999) described the transformation of >50% ciprofloxacin by *Gloeophyllum striatum* after 90 h, with some production of $^{14}CO_2$ from

^{14}C labeled structures and reduction in antibacterial activity. Eleven metabolites were elucidated, including mono- and dihydroxylated congeners, an isatin-type compound (proving elimination of C-2) and metabolites indicating both elimination and degradation of the piperazinyl moiety. In a similar study with *T. versicolor*, Prieto et al. (2011) achieved >90% elimination after 7 d and 98% after 20 h with whole cells and LAC/mediators, respectively. The role of the cytochrome P450 enzymatic complex was also implied in the transformation, and six intermediates were elucidated: three previously described by Wetzstein et al. (1999), plus a new product of the piperazinyl moiety breakdown. The other two metabolites corresponded to dimeric products formed by a C-C covalent bond and followed by several transformations including the breakdown of the piperazinyl group, removal of a cyclopropyl group and hydroxylation.

Metabolism of ciprofloxacin by *Pestalotiopsis guepini* yielded *N*-acetylciprofloxacin (the most abundant metabolite), desethylene-*N*-acetylciprofloxacin, *N*-formylciprofloxacin and 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxilic acid (Parshikov et al., 2001a). Other transformation reports include the regioselective production of *N*-acetylciprofloxacin by *Mucor ramannianus* (Parshikov et al., 1999a) and the optically active 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin by *Trichoderma viride* (Parshikov et al., 2002a).

Partial mineralization of enrofloxacin, ranging from 5% to 53%, was demonstrated by different wood-rotting fungi grown on wheat straw after eight weeks, including several strains of the brown-rot fungus *G. striatum* (the most efficient degrader) and the white-rot fungi *I. lacteus*, *P. chrysosporium*, and *Stropharia rugosoannulata* (Martens et al., 1996). Further work with *G. striatum* by Wetzstein et al. (1997) led to the identification of several metabolites, including 3-, 6- and 8-hydroxylated congeners of enrofloxacin (with replacing of the fluorine by a hydroxyl group in one case); 5,6- (or 6,8-), 5,8- and 7,8-dihydroxylated congeners, prone to autoxidative transformation; products of the cleavage of the heterocyclic core of enrofloxacin (an isatin-type compound and an anthranilic acid derivative) and products of both elimination and degradation of the piperazinyl moiety (1-ethylpiperazine, the 7-amino congener and desethylene-enrofloxacin). Four different degradation routes

were proposed (Figure VII.6), initiated by either oxidative descarboxylation, defluorination, hydroxylation at C-8 or oxidation of the piperazinyl moiety.

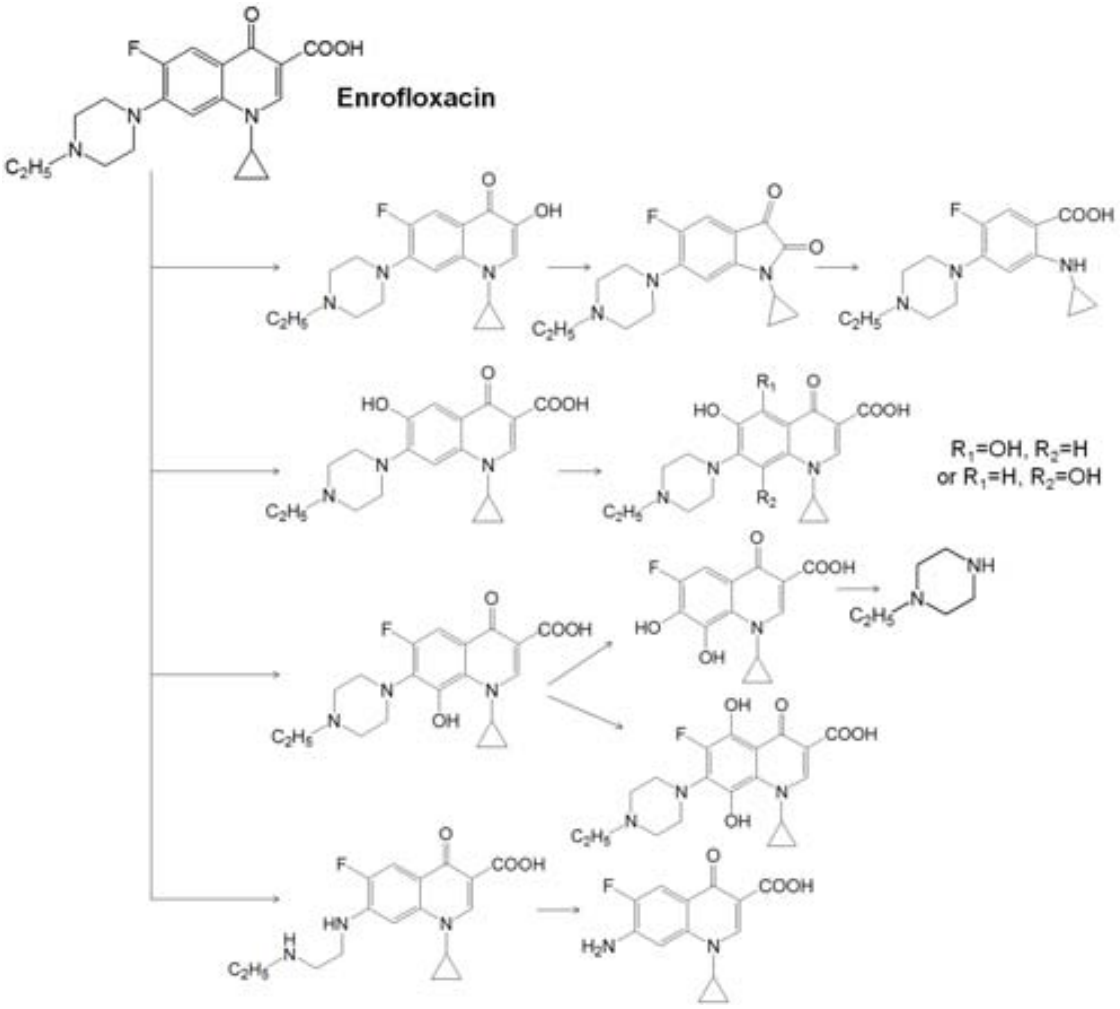

Figure VII.6: Main routes of degradation of enrofloxacin employed by *G. striatum* (Adapted from Wetzstein et al. 1997).

Wetzstein et al. (2006) subsequently described the patterns of metabolites produced from enrofloxacin by seven basidiomycetes indigenous to agricultural sites. Their findings showed similar patterns of major metabolites, but differed considerably from those obtained from *G. striatum*, due particularly to the absence of monohydroxylated congeners and a greater variety of metabolites derived from the modification of the piperazine moiety. Metabolites comprised ethylpiperazine moieties with oxido-, hydroxyl-, oxo- and acetoxy- groups, or showing partial degradation, linked to the unmodified, oxidatively decarboxylated or multiply hydroxylated core of enrofloxacin and to isatin- and anthranilic acid type enrofloxacin

congeners. Metabolites with hydroxylated aromatic rings likely suffered additional ring cleavage to form four potential oxidizable *o*-aminophenol and one catechol-type intermediates. The transformation of this fluoroquinolone was also studied with *M. ramannianus*, with 78% removal of the parent compound after 21 d and the identification of enrofloxacin *N*-oxide, *N*-acetylciprofloxacin and desethylene-enrofloxacin as metabolites, being the former the most abundant (Parshikov et al., 2000).

Norfloxacin transformation has been demonstrated by *P. guepini* (Parshikov et al., 2001a) and *T. viride* (Parshikov et al., 2002a). In the first case, the metabolites identified included *N*-acetylnorfloxacin as the major intermediate, desethylene-*N*-acetylnorfloxacin, *N*-formylnorfloxacin and 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. For *T. viride*, 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin was elucidated as an intermediate. In both reports, the intermediates were analogous to those derived from ciprofloxacin by the same fungi. Similarly, Prieto et al. (2011) showed the degradation of norfloxacin by enzymatic means (LAC with mediators, 33% after 20 h) and whole cells of *T. versicolor* (>90% after 7 d). Three metabolites resulting from the transformation of the piperazinyl moiety were identified, with the accumulation of one of them, 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxilic acid.

Transformation of the fluoroquinolones sarafloxacin to *N*-acetylsarafloxacin and desethylene-*N*-acetylsarafloxacin by *M. ramannianus* (Parshikov et al., 2001b) and flumequine to two diasteroisomers of 7-hydroxyflumequine and 7-oxoflumequine by *C. elegans* (Williams et al., 2007) was also reported. Meanwhile, the quinolone-like antibiotic cinoxacin produced 1-ethyl-1,4-dihydro-3-(hydroxymethyl)[1,3]dioxolo[4,5-*g*]cinnolin-4-one and 1-ethyl-1,4-dihydro-6,7-dihydroxy-3-(hydroxymethyl)cinnolin-4-one when transformed by *Beauveria bassiana* (Parshikov et al., 2002b).

Transformation of quinolones has been reported especially with fungal cells of diverse species, even obtaining partial mineralization, though LAC also proved relative efficiency in the removal of some of them. The abundance of metabolites described, many times analogous from one compound to another, has led to the proposal of

several degradation pathways, which usually involve breakdown of the parental molecules and reactions of hydroxylation, acetylation or formylation, among others.

VII.2.5. Other antimicrobial agents

Triclosan is a broad spectrum antibacterial agent with antifungal and antiviral properties, widely employed in personal care products such as soaps, shampoos, toothpastes and cosmetics (Kim and Nicell, 2006). Fungal-mediated degradation studies have been mainly performed by means of enzymatic processes, although a couple of whole cell transformation reports are available.

Inoue et al. (2010) studied the elimination of triclosan by MnP from *P. chrysosporium*, and LAC from *T. versicolor*. MnP (0.5 nkat mL^{-1}) was the most efficient, achieving a removal of 94% after 30 min and almost complete after 60 min, while LAC (2.0 nkat mL^{-1}) and LAC-HBT removed triclosan at 51% and 66%, respectively. Moreover, the treatment with MnP resulted in the complete loss of bacterial inhibition activity after 30 min and reduced the algal growth inhibition by 90% after 60 min. Similar results were obtained by Kim and Nicell (2006) with LAC from *T. versicolor* after 6 h, with concomitant reduction in toxicity even without mediators.

Immobilized enzymes, particularly LAC, have been employed in the degradation of triclosan. The immobilization of LAC from *Coriolopsis polyzona* through the formation of cross-linked enzyme aggregates (CLEAs) and their subsequent use in a FBR for the removal of endocrine disrupting compounds (Cabana et al., 2007a), produced the complete removal of triclosan, nonylphenol and bisphenol A (5 mg L^{-1} each) at a HRT of 150 min. The application of CLEAs in a perfusion basket reactor (Cabana et al., 2009a) resulted in the continuous elimination of the above mentioned mixture at 85% with a HRT of 325 min during 7 d. Moreover, the system was able to eliminate >95% triclosan and nonylphenol at 100 mg L^{-1} with a HRT of 400 min. The immobilization of VP from *B. adusta* in the form of CLEAs, although successful in the removal of other endocrine disrupting compounds, could only eliminate 26% of triclosan after 10 min in batch experiments (Taboada-Puig et al., 2011). Immobilized

LAC from *C. polyzona* on the diatomaceous earth support Celite ® R-633 (Cabana et al., 2009b) was employed as a catalyst for the removal of triclosan (5 mg L^{-1}) in a packed-bed reactor (PBR). Its operation in repeated batch treatments resulted in the complete elimination of the antimicrobial agent at a contact time of less than 200 min during five cycles. Similarly, the conjugation of LAC from *T. versicolor* with the biopolymer chitosan (Cabana et al., 2011) produced a high-stable solid biocatalyst which degraded triclosan from aqueous solutions (100% after 6 h) with a higher efficiency than free LAC (60% after 6 h).

The identification of TPs derived from triclosan degradation has been determined through experiments with LACs from *G. lucidum* and *C. polyzona* and whole cell cultures of *T. versicolor* and *Pycnoporous cinnabarinus*. Enzymatic degradation with LAC from *G. lucidum* (5000 U L^{-1}) removed 57% triclosan within 24 h, and produced dimers and trimers of the parental compound. The degradation was enhanced by the addition of LAC mediators (HBT or syringaldehyde, SYD), reaching 90% removal, and resulted in the formation of different intermediates: 2,4-dichlorophenol and dechlorinated forms of 2,4-dichlorophenol (Murugesan et al., 2010), thus suggesting two mechanisms of triclosan removal by LAC, oligomerization in the absence of mediators and ether bond cleavage with subsequent dechlorination in the presence of mediators (Figure VII.7). The production of high molecular weight metabolites through a radical polymerization mechanism was also demonstrated by Cabana et al. (2007b) who identified dimers, trimers and tetramers, formed through C-C and C-O bonds in enzymatic degradation with LAC from *C. polyzona*. In this study, the removal of triclosan was 65% with a LAC activity of 100 U L^{-1}, either at 4 h or 8 h of treatment. The same authors also described the production of triclosan oligomers as a result of removal with LAC from *T. versicolor* (Cabana et al., 2011).

Hundt et al. (2000) described the transformation of triclosan by whole cell cultures of *T. versicolor* and identified the conjugates 2-*O*-(2,4,4'-trichlorodiphenyl ether)-β-D-xylopyranoside and 2-*O*-(2,4,4'-trichlorodiphenyl ether)-β-D-glucopyranoside, and in coincidence with the enzymatic treatment described by Murugesan et al. (2010), 2,4-dichlorophenol. Under the same cultivation conditions, *P. cinnabarinus* also produced the glucoside conjugate determined for *T. versicolor* and

an additional product corresponding to the methylation of triclosan, identified as 2,4,4'-trichloro-2'-methoxydiphenyl ether. The conjugates exhibited reduced cytotoxicity and antimicrobial activity than triclosan. Elimination of triclosan by fungal cells was also evaluated by Cajthalm et al. (2009), although the identification of metabolites was not performed. From seven WRF tested, all but one (*B. adusta*) significantly removed the antimicrobial agent within 14 d, with a progressive reduction in the estrogenic activity throughout the process.

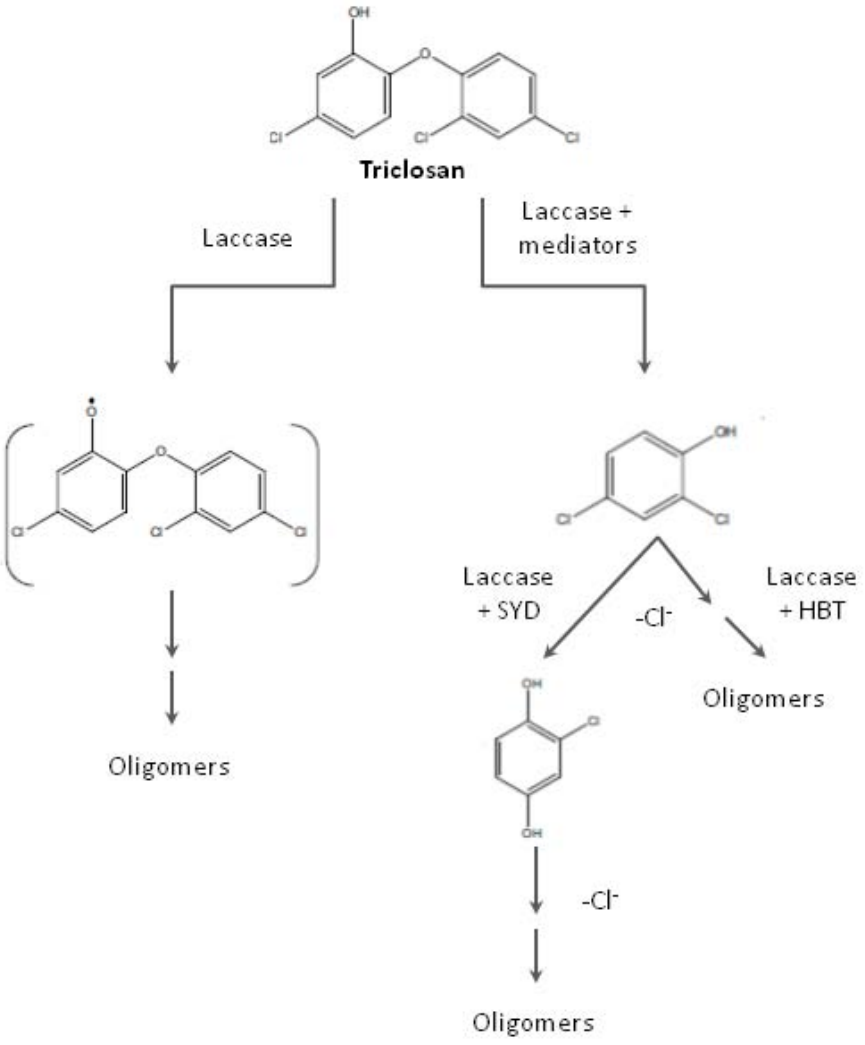

Figure VII.7: Proposed pathways for the degradation of triclosan by *G. lucidum* (Adapted from Murugesan et al., 2010).

Either by enzymatic means or with fungal cells, the transformation of triclosan seems to elapse through oligomerization and the production of 2,4-dichlorophenol. Other different metabolites have been identified depending on the process.

Phenothiazine and its derivates have been used as antihelmintic agents, as well as antiarrhythmic drugs, coronary vasodilators, and antidepressants. Parshikov et al. (1999b) reported the biotransformation of *N*-acetylphenothiazine, a phenothiazine derivate with a *N*-caronyl substituent with three fungi, *Aspergillus niger, Cuninghamella verticillata* and *Penicillium simplicissimum*, usually employed as model organisms in studies of drug bioconversion processes in mammalian systems. In the first step of the degradation all the fungi produced *N*-acetylphenothiazine sulfoxide and phenothiazine, while in a second step the last metabolite was transformed to phenothiazine sulfoxide. Furthermore, *C. verticillata* also could degrade phenothiazine to phenothiazin-3-one and phenothiazine-*N*-glucoside.

Oseltamivir, better known as Tamiflu, is the antiviral worldwide employed for the treatment of influenza (flu). Due to the importance of that drug for treating regular seasonal flu and its potential use in the case of pandemic flu scenarios, it is not strange that oseltamir has also been detected in high concentrations in the environment during flu season. In novel fungal-mediated approaches, Accinelli et al. (2010) evaluated the efficiency of a granular bioplastic formulation which entraps propagules of *P. chrysosporium* for the removal of oseltamivir from wastewater samples. Their results showed a significant increase in the removal, obtaining two times more elimination values after 30 d in the bioremediated wastewater compared to the controls. This work demonstrated the success of the bioplastic matrix to facilitate the adaptation of the fungus to unusual environments such as wastewater. However, the identification of TPs was not carried out and the toxicity was not assessed. Hence, the suitability of the treatment could not be evaluated.

Artemisinin is a naturally occurring sesquiterpene lactone that shows promising attributes as the basis of an anti-malarial agent. However its toxicity and water insolubility limit the application of the drug. This issue could be overcome with the production of semi-synthetic derivatives of artemisinin, such as 7β-hydroxyartemisinin which increase the anti-malarial activity. For this reason, Parshikov et al. (2004, 2005 and 2006) carried out experiments to examine the transformation of artemisinin to 7β-hydroxyartemisinin with different fungi. The authors achieved a 79% conversion with *C. elegans* but secondary TPs were also found: 7β-hydroxy-9α-artemisinin (6%), 4α-

hydroxy-1-deoxoartemisinin (5.4%) and 6β-hydroxyartemisinin (7%). Similarly, three strains of *M. ramannianus* were able to metabolized artemisinin into significant yields of hydroxylated metabolites, in particular 7β-hydroxyartemisinin (88%) and 6β-hydroxyartemisinin (1%), 4α-hydroxy-1-deoxoartemisinin (6%) and 7 α – hydroxyartemisinin (5%). The same research group also assessed the transformation of artemimisin by the fungi *Eurotium amstelodami* and *A. niger* and identified two major TPs, 5β-hydroxyartemisinin and 7β-hydroxyartemisinin from both organisms: 63% and 32% yields respectively, from the extract of *E. amstelodami*, and 80% and 19% respectively, from the extract of *A. niger*. These results suggest that the fungal transformation of artemisinin takes place through the production of hydroxylated compounds.

VII.2.6. β-bloquers

β-blokers comprise a group of therapeutic agents employed for the treatment of cardiac arrhythmias, cardioprotection after myocardial infarction and hypertension. Propanolol, first successful β-blocker developed, and atenolol are among the most commonly used β-blockers for cardiovascular diseases. Due to their long-term use in Europe and North America and their subsequent occurrence in the aquatic environment, they were selected by Marco-Urrea et al. (2010d) to be degraded by biological Fenton-like system mediated by *T. versicolor* (see section 2), developed to degrade emerging contaminants. With an initial pharmaceutical concentration of 10 mg L^{-1}, they achieved degradations above 80% after 6 h of incubation for atenolol and propanol. The main degradation metabolites produced in the redox cycling treatment were identified as hydroxylated derivatives for both compounds. These metabolites were accumulated in the medium and the toxicity was not assessed.

VII.2.7. Estrogens

Degradation of estrogens has been studied especially by enzymatic means, but also there are some reports of transformation by whole fungal cells.

VP from *B. adusta* was shown to completely degrade estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) after 5-25 min, even at low VP activity (10 U L^{-1}) (Eibes et al., 2011). MnP from *P. chrysosporium* and LAC from *T. versicolor* in the presence of HBT degraded E2 and EE2 within 1 h as well, with an 80% reduction in their estrogenic activity. Extending the treatment to 8 h resulted in the total removal of the estrogenic activity (Suzuky et al., 2003). Similar results were obtained with MnP from *P. sordida* and LAC from *T. versicolor* in the degradation of E1, as the estrogen was removed after 1 h of treatment with the complete elimination of estrogenic activity within 2 h (Tamagawa et al., 2006).

The oxidation of E2 was described by Nicotra et al. (2004) in two different LAC-mediated systems: in organic solvents with enzyme from *Myceliopthora* sp. (previously adsorbed on glass beads) and a biphasic system with enzyme from *Trametes pubescens*. In both cases, the production of C-C and C-O dimers occurred, attributed to the generation of oxygen radicals that can delocalize to carbon-located radicals, thus producing reactive monomer intermediates. The dimers could suffer further oxidation leading to the generation of oligomers and polymers (Figure VII.8).

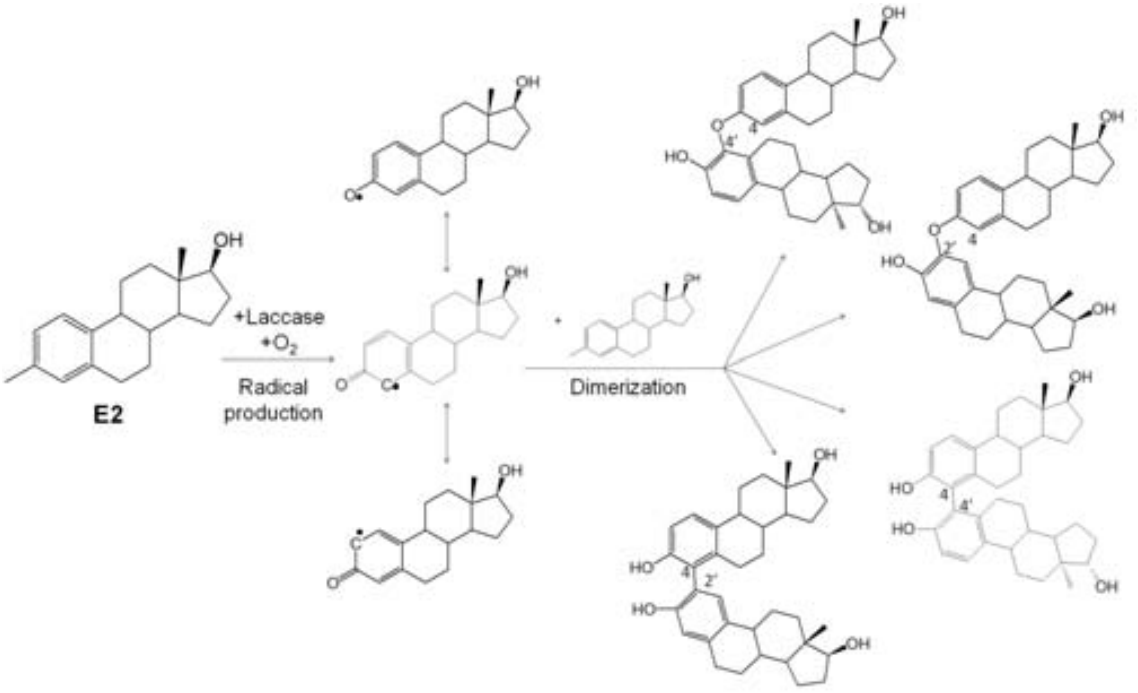

Figure VII.8 Pathway of LAC-mediated oxidation of E2 (Adapted from Cabana et al., 2007a and Nicotra et al., 2004).

Enzymatic degradation was tested with commercial LAC from *M. thermophila* (2000 U L^{-1}). E2 and EE2 were completely degraded even in the absence of mediators after 3 h and 5 h, respectively, and after 1 h in the presence of some mediators. For E1 total removal was achieved in 8 h in the presence of VA and > 70% for the other mediators after 24 h, whereas elimination reached 65% in the absence of mediators (Lloret et al., 2010). The immobilization of this enzyme by encapsulation in a sol-gel matrix (Lloret et al., 2011) was employed for the treatment of a mixture of E1, E2 and EE2 both in a batch stirred tank reactor (BSTR) operating in cycles and a continuous PBR. Removal of estrogens was > 85% in the BSTR and 55%, 75% and 60% for E1, E2 and EE2, respectively in the PBR. Both systems were able to reduce the estrogenic activity of the mixture in 63%. Likewise, the immobilization of VP in the form of CLEAs completely removed E2 and EE2 within 10 min from batch experiments, with a concomitant reduction of estrogenic activity, higher than 60% for both compounds (Taboada-Puig et al., 2011).

The application of LAC for the removal of estrogens from municipal wastewater was assessed by Auriol et al. (2007). Different enzyme levels were tested and a LAC activity of 20 000 U L^{-1} was enough to achieve complete removal of E1, E2, estriol (E3) and EE2 in 1 h both from the wastewater and synthetic water in 1 L batch reactors. This work also aimed at evaluating the wastewater matrix effect on the enzymatic process. By comparing with synthetic water, it was concluded that the wastewater constituents did not have a significant effect on the conversion of the estrogens. The same group evaluated the removal of estrogenic activity from the LAC-catalyzed process and compared it with a horseradish peroxidase treatment (8000-10000 U L^{-1}), which was also able to remove the hormones in 1 h. According to results from the recombinant yeast assay, the LAC process yielded a residue with a slightly lower estrogenic activity (elimination of 97%), compared to horseradish peroxidase (removal of 88%) (Auriol et al., 2008).

Degradation of estrogens has been also demonstrated with whole fungal cells. E2 and EE2 were removed by *T. versicolor* pellets in batch (flasks) and in a continuous FBR (Blánquez and Guieysse, 2008). Removal of both compounds was > 97% within 24 h in batch cultures. The continuous bioreactor was operated for 26 d at a HRT of 120 h,

and achieved complete removal of E2, added at different concentrations ranging from 3 mg L^{-1} to 18.8 mg L^{-1}, and simultaneous complete removal of E2 and EE2 added after biomass renovation (day 19). Degradation was ascribed to extracellular LAC. Likewise, Tamagawa et al. (2006) reported 98% removal of E1 after 5 d by *P. sordida* and attributed the process to the effect of the extracellular ligninolytic enzymes released during the treatment. Cajthalm et al. (2009) studied the degradation of EE2 (among other endocrine disrupting compounds) by eight ligninolytic fungal strains. *I. lacteus*, *P. cinnabarinus* and *P. ostreatus* were the most efficient degraders, as they reduced EE2 below detection limit within 3 d, followed by *T. versicolor* within 7 d and *B. adusta* and *Dichomitus squalences* within 14 d. Only *P. crysosporium* and *Phanerochaete magnoliae* failed to completely remove the synthetic estrogen. EE2 degradation was accompanied by a decrease in the estrogenic activity of the solution, except in the case of *P. magnoliae*.

The fungal transformation of estrogens, by enzymes or cells, seems to produce metabolites with reduced estrogenic activity in short periods, usually days or even few hours. However, only oligomeric products have been identified in enzymatic processes and a lack in the identification of metabolites derived from whole cell fungal systems is still observed.

VII.2.8. Iodinated contrast media

X-ray contrast agents, as triiodinated benzoates, permit visualization of the details of the internal structure of organs that would otherwise not be apparent. Given that iodinated X-ray contrast agents are very stable and chemically inert, they are excreted untransformed by humans and are not degraded in their subsequent pass through WWTPs.

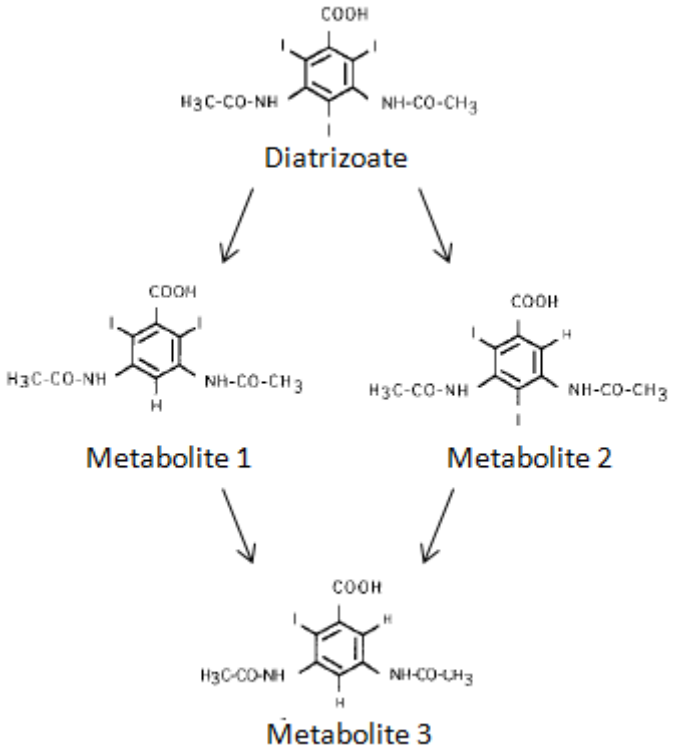

Figure VII.9 Proposed transformation of diatrizoate by *T. versicolor* (Adapted from Rode and Müller 1998).

The degradation of triiodinated benzoates by *T. versicolor* was investigated by Rode and Müeller (1998).The authors reported a removal of diatrizoate of approximately an 80% after 14 d. During the time course experiments, they found three TPs. In a first step, diatrizoate was degraded to 3,5-di(acetamido)-2,6-diiodobenzoate (metabolite 1) and 3,5-di(acetamido)-4,6-diiodobenzoate (metabolite 2), which indicates that the degradation evolves through the successive release of iodide ions (Figure VII.9). A third byproduct identified as 3,5-di(acetamido)-2-monoiodobenzoate (metabolite 3) was formed by a second deiodination of the former metabolites. Degradation yields of 58, 65 and 80% were also reported after 14 d when aminotrizoate, acetrizoate and iodipamide were incubated with *T. versicolor*, respectively. During the degradation of iodipamide five TPs were detected, while in the case of acetrizoate, which has one acetamido group in the aromatic ring, the HPLC chromatograms showed only one metabolite. No aromatic metabolites were detected in the experiments with aminotrizoate, maybe due to the polymerization of the highly reactive amino group, which generates undetectable products in HPLC, or the direct

cleavage of the ring structure. Nevertheless, the TPs of the three triiodinated compounds were not identified.

Degradation of iopromide, a nonionic X-ray contrast agent, and its precursor aminotriiodoisophthalic acid were also assessed with *T. versicolor*. Engels-Matena (1996) reported almost complete degradation (90%) of iopromide over 15 d and detected 14 metabolites from iopromide. From all the TPs, 5-methoxyacetylamino-4-monoiodoisophthalic acid (2,3-dihydroxy-propyl) diamide (metabolite 1), 5-methoxyacetylamino-4(6)-monoiodoisophthalic acid [(2,3-dihydroxy-propyl)-methyl)] diamide (metabolite 2) and 5-methoxyacetylamino-2,6-diiodoisophthalic acid [(2,3dihydroxy-N-methyl-propyl)-2,3-dihydroxy-propyl)] diamide (metabolite 3) were recognized as the main metabolites (Figure VII.10). Metabolite 1 was deiodinated at the 2 and 6 positions of the aromatic ring and demethylated on one side chain. Metabolite 2 was twice deiodinated and demethylated, like metabolite 1, and additionally depropylated. The authors observed that when metabolite 2 appeared, the increase in concentration of metabolite 1 slowed down. For this reason, they suggested that the metabolite 2 was formed by depropylation of metabolite 1. Metabolite 3 was monodeiodinated at the 4 position on the aromatic ring. Regarding aminotriiodoisophthalate, which is the matrix moiety of iopromide without the side chains, they observed a degradation of only 50% after 14 d, with the subsequent production of only one metabolite. Finally, Engels-Matena indicates that the reductive deiodination of iodinated X-ray contrast agents is a general biodegradative pathway of *T. versicolor* and seems to occur prior to the cleavage of the ring structure.

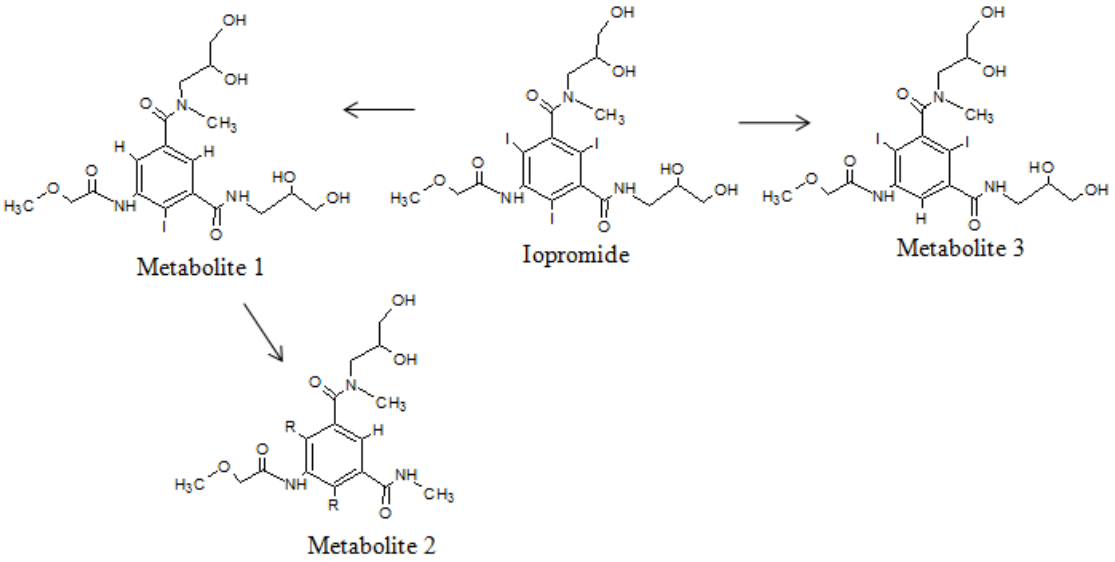

Figure VII.10 Identified metabolites from iopromide degradation by *T. versicolor* and proposed degradation scheme (Adapted from Engels-Matena, 1996).

The role of extracellular enzymatic systems in the degradation of triiodinated aromatics compounds was demonstrated by the same authors. Degradation yields between 87% to 93% for diatrizoate, iodipamide and acetrizoate, and between 68% to 73% for aminotrizoate and aminotriiodoisophthalate were observed in *in vitro* experiments with extracellular enzyme concentrate of *T. versicolor* in the presence of $MnSO_4$ and malonate. The identification of the same metabolites in the whole cell cultures supports the evidence that their production from the triiodinated aromatic compounds is caused by the extracellular enzymes of *T. versicolor*. Engels-Matena (1996) carried out similar experiments with commercial peroxidase, LAC, tyrosinase, LiP and MnP from *P. chrysosporium*. The three former enzymes failed to degrade all the tested compounds, whereas LiP achieved the polymerization of the amino compounds aminotrizoate and aminotriiodoisophthalic acid. Only MnP was able to degrade all the target drugs, remarking its possible participation in the transformation of triiodinated aromatic compounds by WRF.

VII.3. Concluding remarks

Fungi are attractive candidates for designing effective bioremediation strategies of pharmaceuticals because of the unspecificity of its oxidative enzymatic system that includes LME but also intracellular enzymes such as cytochrome P450. In particular, fast degradation, from minutes to few days, has been demonstrated for estrogens, β-blockers, some anti-inflammatory drugs, antibiotics and other anti-microbial agents, while iodinated contrast agents and psychiatric drugs are removed at slower rates. The participation of LMEs and cytochrome P450 has been shown in the transformation of members from every family of drugs, except in lipid regulators, in which the LMEs were not involved in the process.

According to the metabolites identified, the most commonly reactions involved in the transformation of pharmaceuticals by fungi include hydroxylations, formylations, deaminations and their combinations (anti-inflammatory drugs, psychiatric drugs, antibiotics, β-blockers, lipid regulators and other anti-microbial agents); dehalogenations (iodinated contrast media and triclosan) and oligomerizations (estrogens and triclosan). Analogous TPs identified for members of the same families (quinolones and sulfonamides) suggest common degradation pathways for similar molecules. Mineralization has been barely demonstrated, only suggested for some anti-inflammatory drugs (diclofenac and ketoprofen) and quinolones. In some cases, the accumulation of TPs has led to an increase of the toxicity (ibuprofen, carbamazepine and clofibric acid). Therefore, the assessment of this parameter is essential to evaluate the suitability for the potential application of these processes in the treatment of effluents.

Moreover, the fungal treatments have resulted in the reduction of estrogenic activity (estrogens and triclosan) and antimicrobial activity (triclosan). The use of different reactor configurations (continuous and batch fluidized bed, packed bed, perfusion basket reactors) for anti-inflammatory, psychiatric drugs, lipid regulators and triclosan removal, has proved the possibility of scaling up the pharmaceutical degradation process.

However, most of the published studies to date on removal of pharmaceuticals by fungi were carried out in synthetic liquid media under controlled conditions of pH, temperature and absence of competitors that allow demonstrating the ability of the tested fungi to degrade the target pharmaceutical. Despite of great promise of fungi as removal agents, a number of challenges remain to be surmounted in using them at larger field scale, i.e. competition with autochthonous microorganisms, non optimal pH, presence of inhibitors in wastewaters, etc. To date, these aspects have been barely studied and future research efforts on fungal bioremediation technology should shed light on them to translate our basic knowledge on fungi into cost-effective practical bioremediation applications.

Table VII.1: Summary of pharmaceutical degradation by whole cell fungi and their TP.

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
Anti-inflammatories/analgesic drugs	Diclofenac	*B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	Total degradation in 7 d		Rodarte-Morales et al., 2011a
		P. chrysosporium			Completely disappeared in 4 d.		
		Bjerkandera sp.			Total degradation in 4 d		
		P. chrysosporium	Degradation in an aerated fed-batch bioreactor	1 mg L^{-1}	Diclofenac was added every 2 d. Every pulse was completely removed after 23 h.		Rodarte-Morales et al., 2011b
		P. sordida	Mycelium incubation in flask shaked at 150 rpm and 30ºC	30 mg L^{-1}	Completely disappeared after 4 d.	4-hydroxydiclofenac; 5-hydroxydiclofenac; 4',5-dihydroxydiclofenac	Hata et al., 2010a
		T. versicolor	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25ºC	10 mg L^{-1} and 45 µg L^{-1}	Complet degradation after 1 h in both cases	4'-hydroxydiclofenac; 5-hydroxydiclofenac	Marco-Urrea et al., 2010a
	Fenoprofen	*T. versicolor*	Incubation at 30ºC in shaken condition for 48h	10 µg L^{-1}	100 % removed		Tran et al., 2010
	Ibuprofen	*B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	Total degradation in 7 d		Rodarte-Morales et al., 2011a
		P. chrysosporium			Total degradation in 4 d		
		Bjerkandera sp.			Total degradation in 4 d		
		P. chrysosporium	Degradation in an aerated fed-batch bioreactor	1 mg L^{-1}	Diclofenac was added every 2 d. Every pulse was completely removed after		Rodarte-Morales et al., 2011b

				23 h.		
	I. lacteus	Cultures were incubated in serum bottles shaked at 135 rpm and 25ºC during 7 d	10 mg L^{-1}	100% before 7 d		Marco-Urrea et al., 2009
	G. lucidum			100% before 7 d		
	P.chrysosporium			70-88% after 7 d		
	T. versicolor			Complete degradation after 1h	1-hydroxy ibuprofen; 2-hydroxy ibuprofen; 1,2-dihydroxy ibuprofen	
Indomethacin	*T. versicolor*	Incubation at 30ºC in shaken condition for 48h	10 µg L-1	100 % removed		Tran et al., 2010
Ketoprofen	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25ºC	10 mg L^{-1} and 40 µg L^{-1}	100% removed after 24h and 5h respectively	2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid; 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid; 2-(3-benzoyl-4-hydroxyphenyl)-propanoic acid	Marco-Urrea et al., 2010c
Mefenamic	*P. sordida*	Mycelium incubation in flask shaked at 150 rpm and 30ºC	10^{-4} M	90% after 6 d of treatment.	3'-hydroxymethylmefenamic acid; 3'-hydroxymethyl-5-hydroxymefenamic acid; 3'-hydroxymethyl-6'-hydroxymefenamic acid; 3'-carboxymefenamic acid	Hata et al., 2010a

	Naproxen	*B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	100% after 7 d		Rodarte-Morales et al., 2011a
		P. chrysosporium			100% after 4 d		
		Bjerkandera sp.			100% after 7 d		
		P. chrysosporium	Degradation in an aerated fed-batch bioreactor	1 mg L^{-1}	Diclofenac was added every 2 d. Every pulse was completely removed after 23 h.		Rodarte-Morales et al.,2011b
		T. versicolor	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25ºC	10 mg L^{-1} and 55 µg L^{-1}	Complete degradation after 6h and 5h respectively	2-(6-hydroxynaphthalen-2-yl)propanoic acid; 1-(6-methoxynaphthalen-2-yl)ethanone	Marco-Urrea et al., 2010c
	Propyphenazone	*T. versicolor*	Incubation at 30ºC in shaken condition for 48h	10 µg L^{-1}	75% removed at 2 d		Tran et al., 2010
Antibiotics	Cinoxacin	*B. bassiana*	Experimental cultures in flasks were incubated at 28ºC with rotary shaking at 180 rpm during 20 d.	20 mM	52.7% removed after 20 d	1-Ethyl-1,4-dihydro-3-(hydroxymethyl)-[1,3]dioxolo[4,5-g]cinnolin-4-one1-Ethyl-1,4-dihydro-6,7-dihydroxy-3-(hydroxymethyl)cinnolin-4-one	Parshikov et al 2002a

Ciprofloxacin	*G. striatum*	Erlenmeyer flask containing 30 ml of medium and fungi were incubated at 150 rpm at room temperature for 13 weeks	10 mg L^{-1}	33% after 13 weeks.	Monohydroxylated congeners; dihydroxylated congeners	Parshikov et al 2001a
	M. Ramannianus	Cultured incubated for 14 d at 28ºC with shaking at 200 rpm	300 µM	89.9±0.4% removed after 14 d	1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-acetyl-1-piperazinyl)-3-quinolinecarboxylic acid	Parshikov et al 1999a
	P. guepini	Incubation of mycelium for 18 d	300 µM	67.8% removed after 18 d	N-acetylciprofloxacin (52%) Desethylene-N-acetylciprofloxacin (9.2%); N-formylciprofloxacin (4.1%); 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (2.3%)	Parshikov et al 2001a
	T. viride	Incubation of mycelium for 16 d	300 µM	31%had been transformed to the prduct	4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin	Parshikov et al 2002b
	P. chrysosporium	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastewater	10 µg mL^{-1}	80% after 30 d incubation		Accinelli et al., 2010

Ciprofloxacin	*T. versicolor*	Pellets incubated at 30 ºC under orbital agitation	2 mg L^{-1}	> 90% after 7 d	7-((2-aminoethyl)amino)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 1-cyclopropyl-6-fluoro-8-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid; 7-((2-acetamidoethyl)amino)-1-cyclopropyl-6-fluoro-8-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; two dimeric products formed by C-C covalent bond and followed by several transformations	Prieto et al., 2011
Enrofloxacin	*G. striatum*	Wood-rotting fungi grown on wetted wheat straw	3.3 µg mL^{-1}	53.3%± 1.2% in 8 weeks		Martens et al., 1996
	S.rugosoannulata			5.1%± 0.6% in 8 weeks		
	P. chrysosporium			25.6%± 0.6% in 8 weeks		
	I. lacteus			13.7%± 0.8% in 8 weeks		
	M. ramannianus	Cultures grown in sucrose-peptone broth were dosed with enrofloxacin	253 µM	22% of the enrofloxacin remained after 21 d	Enrofloxacin N-oxide (62%) N-acetylciprofloxacin (8%); Desethylene-enrofloxacin (3.5%);	Parshikov et al 2000

	Several basydiomycetes	Static cultures inoculated with mycelium	28.5 mg L^{-1}	n.d.	61 different compounds; see text for more details	Wetzstein et al., 2006
	G. striatum	Mycelia suspended in a defined liquid medium with contaminant were shaked at 150 rpm during 8 weeks	10 mg L^{-1}	Production of 27.3 % $^{14}CO_2$ from [^{14}C] enrofloxacion	3-,6-,and 8- hydrolated congeners of enrofloxacin 5,6- (or 6,8-), 5,8-, and 7,8-dihydroxylated congeners isatin-type compound Anthranilic acid derivative 1-ethylpiperazine; Desethylene-enrofloxacin	Wetzstein et al., 1997
Erythromycin	*P. chrysosporium*	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium*	10 µg mL^{-1}	98% after 30 d.		Accinelli et al., 2010
Norfloxacin	*P. guepini*	Incubation of mycelium for 18 d	313 µM	68.9% removed after 18 d	N-acetylnorfloxacin (55.4%); desethylene--n-acetylnorfloxacin (8.8%); N-for-mylnorfloxacin (3.6%); 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (2.1%)	Parshikov et al 2001a
	T. viride	Incubation of mycelium for 16 d	313 µM	42%had been transformed to the prduct	4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin	Parshikov et al 2002b
	T. versicolor	Pellets incubated at 30 ºC under orbital agitation	2 mg L^{-1}	> 90% after 7 d	7-((2-aminoethyl)amino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 7-((2-scetamidoethyl)amino)-1-	Prieto et al., 2011

					ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	
Flumequine	*C. elegans*	Cultures at 28 ºC and shaking	308 µM	n.d.	7-hydroxyflumequine (two diastereomers); 7-oxoflumequine	Williams et al., 2007
Sarafloxacin	*M. ramannianus*	Cultures grown in sucrose-peptone broth were dosed with sarafloxacin	260 µM	59% of the starting material remained.	N-acetylsarafloxacin; Desethylene-N-acetylsarafloxacin	Parshikov et al 2001b
Sulfamethazine	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25ºC	9 mg L^{-1}	> 95% after 20 h	N-(4,6-dimethylpyrimidin-2-yl)benzene-1,4-diamine (desulfonated sulfamethazine); N-(4,6-dimethylpyrimidin-2-yl)-4-(formylamino)benzenesulfonamide (formylated sulfamethazine)	García-Galán et al., 2011
	T. versicolor	Continuous degradation in an air pulsed fluidized bed bioreactor	5 mg L^{-1}	94% at HRT 72 h		Rodríguez-Rodríguez et al., 2012b
Sulfathiazole	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25ºC	11 mg L^{-1}	100% after 7 d	formylated sulfathiazole; desulfonated-desaminated-hydroxylated sulfathiazole	Rodríguez-Rodríguez et al., 2012b
		Continuous degradation in an air pulsed fluidized bed bioreactor	5 mg L^{-1}	> 95% at HRT 72 h		

	Sulfapyridine	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25ºC	9 mg L^{-1}	100% after 48 h	desulfonated sulfapyridine; formylated sulfapyridine; formylated-desulfonated sulfapyridine; hydroxylated sulfapyridine; a hydroxy-formyl intermediate after the loss of the pyridine ring.	Rodriguez - Rodríguez et al., 2012b
			Continuous degradation in an air pulsed fluidized bed bioreactor	5 mg L^{-1}	> 99% at HRT 72 h		
	Sulfamethoxazol	*P. chrysosporium*	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastwater	10 µg mL^{-1}	98% after 30 d.		Accinelli et al., 2010
		B. adusta	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	Complete degraded before 14 d		Rodarte-Morales et al., 2011a
		P. chrysosporium			80% degradation after 7 d		
		Bjerkandera sp.			Complete degraded before 14 d		
Other antimicrobial agents	N-acetylphenothiazine	*A. nigen*	Cultures were dosed and then incubated for 72 h at 28ºC with shaking at 200 rpm.	20 mg ml^{-1}	Residual N-Acetylphenothiazine	N-acetylphenothiazine; sulfoxidephenothiazine sulfoxide	Parshikov et al 199b
		C. verticillata				N-acetylphenothiazine sulfoxide; phenothiazine sulfoxide; phenothiazin-3-one; phenothiazine N-glucoside;	
		P.simplicissimum				N-acetylphenothiazine sulfoxide; phenothiazine sulfoxide; Phenothiazine	

Triclosan	*I. lacteus*	Static culture inoculated with mycelium	2.5 mg L^{-1}	> 95% after 14 d		Cajthalm et al., 2009
	B. adusta			> 95% after 14 d		
	P. chrysosporium			> 95% after 14 d		
	P. magnolia	Static culture inoculated with mycelium	2.5 mg L^{-1}	90% after 14 d		
	P. ostreatus			95% after 14 d		
	T. versicolor			91% after 14 d		
	P. cinnabarinus	Static culture inoculated with mycelium	2.5 mg L^{-1}	> 95% after 14 d		
	D. squalens			> 95% after 14 d		
	T. versicolor			> 95% after 14 d	2,4-dichlorophenol; the conjugates 2-*O*-(2,4,4'-trichlorodiphenyl ether)-β-D-xylopyranoside and 2-*O*-(2,4,4'-trichlorodiphenyl ether)-β-D-glucopyranoside	Hundt et al., 2000
	P. cinnabarinus	Mycelium inoculation and shaking at 30 ºC	0.25 mM	n.d.	2,4,4'-trichloro-2'-methoxydiphenyl ether; the conjugate 2-O-(2,4,4'-trichlorodiphenyl ether)-β-D-glucopyranoside	
Artemisinin	*E. amstelodami*	Cultures were incubated at 28ºC in environmental shakers during 14 d.	500 mg L^{-1}	Residual artemisinin afer 14 d	5β-hydroxyartemisinin; 7β-hydroxyartemisinin	Parshikov et al., 2006
	Aspergillus nigen			Residual artemisinin afer 14 d	5β-hydroxyartemisinin; 7β-hydroxyartemisinin	

		M. Ramannianus			Completely degraded after 14 d	6β-hydroxyartemisinin; 7β-hydroxyartemisinin; 7α-hydroxyartemisinin; 4α-hydroxy-1-deoxoartemisinin	Parshikov et al 2005
		C. elegans			96.5 % degradation after 14 d	6β-hydroxyartemisinin; 7β-hydroxyartemisinin; 7α-hydroxy-9α-artemisinin 4α-hydroxy-1-deoxoartemisinin	Parshikov et al 2004
	Oseltamivir	*P. chrysosporium*	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastwater	10 μg mL^{-1}	50% after 16 d incubation		Accinelli et al., 2010
B-blockers	Atenolol	*T. versicolor*	Degradation of contaminant by induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	80% reached after 6 h of incubation.	Atenolol hydroxylated derivative (P282)	Marco-Urrea et al., 2010d
	Propanolol	*T. versicolor*	Degradation of contaminant by induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	50% in 1 h and 80% after 6 h incubation.	Hydroxy propranolol (P275)	Marco-Urrea et al., 2010d
Lipid regulator	Clofibric acid	*I. lacteus* *P. chrysosporium* *G. lucidum*	Cultures were incubated in serum bottles shaked at 135 rpm and 25ºC during 7 d	10 mg L^{-1}	Low degradation Low degradation Low degradation		Marco-Urrea et al., 2009

		T. versicolor			91% after 7 d of the treatment		
		T. versicolor	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	30 µg L^{-1}	Completely degraded after 4 d		Not yet published
		T. versicolor	Continuous degradation in an air pulsed fluidized bed bioreactor	160 µg mL^{-1}	80 % of the inflow concentration was reduced at the steady state. 16.5 µg removed g^{-1} d.w d^{-1}	2-(4-chlorophenoxy)-2-(hydroxymethyl) propanoic	
		T. versicolor	Degradation of contaminant by induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	80% reached after 6 h of incubation.	Clofibric hydroxylated derivative	Marco-Urrea et al., 2009
	Gemfibrizil	*T. versicolor*	Incubation at 30ºC in shaken condition for 48h	10 µg L^{-1}	70% degraded after 2 d		Tran et al., 2010
Psychiatric drugs	Carbamazepine	*P. chrysosporium*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	Complete removed before 14 d		Rodarte-Morales et al., 2011a
		Bjerkandera sp.			Complete removed before 14 d		
		B. adusta			Complete removed at 14 d		
		T. versicolor	Incubation at 30ºC in shaken condition for 48h	10 µg L^{-1}	75% degraded after 2 d		Tran et al., 2010
		P. chrysosporium	Cultures were incubated in serum bottles shaked at 135 rpm and 25ºC during 7 d	10 mg L^{-1}	<10% after 7 d incubation		Marco-Urrea et al., 2009
		G. lucidum			47% after 7 d incubation		
		T. versicolor			58% after 7 d treatment		
		I. lacteus			<10 % after 7 d incubation		
		P. ostreatus	Incubation in Erlenmeyer flasks at 28 ºC in the dark	10 mg L^{-1}	60 % degraded after 17 d		Golan-Rozen et al., 2011

	P. ostreatus			48 % after 17 d		
Carbamazepine	*U. ramanniana*			25 % removed after 25 d	11-epoxy-carbamazepine; 2-hydroxy-carbamazepine; 3-hydroxy-carbamazepine	Kang et al., 2008
	C. elegans			45 % of degradation after 25 d	11-epoxy-carbamazepine; 2-hydroxy-carbamazepine; 3-hydroxy-carbamazepine	
	P. ostreatus			Completely degradation after 10 d		
	T. versicolor	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	10 mg L^{-1} and 50 µg L^{-1}	High concentrations 94 % elimination after 6 d and in low 61 % after 7 d.	Acridine; acridone; 10,11-dihydro-10,11-dihydroxy-carbamazepine; 11-epoxy-carbamazepine	Jelic et al., 2011
	T. versicolor	Degradation in an air pulsed fluidized bed bioreactor operated in batch and continuous	200 µg mL^{-1}	Completely degraded after 2 d in batch mode. In continuous operation, 54 % of the inflow concentration was reduced at the steady state. 11.9 µg removed g^{-1} d.w d^{-1}	11-epoxy-carbamazepine	
	T. versicolor	Induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	50% in 1 h and 80% after 6 h incubation.	Two hydroxylated isomers of CBZ (P254A and B)	Marco-Urrea et al., 2009
Diazepam	*P. chrysosporium* *Bjerkandera* sp. *B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	57% removed in 5 d. 54% removed in 5 d. 56% removed in 5 d.		Rodarte-Morales et al., 2011a
Fluoxetine	*P. chrysosporium* *Bjerkandera* sp.	Erlenmeyers flasks containing defined medium and contaminant were statically	1 mg L^{-1}	< 10 % in 2 weeks 23 % after 14 d		Rodarte-Morales et al., 2011a

			incubated at 30ºC for 2 weeks				
		B. adusta			< 10 % in 2 weeks		
	Citalopram	*B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30ºC for 2 weeks	1 mg L^{-1}	Complete degradation before 14 d		Rodarte-Morales et al., 2011a
		P. chrysosporium			Complete degradation before 14 d		
		Bjerkandera sp.			58% removal after 4 d		
Estrogens	17B-estradiol	*T. versicolor*	Continuous degradation in an bioreactor	18.8 mg L^{-1}	100% at a HRT 120 h		Blánquez and Guieysse, 2008
	17a-ethinylestradiol	*T. versicolor*		7.3 mg L^{-1}	100% at a HRT 120 h		
		I. lacteus	Static culture inoculated with mycelium	2.5 mg L^{-1}	100% after 14 d		Cajthalm et al., 2009
		B. adusta			100% after 14 d		
		P. chrysosporium			30% after 14 d		
		P. magnoliae			70% after 14 d		
		P. ostreatus			100% after 14 d		
		T. versicolor			100% after 14 d		
		P. cinnabarinus			100% after 14 d		
		D. squalens			100% after 14 d		
X-ray contrast media	Diatrizoate	*T. versicolor*	Flasks scale at 30 ºC	1 mM	80 % after 14 d	3,5-di(acetamido)-2,6-diiodobenzoate; 3,5-di(acetamido)-4,6-diiodobenzoate; 3,5-di(acetamido)-2-monoiodobenzoate	Rode and Müller 1998
	Iodipamide	*T. versicolor*			80 % after 14 d	5 metabolites detected	

Aminotrizoate	*T. versicolor*		58 % after 14 d	No metabolites detected	
Acetrizoate	*T. versicolor*		65 % after 14 d	1 metabolite detected	
Aminotriiodoisophthalic acid	*T. versicolor*		50 % after 14 d	1 metabolite detected	
Aminotriiodoisophthalic acid	*T. versicolor*		50 % after 14 d	1 metabolite detected	Engels-Matena 1996
Iopromide	*T. versicolor*	1mM	90 % after 15 d	Deiodation of the principal ring.	

Table VII.2: Summary of pharmaceutical degradation by fungal enzyme and their TP.

Family drug	Drug	Fungal enzyme	Treatment	Initial concentration	Removal rate	Metabolites	References
Anti-inflammatories/analgesic drugs	Diclofenac	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 25 min	4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol	Eibes et al., 2011
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	83-100% after 24 h with mediators, 83% without mediators		Lloret et al., 2011
		LiP from *P. chrysosporium*	Flask scale	5 mg L^{-1}	100% after 2 h		Zhang and Geiβen, 2010
		LAC from *T.versicolor*	Flask scale	40 mg L^{-1}	> 95% after 4.5 h		Marco-Urrea et al., 2010a
		LAC from *T.versicolor*	Flask scale	10 µg L^{-1}	> 90% in 30 min		Tran et al., 2010
	Fenoprofen	LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	> 90% in 3 h		Tran et al., 2010
	Ibuprofen	LAC from *T. versicolor*/ MnP from *Bjerkandera sp*	Flask scale	10 mg L^{-1}	Negligible after 24 h, even with mediators		Marco-Urrea et al., 2009
		LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	~35% in 3 h		Tran et al., 2010
	Indomethacin	LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	> 90% in 30 min		Tran et al., 2010
	Ketoprofen	LAC from *T. versicolor*	Flask scale	10 mg L^{-1}	Negligible after 20 h, even with mediators		Marco-Urrea et al., 2010c
		LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	~ 50% in 3 h		Tran et al., 2010

	Naproxen	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	80% after 7 h		Eibes et al., 2011
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	36-68% after 24 h with mediators		Lloret et al., 2010
		LAC from *T. versicolor*	Flask scale	20 mg L^{-1}	> 95% after 30 h with HOBT, < 10% without mediator		Marco-Urrea et al., 2010b
		LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	> 90% in 30 min		Tran et al., 2010
	Propyphenazone	LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	~ 25% in 3 h		Tran et al., 2010
Antibiotics	Tetracycline	LiP from *P. chrysosporium*	Flask scale	50 mg L^{-1}	> 99% after 30 min, only in the presence of veratryl alcohol		Wen et al., 2009
		LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 1 h with mediators		Suda et al., 2012
	Oxytetracycline	LiP from *P. chrysosporium*	Flask scale	50 mg L^{-1}	> 99% after 30 min, only in the presence of veratryl alcohol		Wen et al., 2009
		LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 1 h with mediators		Suda et al., 2012
	Chlortetracline	LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 15 min with mediators		Suda et al., 2012
	Doxycycline	LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 15 min with mediators		Suda et al., 2012
	Sulfadimethoxine	LAC from *T. versicolor*	Flask scale	10^{-3} M	75.1% after 15 d	4-(6-imino-2,4-dimethoxypyrimidin-1-yl)aniline; additional metabolites were preliminary assigned	Schwarz et al., 2010

					but not confirmed	
	Sulfamethazine	LAC from *T. versicolor*	Flask scale	20 mg L^{-1}	22% after 50h without mediators, 93-100% with mediators	*N*-(4,6-dimethylpyrimidin-2-yl)benzene-1,4-diamine (desulfonated sulfamethazine); *N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide (desaminated sulfamethazine); N_1-hydroxysulfamethazine or N_4-hydroxysulfamethazine (hydroylated sulfamethazine)	García-Galán et al., 2011
	Sulfamethoxazole	VP from *B. adusta*	Flask scale	25 mg L^{-1}	80% after 7 h	3-amino-5-methylisoazole	Eibes et al., 2011
	Sulfanilamide	LAC from *T. versicolor*	Flask scale	10^{-3} M	10.0% after 15 d	Aniline was preliminary assigned but not confirmed	Schwarz et al., 2010
	Sulfapyridine	LAC from *T. versicolor*	Flask scale	10^{-3} M	95.6% after 15 d	Aniline; 4-(2-imino-1-pyridyl)aniline; additional metabolites were preliminary assigned but not confirmed	Schwarz et al., 2010
		LAC from *T. versicolor*	Flask scale	20 mg L^{-1}	75-98% with mediators	Desulfonated sulfapyridine; a formyl intermediate after the	Rodríguez-Rodríguez et al., 2012b

						loss of the pyrimidine ring	
	Sulfathiazole	LAC from *T. versicolor*	Flask scale	16 mg L^{-1}	82-100% with mediators	Desulfonated sulfapyridine; a formyl intermediate after the loss of the thiazole ring	Rodríguez-Rodríguez et al., 2012b
	Ciprofloxacin	LAC from *T. versicolor*	Flask scale	10 mg L^{-1}	16% after 20 h; 97,7% with mediators		Prieto et al., 2011
	Norfloxacin	LAC from *T. versicolor*	Flask scale	10 mg L^{-1}	0% after 20 h, 33,7% with mediators		Prieto et al., 2011
Other antimicrobial agents	Triclosan	MnP from *P. chrysosporium*	Flask scale	28.95 mg L^{-1}	100% after 90 min		Inoue et al., 2010
		LAC from *T. versicolor*	Flask scale	28.95 mg L^{-1}	66% after 90 min with mediators, 10-51% without mediators		
		LAC from *C. polyzona*	PBR with immobilized LAC; repeated batch cycles	5 mg L^{-1}	> 90% after 200 min		Cabana et al., 2009a
		LAC from *C. polyzona*	Continuous perfusion basket reactor with CLEAs	5 mg L^{-1} ; 100 mg L^{-1}	85% at HRT 325 min		Cabana et al., 2009b
		LAC from *T. versicolor*	Flask scale	5.8 mg L^{-1}	100% after 4 h without mediators, after 30 in with mediators		Kim and Nicell 2006
		LAC from *C. polyzona*	Flask scale	5 mg L^{-1}	65% after 4 h without mediator,	Oligomers (dimers, trimers and tetramers)	Cabana et al., 2007b

				50% after 1 h with mediators		
		LAC from *G. lucidum*	Flask scale	0.2 mmol L^{-1}	56.5% after 24 h without mediators, 90% with mediators; reduction in toxicity demostrated by bacterial inhibition methods	Oligomers in the absense of mediators (dimers and trimers of triclosan); 2,4-dichlorophenol (2,4-DCP) and dechlorinated forms of 2,4-DCP in the presence of mediators	Hundt et al., 2000
		LAC from *T. versicolor*	Conjugates laccase-chitosan	5 mg L^{-1}	100% after 6 h (only 60% with free LAC)	Oligomers and dechlorinated oligomers	Cabana et al., 2011
		LAC from *C. polyzona*	Continuous FBR with CLEAs	5 mg L^{-1}	100% at HRT 150 min		Cabana et al., 2007a
		VP from *B. adusta*	Flask scale with CLEAs	10 mg L^{-1}	26% after 10 min	VP was co-aggregated with glucose oxidase	Taboada-Puig et al., 2011
Psychiatric drugs	Carbamazepine	LAC from *T. versicolor*	Flask scale with repeated additions of LAC or LAC + HBT	2×10^{-5} M	60% after 48 h, only with HBT	10,11-dihydro-10,11-epoxycarbamazepine, 9(10H)acridone	Hata et al., 2010b
		MnP from *P. chrysosporium*	Flask scale	2×10^{-5} M	14% after 24 h		
		VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	Negligible after 7 h		Eibes et al., 2011
		LiP from *P. chrysosporium*	Flask scale	5 mg L^{-1}	< 10% in 2 h		Zhang and Geiβen 2010
		LAC from *T. versicolor*/ MnP from *Bjerkandera*	Flask scale	10 mg L^{-1}	Negligible after 24 h, even with mediators		Marco-Urrea et al., 2009

		sp				
		LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	~ 35% in 3 h	Tran et al., 2010
	Citalopram	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	18% after 7 h	Eibes et al., 2011
	Fluoxetine	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	< 10% after 7 h	Eibes et al., 2011
Lipid regulators	Clofibric acid	LAC from *T. versicolor*/ MnP from *Bjerkandera* sp.	Flask scale	10 mg L^{-1}	Negligible after 24 h, even with mediators	Marco-Urrea et al., 2009
		LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	~ 20% in 3 h	Tran et al., 2010
	Gemfibrozil	LAC from *T. versicolor*	Flask scale	10 µg L^{-1}	~ 80% in 3 h	Tran et al., 2010
Estrogens	17a-ethinylestradiol	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 15 min	Eibes et al., 2011
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	100% after 5 h without mediators; 1-3 h depending on mediator	Lloret et al., 2010
		MnP from *P. chrysosporium*	Flask scale	10^{-7} M	> 95% after 1h; estrogenic activity completely removed after 8 h	Suzuki et al., 2003
		LAC from *T. versicolor*	Flask scale	10^{-7} M	> 95% after 1h with mediators; estrogenic activity completely removed after 8 h	
		LAC from *T. versicolor*	Stirred batch reactor with the	100 ng L^{-1}	100% after 1 h	Auriol et al., 2007

		compound spiked in wastewater				
	LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L-1	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC		Auriol et al., 2008
	LAC from *M. thermophila*	BSTR working in cycles/ Continuous PBR with immobilized enzyme	5 mg L-1	>94%/60%; reduction in toxicity detected		Lloret et al., 2011
	VP from *B. adusta*	Flask scale with CLEAs	10 mg L^{-1}	93,1% after 10 min; 100% free VP		Taboada-Puig et al., 2011
17B-estradiol	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 15 min		Eibes et al., 2011
	LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	100% after 3 h without mediators; 1-3 depending on mediator		Lloret et al., 2010
	LAC from *T. pubescens*	Flask scale, biphasic system (buffer/AcOEt)	5 g L^{-1}	n.d.	Two C-C and two C-O dimeric products	Nicotra et al., 2004
	LAC from *Myceliophthora* sp.	Flask scale, adsorbed on glass beads in organic solvents (dioxane/water	10 g L^{-1}	n.d.	Two C-C and two C-O dimeric products	

		saturated toluene)			
	MnP from *P. chrysosporium*	Flask scale	10^{-7} M	> 95% after 1h; estrogenic activity completely removed after 8 h	Suzuki et al., 2003
	LAC from *T. versicolor*	Flask scale	10^{-7} M	> 95% after 1h with mediators; estrogenic activity completely removed after 8 h	
	LAC from *Trametes* sp.	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h	Auriol et al., 2007
	LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC	Auriol et al., 2008
	LAC from *M. thermophila*	BSTR working in cycles/ Continuous PBR with immobilized enzyme	5 mg L^{-1}	>95%/75%; reduction in toxicity detected	Lloret et al., 2011
	VP from *B. adusta*	Flask scale with CLEAs	10 mg L^{-1}	90,1% after 10 min; 100% free VP	
Estriol	LAC from *Trametes* sp.	Stirred batch reactor with the compound spiked in	100 ng L^{-1}	100% after 1 h	Auriol et al., 2007

		wastewater			
	LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC	Auriol et al., 2008
Estrone	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 15 min	Eibes et al., 2011
	LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	37-100% after 24 h with mediators, 65% without mediators	Lloret et al., 2010
	MnP from *P. sordida*	Flask scale	10^{-5} M	100% after 1 h	Tamagawa et al., 2006
	LAC from *P. sordida*	Flask scale	10^{-5} M	100% after 1 h	
	LAC from *Trametes* sp.	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h	Auriol et al., 2007
	LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC	Auriol et al., 2008
	LAC from *M. thermophila*	BSTR working in cycles/ Continuous PBR with immobilized enzyme	5 mg L^{-1}	>87%/55%; reduction in toxicity detected	Lloret et al., 2011

Iodinated contrast media	Diatrizoate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	87%/29%/0%/0%	3,5-di(acetamido)-2,6-diiodobenzoate 3,5-di(acetamido)-4,6-diiodobenzoate 3,5-di(acetamido)-2-monoiodobenzoate	Rode and Müller et al., 1998
	Iodipamide	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	90%/16%/0%/0%		
	Acetrizoate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	93%/27%/0%/0%		
	Aminotrizoate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	68%/0%/60%/0%		

Aminotriiodoiso-phthalate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	73%/18%/34%/0%		Engels-Matena, 1996
Iopromide	Extracellular extract from *T. versicolor*	Flask scale	1 mM	98%	5-methoxyacetylamino-4-monoiodoisophthalic acid (2,3-dihydroxy-propyl) diamide. 5-methoxyacetylamino-4(6)-monoiodoisophthalic acid [(2,3-dihydroxy-propyl)-methyl)] diamide. 5-methoxyacetylamino-2,6-diiodoisophthalic acid [(2,3dihydroxy-N-methyl-propyl)-2,3-dihydroxy-propyl)] diamide.	

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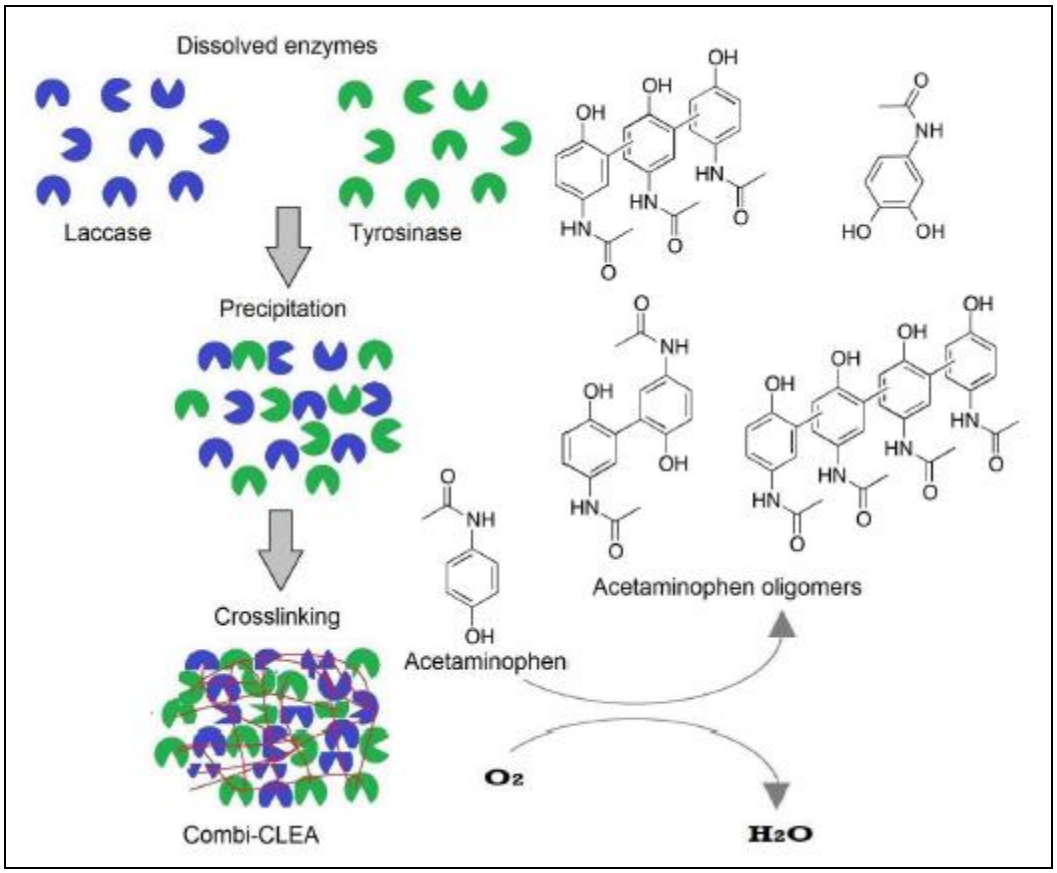
SECTION 2.2:

Effluents Treatment

Chapter VIII:

Enzymatic removal of pharmaceuticals from municipal and hospital wastewaters using combined cross-linked laccase and tyrosinase aggregates

Part of this work have been submitted as: **Sidy Ba, Lounes Haroune[2], Carles Cruz-Morató, Chloé Jacquet, Imad E. Touahar, Jean-Phillipe Bellenger, Claude Y. Legault, J. Peter Jones, Hubert Cabana. Enzymatic transformation of acetaminophen from municipal and hospital wastewaters using combined cross-linked laccase and tyrosinase aggregates. STOTEN**



Abstract

Acetaminophen is one of the ubiquitous pharmaceuticals detected in wastewaters at low concentrations (ng/L-μg/L) throughout the world. It has been found that contaminants at such low concentrations are generally unsatisfactorily removed from sewage treatment plants (STP). In the present study, samples of municipal and hospital wastewaters analyzed using a UPLC-MS/MS showed the presence of acetaminophen at 57.5-77.4 μg/L in the former and at 90.2 μg/L in the latter. Two enzymes, laccase and tyrosinase, have been insolubilized as combination of crosslinked enzyme aggregates (combi-CLEA) using chitosan, a renewable and biodegradable polymer, as crosslinker and subsequently applied to the wastewaters samples for the transformation of acetaminophen. The combi-CLEA, with specific activity of 12.3 U/g for laccase and 167.4 U/g for tyrosinase, exhibited high enzymatic activity at pH 5-8 and temperature 5-30 °C, significant resistance to denaturation and no diffusional restriction to its active site based upon the Michaelis-Menten kinetic parameters. Application of the combi-CLEA in batch mode achieved acetaminophen transformations of more than 80% to nearly 100% from the municipal wastewater and of more than 90 % from the hospital wastewater. HPLC analysis of the metabolites resulting from the enzymatic transformation of acetaminophen showed the formation of its oligomers as dimers, trimers and tetramers due to the laccase and 3-hydroxyacetaminophen due to the tyrosinase.

Keywords: Laccase; Tyrosinase; Combi-CLEA; Wastewaters; Phenolic Compounds; Acetaminophen.

XII.1. Introduction

Recently, numerous active molecules of pharmaceuticals have been detected in aquatic systems throughout the world (Kolpin et al. 2002; Richardson et al. 2005; Sacher et al. 2008). Wastewater effluents discharge has been identified as a major source of entry of these pharmaceuticals into water bodies (Verlicchi et al. 2012) exposing the inability of conventional sewage treatment plants (STP) to effectively remove many of these contaminants. In fact, STP are not designed for the treatment of these contaminants found at very low concentrations (ng/L-µg/L) (Caliman and Gavrilescu. 2009). Many pharmaceuticals remain persistent and biologically active with, for some, reported toxicity and/or endocrine disrupting properties causing adverse effects on aquatic species under low concentrations (Crane et al. 2006). It is also recognized that the most prevalent pharmaceuticals in effluent of wastewaters are molecules of drugs most frequently prescribed or purchased over-the-counter (Wu et al. 2012) including acetaminophen, an active agent used in the formulation of hundreds of medicines.

Acetaminophen is a phenolic compound known under different brand names used worldwide as minor pain and fever reducer. Acetaminophen has widely been detected in hospital wastewaters (one of its main sources of discharge) where it can surpass 150 µg/L (Wu et al. 2012). Although complete removal of acetaminophen from STP has been found in some studies (Verlicchi et al. 2012), there are several reported concentrations of this contaminant in both surface waters and outflows of STP in the range of 243 to 338 ng/L (Gros et al. 2012), and in the intake of raw surface water and groundwater used for public drinking water supply at 163 ng/L to 1.89 µg/L (Boleda et al. 2011; Fram and Belitz. 2011). Nonetheless, there is no ecotoxicological effects of acetaminophen reported to date to our knowledge. However, in an experiment, reaction of acetaminophen with hypochlorite simulating wastewater disinfection formed at least 11 distinct disinfection by-products including 1,4-benzoquinone and N-acetyl-p-benzoquinone imine, both known to be toxic compounds (Bedner and MacCrehan. 2006). This is an evidence of the potential for acetaminophen or its by-products to exhibit side effects on aquatic ecosystems that cannot be ignored or

underestimated. Acetaminophen is an active chemical among the many pharmaceuticals whose immediate effects could escape detection if they are subtle (Daughton and Ternes. 1999). Also, it was suggested that for pharmaceuticals with molecules designed to be biologically active, it cannot be excluded that they affect sensitive aquatic organisms even at concentrations in the order of ng/L to µg/L (Huber et al. 2005). The continuous discharge of acetaminophen and its by-products to water bodies, where they can interact with aquatic organisms, deserves particular investigation on the basis of precautionary principle for effective treatment of this phenolic compound.

In several experiments laccase (EC 1.10.3.2) and tyrosinase (EC 1.14.18.1), two oxidoreductases widely distributed in plants, fungi, and other organisms, have been proven to enzymatically remove phenolic and non-phenolic aromatic compounds from polluted waters (Husain and Jan. 2000). Both laccase and tyrosinase are non-substrate specific copper-containing phenoloxidases requiring readily available dioxygen as sole cofactor for the catalytic oxidation of phenolic contaminants. Their oxidation reaction leads to a release of water as by-product and free reactive phenoxy radicals (for laccase) or quinones (for tyrosinase) that subsequently polymerize and precipitate (Atlow et al. 1984; Bollag. 1992) thus becoming easier to separate from the reaction solution. Moreover, the polymerization causes the inactivation of the reactive phenol or quinone functional groups to prevent them from reacting with living cells for instance. However, when applied in their free form for the treatment of contaminants present in solution, enzymes face major operational shortcomings such as rapid denaturation, lack of reusability, and requirement of large quantities which will impact the overall cost of their use (Cabana et al. 2007b; Sheldon. 2007). Insolublization of enzymes as combined or simple crosslinked enzyme aggregates (combi-/CLEA) is of one the most effective techniques used to circumvent these drawbacks (Sheldon. 2007; Taboada-Puig et al. 2011). The technique consists of covalently binding the free enzymes between themselves with the aid of a crosslinking reagent (glutaraldehyde in most cases) to yield a stable and reusable biocatalyst.

To our knowledge, no previous work has been published on the removal of acetaminophen from real wastewaters using combi-CLEA of laccase and tyrosinase.

However, the removal of other non-pharmaceutical phenolics, (bisphenol A and nonylphenol) in solution using combi-CLEA of versatile peroxidase and glucose oxidase was reported recently (Taboada-Puig et al. 2011). The objective of this work was to, first, insolubilize fungal laccase (active in acidic pH) and mushroom tyrosinase (active in neutral to alkaline pH) as combi-CLEA to form a stable biocatalyst with an expanded oxidative pH spectrum. A coupling of chitosan with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC) was used as crosslinking agent due to the remarkable affinity of chitosan to proteins and to its biodegradability to innocuous products (Krajewska. 2004). The second objective was to characterize the combi-CLEA (pH, temperature, kinetics, stability). The final objective was to apply the biocatalyst to the transformation of acetaminophen from both municipal and hospital wastewaters followed by the identification of the transformation products (metabolites).

XII.2. Materials and Methods

XII.2.1. Materials

Trametes versicolor laccase (TvL) with a specific activity of 22.4 U/mg-solid, mushroom tyrosinase (Tyr) with a specific activity of 3610 U/mg-solid, chitosan from crab shells (65% deacetylation and molecular weight of 750 kDa), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3,4-dihydroxy-*L*-phenylalanine (L-DOPA) and acetaminophen (≥99.0% purity) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA) . All other chemicals were of analytical grade.

XII.2.2. Preparation of Combi-CLEA and its Yield Estimation

Combination of 0.3 U of free TvL and 0.7 U of free Tyr was dissolved in deionized water to a total activity of 1 U/mL according to a proven procedure of previous work in our laboratory (Arsenault et al. 2011; Ba et al. 2012). Aggregation was performed by precipitating the free enzymes in a solution of ammonium sulfate (500 g/L) for 1 h followed by addition of solutions of chitosan (1 g/L) and EDAC (100 mM). Phosphate buffer at 0.1 M and pH 5 completed the total solution to the final volume of 100 mL. Solution was then stored undisturbed at 4°C for 48 h to allow complete crosslinking

reaction followed by combi-CLEA extraction by centrifugation at 10 000g for 5 min and 4°C. The aliquots were then washed three times with deionized water and subsequently used for experimentation.

The yield of the combi-CLEA was estimated based on the activity balance of the amounts of free laccase and tyrosinase used and combi-CLEA produced according to the following equation:

$$Y\ (\%) = \frac{Total\ Unit\ of\ combi-CLEA\ produced}{Total\ Unit\ of\ free\ TvL\ and\ Tyr\ used} \times 100$$

XII.2.3. Enzyme activity assays

The activity measurements of free enzymes and combi-CLEA were conducted by measuring the initial reaction rate of substrate oxidation with a double-beam UV–Vis spectrophotometer (SpectraMax Plus 384, Molecular Devices Corp., Sunnyvale, CA). Laccase activity was determined by monitoring the oxidation of 1 mM ABTS to its cation radical ($ABTS^{\cdot+}$) at 420 nm (ε = 36000 $M^{-1}cm^{-1}$) (Bourbonnais and Paice. 1990). Tyrosinase activity was determined by monitoring the oxidation of 5 mM *L*-DOPA to dopachrome at 475 nm (ε = 3600 $M^{-1}cm^{-1}$) (Edwards et al. 1999). Both substrates were mixed with 0.1 M citrate-phosphate pH 3-6, 0.1 M sodium-phosphate pH 7-8 and 0.1 M boric acid-hydroxide pH 9 buffers for activities measurement. One unit (U) of activity is defined as the amount of enzyme (TvL or Tyr) that catalyzes the conversion of substrate (ABTS or *L*-DOPA) into colored products (green for $ABTS^{\cdot+}$ or orange for dopachrome) causing an increase in absorbance at a rate of 1 µmol per min.

Throughout the paper, combi-CLEA-Lac refers to the combi-CLEA due to its laccase content and combi-CLEA-Tyr refers to the combi-CLEA due to its tyrosinase content.

XII.2.4. Determination of pH and temperature optima

To determine the optimum pH of the free and insolubilized enzymes, their activities were measured in the pH range 3–9 at 20 °C following the activity assay described above. The effect of temperature on the biocatalysts was determined by measuring their activities in the temperature range 5-60 °C at pH 4 or 7 using ABTS or *L*-DOPA, respectively. The results for optimum temperature and pH were given in relative form with the highest value being 100% activity.

XII.2.5. Determination of kinetic parameters

Kinetic assays for the free enzymes and combi-CLEA were carried out at 20 °C and pH optimum for each biocatalyst by measuring the appearance of the product in the reaction medium (substrate, buffer, and biocatalyst). The concentrations of substrates were varied in the range 0.05-10 mM in pH 7 buffer for *L*-DOPA and in the range 0.05-1 mM in pH 4 buffer for ABTS. The kinetic parameters were determined by nonlinear regression of reaction rate *vs* substrate concentrations according to the Michaelis-Menten relationships using SigmaPlot 12 (**Systat Software Inc.,** San Jose, CA).

XII.2.6. Stability of combi-CLEA against denaturation

The thermal stability study was carried out by incubating samples of the biocatalysts in a thermostatic bath (Isotemp 2100 water bath, Fisher Scientific) and their activities measured periodically following the activity assay. The results were expressed in relative form with the initial measurement assigned 100% activity.

The stability of free enzymes and combi-CLEA against denaturation were tested in the presence of deactivating reagents $CaCl_2$, $CuCl_2$, $ZnCl_2$, NaN_3, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS) and hydrophilic organic solvents acetone, acetonitrile, dimethyl sulfoxide (DMSO), and methanol. All these chemicals are known to either denature proteins or inhibit metalloenzymes by binding the copper ions at their active site (Cabana et al. 2007c; Selinheimo et al. 2007; Xu et al. 2011). Activity of 250 U/L of each biocatalyst was incubated for 1 h in solution of every denaturant and deionized water (used as control) and assayed at 20 °C and

optimum pH of the biocatalyst for residual activity. The results for stability were given in residual form with the value in deionized water being 100% activity.

XII.2.7. *Elimination of acetaminophen in wastewater*

Two samples of municipal wastewater (MWW1 and MWW2, pH 7.3) taken at different periods from the influent of a STP in Magog (QC, Canada) and sample of hospital wastewater (HWW, pH 7.7) from the effluent of the University of Sherbrooke Hospital Center in Sherbrooke (QC, Canada) were analyzed for the presence of acetaminophen. Milli-Q ultrapure water (mLQW) (18.2 MΩ·cm at 25 °C, TOC < 10 ppb) from our laboratory was also used for comparison purpose with the real wastewaters. Transformation of acetaminophen from the samples was performed in batch mode at 20 °C in 125 mL Erlenmeyer flask with orbital shaking at 150 rpm. Both mLQW and MWWs were spiked with 100 µg/L of acetaminophen (as this value is within the concentration range of pharmaceuticals found in STP) before application of biocatalysts (combi-CLEA, free TvL or Tyr) to a final concentration of 50 U/L. The HWW samples were not spiked with acetaminophen and biocatalysts were applied to a final concentration of 200 U/L. The pH of MWW2 was adjusted to 4 in some cases to favor the activity of laccase in the transformation process. In all other instances no adjustment was made to the pH of the wastewater samples.

XII.2.8. Extraction of acetaminophen

At different times, 10 mL of sample were taken and the pH of the solution was adjusted to below 2 with 5% (v/v) formic acid before addition of 4 g of $MgSO_4$ and 1 g of NaCl and thoroughly mixed. 10 mL of ethyl acetate was then added to the mixture and vortexed for 2 min before centrifugation at 3500 rpm and 4 ºC for 10 min to separate the organic phase containing acetaminophen and the water phase. Organic phase was then gently transferred with no particle or drop of water to glass vial and subsequently evaporated to dryness under a gentle stream of nitrogen prior to resuspension in 1 mL of a solution of water-methanol 50-50% (v/v) and 0.1% (v/v) formic acid. The mixture was then sonicated for 5 min and filtered through 0.22 µm PTFE membrane filters before transfer into UPLC vial for quantitative analysis.

XII.2.9. UPLC-MS/MS analysis of acetaminophen

Analyses of acetaminophen were performed on an Acquity UPLC XEVO TQ mass spectrometer (Waters Corporation, Milford, MA). An Acquity UPLC HSS-T3 column (100 mm x 2.1 mm, 1.8 µm) (Waters Corporation, Milford, MA) was used. The solvent flow rate was set to 0.40 mL/min and the column temperature was kept at 35 ºC. The sample volume injected was 5 µL. Mobile phase was 0.20% formic acid/water (A) and 0.20% formic acid/methanol-acetonitrile (72-25 v/v) (B) (LC/MS grade). The adopted elution gradient started with 5% of eluent B, increasing to 90% in 8 min and then back to initial conditions in 4 min. The mass spectrometry analysis was performed using a positive electrospray ionization (ESI+) source in Multi-Reaction-Monitoring mode. The optimized parameters were obtained by direct infusion of the analytical standard solution at 10 µg/mL as follows: desolvation gas (nitrogen), 700 L/h; cone gas (nitrogen), 50 L/h; collision gas (nitrogen), 0.22 mL/min; capillary voltage 2.5 kV; source temperature, 150 ºC and desolvation temperature 550 ºC. Two daughter traces (transitions) were used. The most abundant transition, *m/z* = 110.4, was used for quantification, whereas the second most abundant, *m/z* = 92.5, was used for confirmation. A cone voltage of 25.0 V was used for both transitions, while a collision energy of 20.0 and 15.0 V was used for *m/z* = 92.5 and *m/z* = 110.4, respectively. The concentration of acetaminophen was determined by comparing the peak areas obtained with those of standard solutions of known concentrations. Afterward, the possible transformation products of acetaminophen resulting from the enzymatic transformation by combi-CLEA were monitored.

XII.2.10. UPLC analysis of laccase and tyrosinase-oxidation products of acetaminophen.

In order to identify the possible transformation products of acetaminophen resulting from its oxidation reactions with the combi-CLEA of laccase and tyrosinase, experiments were carried out in batch mode by reacting 10 mL of a mixture of 5 mg/L of acetaminophen with 1000 U/L of each of the two enzymes separately and in combination. These high final concentrations of acetaminophen and enzymes activities aimed at ensuring rapid transformation of the drug and subsequent generation of its by-products at significant

quantity for easy detection (Cabana et al. 2007a). All solutions of enzymes and acetaminophen were prepared with Milli-Q ultrapure water. The enzymatic reactions were set at room temperature (20 °C) and pH 7. Duplicate samples of reacting solutions were monitored every 30 min from time 0 (immediately after mixture) to 6 hours. Furthermore, blank samples of acetaminophen, laccase, and tyrosinase were also analyzed separately as controls.

Samples were analyzed by UPLC/MS method. A mass spectrometer (MS) was first run in scan mode between m/z ratios of 100 and 700. Afterward, MS chromatography was selectively acquired on the ions of interest. The MS was operated at ESI+ under the following conditions: capillary voltage, 2.5 kV; cone voltage, 30 V; desolvation temperature, 550 °C; gas desolvation (N_2), 800 L/h; cone gas, 50 L/h; and collision gas (Ar), 0.22 mL/h. Full description of the methodology regarding the UPLC-MS analysis of the transformation products is provided in the Supplementary data Information (SI).

XII.3. Results and discussion

XII.3.1. Insolubilization of Tyr and TvL and yield of combi-CLEA

Laccase and tyrosinase were successfully insolubilized as combnation of crosslinked enzyme aggregates. Results in Table VIII.1 indicate specific activities of 12.3 U/g and 167.4 U/g of TvL and Tyr present in the combi-CLEA, respectively. These specific activities of the two enzymes correspond to activity yields of 10% and 61.8%, respectively, estimated from activity balance and subtracting the loss of activity in the supernatant. Although relatively low, these results of specific activities and yields are in the same range of results found elsewhere for CLEA with various enzymes using the most common crosslinker, glutaraldehyde (Cabana et al. 2007c; Shah et al. 2006; Wilson et al. 2006). Therefore, the use of the hydrophilic biopolymer, chitosan, did not reduce significantly the crosslinking efficiency in consistence with results found by Arsenault et al. (Arsenault et al. 2011) who used chitosan and EDAC as crosslinker to produce *Coriolopsis polyzona* laccase-based CLEA.

Table VIII.1. Specific activity and activity yield of combi-CLEA

Biocatalyst	Substrate	Specific activity (U/g)	Yield (%)
Combi-CLEA-Lac	ABTS	12.3	10.6
Combi-CLEA-Tyr	L-DOPA	167.4	61.8

XII.3.2. Effect of pH on activities of biocatalysts

The activity profiles as function of pH of the biocatalysts are given in Figure VIII.1. The pH optima for free TvL and Tyr are found at 4 and 7, respectively while their combi-CLEA counterparts are found at pH 5 and pH 8, respectively. The relative activity of combi-CLEA-Lac decreased from nearly 80% at pH 3 to 50% at pH 4 then peaked to 100% at pH 5 before steeply decreasing to 20% and less in the pH range 6-7. Such ups and downs trends of pH profile for insolubilized TvL using chitosan as crosslinker have been reported and are believed to be associated with a change in the microenvironment surrounding the laccase as a result of its aggregation and conjugation with the chitosan (Hassani et al. 2013). The relative activity profile of combi-CLEA-Tyr increased steadily from 10% at pH 4 to its optimum 100% at pH 8 followed by a steep decrease to 11% at pH 9. Overall, the pH profiles of the combi-CLEA (combi-CLEA-Lac and combi-CLEA-Tyr) are broader than those of the individual free enzymes as a consequence of the association of the two enzymes and the shift of their pH optima toward more alkaline side. Such shifts in enzymes pH after immobilization/insolubilization have been widely reported and ascribed to the change in both the conformation of the enzyme induced by the covalent bonding and to the microenvironment upon immobilization/insolubilization (Hassani et al. 2013; Kalkan et al. 2012; Sangeetha and Abraham. 2008). The insolubilized enzymes exhibited high relative activity between their individual enzymes pH optima. This latter aspect of the result is very important for the application of the combi-CLEA given that most wastewaters and natural waters have their typical pH values within the range 5-8.

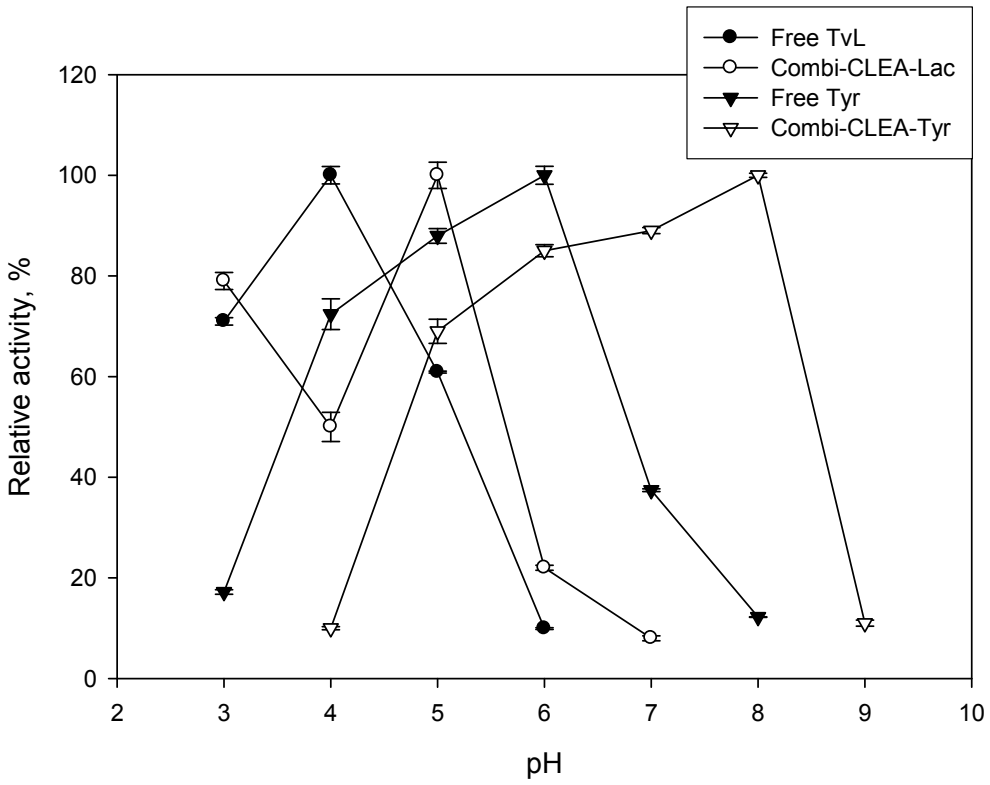
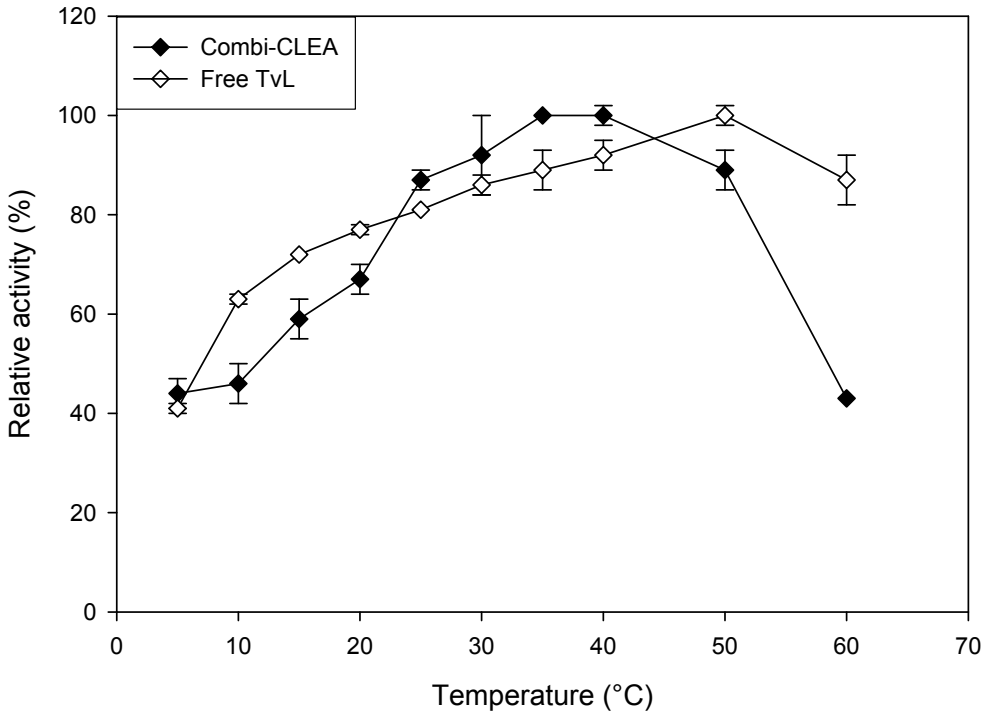


Figure VIII.1. Activity profile as function of pH for free and insoluble TvL, and free and insoluble Tyr at 20 °C. Each datum point represents the mean of triplicate values ± std dev**.**

XII.3.3. Effect of temperature on activities of biocatalysts

The results for temperature effect on the biocatalysts are provided in FigureVIII.2. As given in Figure VIII.2a, optimum temperature was found at 40 °C for combi-CLEA-Lac and 50 °C for free TvL. Both biocatalysts activities increased proportionally with temperature to reach their optimum before steeply decreasing to 60 °C in the case of the combi-CLEA-Lac while the decrease for the free TvL was less pronounced. This trend is similar to that found by D'Annibale et al. (D'Annibale et al. 1999) for free laccase and its counterpart immobilized on chitosan with the exception that the later displayed higher optimum temperature than the former. On the other hand, Figure VIII.2b shows optimum temperature at 20 °C for combi-CLEA-Tyr which is considerably lower than the 40 °C found for free Tyr. Yet, the shift of relative activity for immobilized tyrosinase to lower temperature (30°C) *versus* its free counterpart (40 °C) has been reported (Labus et al. 2011). Similarly to both free and insoluble laccases,

relative activity of free Tyr progressively increased with temperature to its maximum before decreasing. In contrast, the activity of combi-CLEA-Tyr slightly increased between 5 and 20 °C followed by a steady decrease as temperature increases before stabilizing between 40 and 60 °C. Overall, the insolubilization had positive temperature effect on both TvL and Tyr considering the high relative activities (40% to higher) found for combi-CLEA in the temperature range 5-30 °C which corresponds to typical seasonal wastewater temperature range.



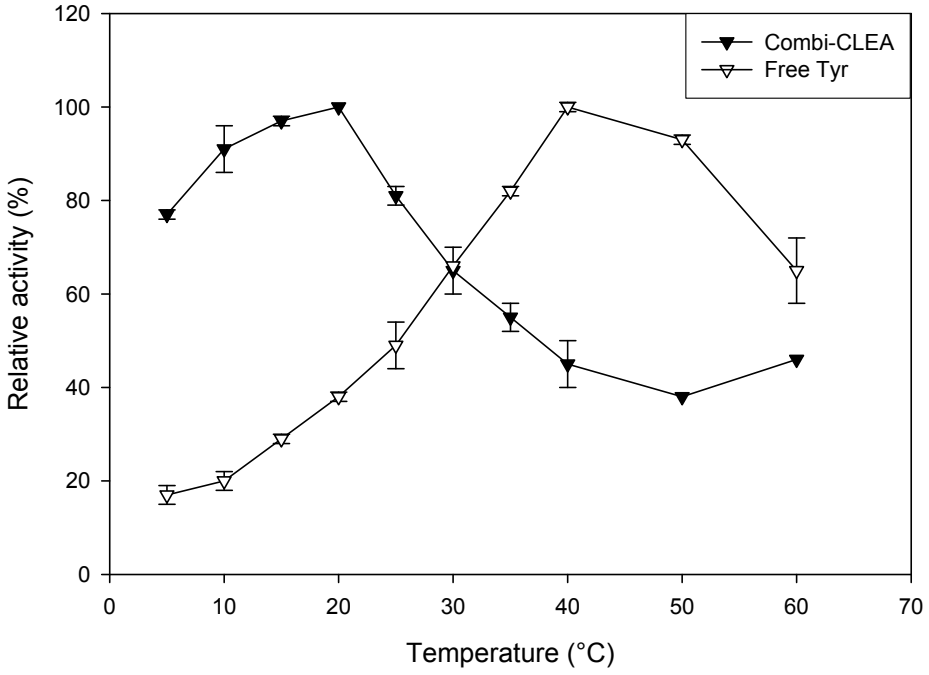


Figure VIII.2. Activity profile as function of temperature for free and insoluble TvL in 0.1 citrate-phosphate buffer pH 4 (a) and for free and insoluble Tyr in sodium-phosphate buffer pH 7 (b). Each datum point represents the mean of triplicate values ± std dev.

XII.3.4. Michaelis-Menten kinetic parameters of biocatalysts

Calculations of Michaelis-Menten constants for combi-CLEA relatively to the free enzymes are provided in Table VIII.2. No difference was found between the K_M values of free Tyr and combi-CLEA-Tyr (1.556 ± 0.037 mM and 1.558 ± 0.126 mM, respectively). This result indicates that diffusion limitation of substrate to the combi-CLEA-Tyr did not occur as a result of insolubilization unlike other immobilization techniques where conformation change of the immobilized enzyme induced steric hindrance or alterations of the enzyme active site resulting in an increase of the K_M value (Nicolucci et al. 2010). In comparison with free TvL, a higher affinity of combi-CLEA-Lac was found for ABTS as indicated by a decrease in the K_M value (0.052 ± 0.008 mM and 0.036 ± 0.002 mM, respectively). It may imply that interaction between enzyme and substrate may have been strengthened by a suitable orientation of the

enzyme active site toward the substrate (Sangeetha and Abraham. 2008). Lower K_M values for combi-CLEA/ CLEA compared to their free enzyme counterparts had been reported (Aytar and Bakir. 2008; Dalal et al. 2007; Taboada-Puig et al. 2011). The turnover numbers K_{cat} for the biocatalysts showed better results for both combi-CLEA-Tyr and combi-CLEA-Lac compared to their free enzyme counterparts (1.161 ± 0.078 U/L.µM *vs* 0.869 ± 0.075 U/L.µM for tyrosinase and 9.514 ± 0.929 U/L.µM *vs* 4.965 ± 1.117 U/L.µM for laccase). Likewise, the catalytic efficiencies K_{cat}/K_M were higher for the combi-CLEA than the free enzymes (0.075 U/L.µM^2 *vs* 0.056 U/L.µM^2 for tyrosinase and 0.268 U/L.µM^2 *vs* 0.099 U/L.µM^2 and for laccase). These results are in agreement with those reported in the literature for catalytic enhancement of insolubilized laccase through higher values of K_{cat} and K_{cat}/K_M (Aytar and Bakir. 2008; Cabana et al. 2007c; Hassani et al. 2013).

Table VIII.2. Michaelis-Menten kinetic parameters of biocatalyst for the oxidation of L-DOPA (free Tyr and combi-CLEA) at pH 7, 20 °C and ABTS (free TvL and combi-CLEA) at pH 4, 20 °C[a]

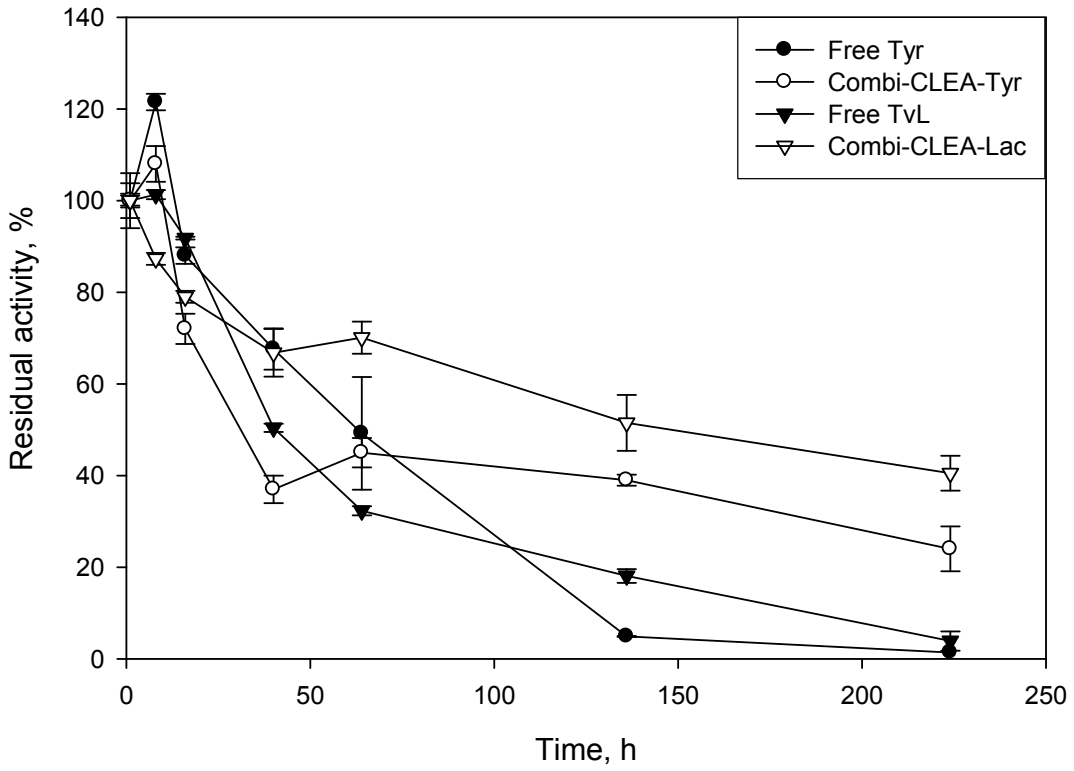
Biocatalyst	K_M mM	K_{cat} U/L.µM	K_{cat}/K_M U/L.µM^2	R^2
Free Tyr	1.556 ± 0.037	0.869 ± 0.075	0.056	0.968
Combi-CLEA-Tyr	1.558 ± 0.126	1.161 ± 0.078	0.075	0.998
Free TvL	0.052 ± 0.008	4.965 ± 1.117	0.099	0.839
Combi-CLEA-Lac	0.036 ± 0.002	9.514 ± 0.929	0.268	0.899

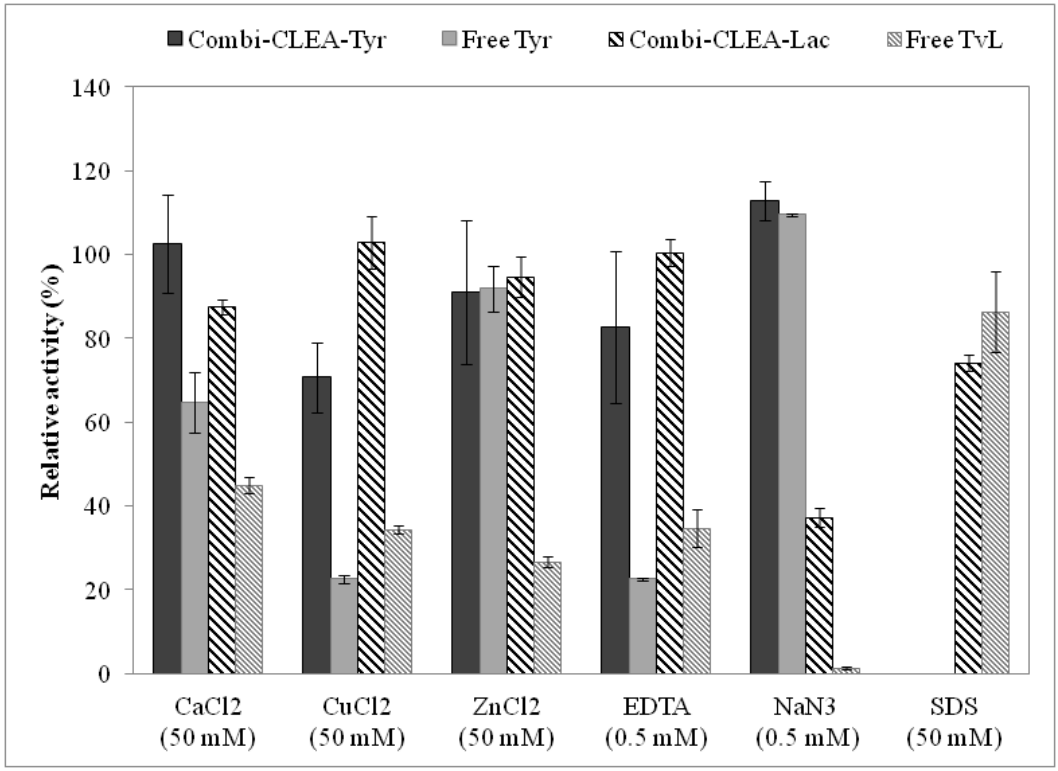
[a]Values represent the mean of triplicate measurements ± standard deviation

XII.3.5. Thermal and chemical stability of biocatalysts

The results of thermal and chemical stabilities of the biocatalyst are presented in Figure VIII.3. Results for thermal stability (Figure VIII.3a) show an initial surge of the biocatalysts residual activities after 8 h of incubation, except for combi-CLEA-Lac, followed by a steady decrease of the residual activities. Both insolubilized enzymes showed better thermal stability than their free enzyme counterparts over the course of the incubation period. The residual activity of free TvL was less than 5% at the end

of incubation period while that of the combi-CLEA-Lac remained significantly high at slightly more than 40%. Likewise, at the same incubation period while the free Tyr exhibited nearly no residual activity, that of the combi-CLEA-Tyr was about 25%. The insolubilization had a positive effect on the enzymes with regard to thermal stability as reported in several other studies (Arsenault et al. 2011; Ba et al. 2012; Dalal et al. 2007). Enhancement of thermal stability of enzymes through immobilization/insolubilization has been associated with the rigidification of the tertiary structure of the enzyme molecules due to multipoint attachment by covalent bonds between enzyme molecules to reduce its conformational change inducing inactivation (Mateo et al. 2007).





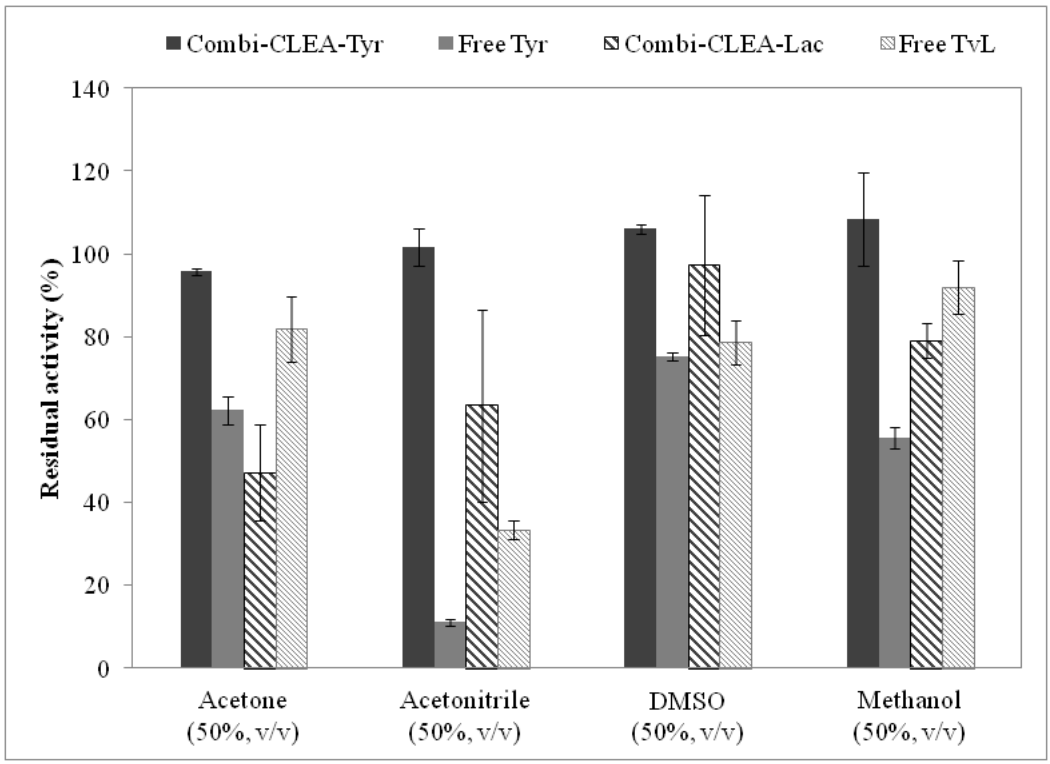


Figure VIII.3. Effect of temperature (a), denaturing ionic compounds (b) and solvents (c) to free and insoluble TvL and Tyr at their optimum pHs and 20 °C. Each datum bar represents the mean of triplicate values ± std dev.

Figure VIII.3b provides residual activities of free enzymes and combi-CLEA exposed to high concentrations (50 mM) of halide salts ($CaCl_2$, $CuCl_2$, $ZnCl_2$) and anionic surfactant (SDS), significant concentrations of sodium azide (0.5 mM NaN_3), and chelator (0.5 mM EDTA). In all cases, with exception for SDS, combi-CLEA withstood denaturation better than the free enzymes with some minor discrepancies from one compound to another. With the three halide salts, combi-CLEA exhibited residual activity of 80-103% except with $CuCl_2$ at pH 7 where that value still remained high at 71%. In contrast, the residual activities for free TvL and Tyr varied between 23% and 65% in all the salts excluding free Tyr with $ZnCl_2$ where it unexpectedly peaked at 92%. The results for EDTA showed 83 and 100% residual activities for combi-CLEA-Tyr and combi-CLEA-Lac, respectively compared to only 23% and 35% for free Tyr and TvL, respectively. These results for combi-CLEA are in agreement with results for tyrosinase CLEA and laccase CLEA found to retain more than 80% residual activity in both low (150 μM) and high (150 mM) concentrations of EDTA (Cabana et al. 2007c; Xu et al. 2011). The results for NaN_3 contrast small activation of free and insolubilized tyrosinase (109 and 113% residual activity, respectively) with considerable inhibition of insolubilized laccase (37% residual activity) to nearly complete inhibition for free TvL (only 1% residual activity). The results for Tyr are somewhat consistent with results reported (Xu et al. 2011) where both free and Tyr CLEA kept about 70% residual activity in 0.5 mM NaN_3; but in contradiction with result where free Tyr was completely inactivated though in much higher concentration of 10 mM NaN_3 (Selinheimo et al. 2007). Likewise, the results for free and insolubilized laccase are in accordance with those reported elsewhere (Cabana et al. 2007b) where free laccase was completely inactivated whereas the CLEA had residual activity of 40-50% in 150 μM NaN_3. Results with SDS showed complete inactivation of both free and insolubilized tyrosinase most probably due to the high concentration of the inhibitor. Surprisingly, both free and insolublized laccase exhibited high resistance to inhibition (86% and 74% residual activity, respectively) by SDS despite its high concentration. Indeed, it has been demonstrated that the enzyme active site is not affected by low concentration (≤1 mM) of SDS and would rather undergo activation (Gandía-Herrero et al. 2005) up to

an optimum micellar concentration of SDS after which only the biocatalyst is inactivated as the concentration increases (Moore and Flurkey. 1990).

The effect of hydrophilic miscible organic solvents known to affect enzymes is presented in Figure VIII.3c. Combi-CLEA-Tyr appears to greatly resist inhibition against all four solvents with marginal activation with acetonitrile, DMSO, and methanol whereas the free Tyr was meaningfully inactivated (25 to 45 activity reduction) with as much as 89% activity reduction in the case of acetonitrile. The results for free and insoluble laccase exposition to the solvents do not follow the consistent trend seen for tyrosinase. The residual activities for the free laccase were lower than those for their combi-CLEA counterpart for acetonitrile (33% *vs* 63%) and DMSO (79% *vs* 97%). Conversely, residual activities for the free laccase were higher than those for their combi-CLEA counterpart in the presence of acetone (82% *vs* 47%) and methanol (92% *vs* 70%). In all cases the combi-CLEA residual activities were significantly higher despite the high concentrations (50% v/v) of the solvents compared to results reported under similar conditions (Xu et al. 2011).

The combi-CLEA displayed considerable denaturation-resistance to temperature and various inhibitors compared to the free enzymes, demonstrating the stabilization effect of the insolubilization. It could be due to some sort of conformation strengthening and access limitation of denaturing molecules to the biocatalyst active site as a consequence of the rigidification or to steric hindrance of the enzymes-chitosan molecular structures composite resulting from aggregation and crosslinking. Although it is unlikely to find these denaturants at such high concentrations in wastewaters, it was still relevant in our viewpoint to have an insight of the behavior of the combi-CLEA when exposed to harsh aqueous conditions for application purposes.

XII.3.6. Acetaminophen detection in wastewater and its transformation with biocatalysts

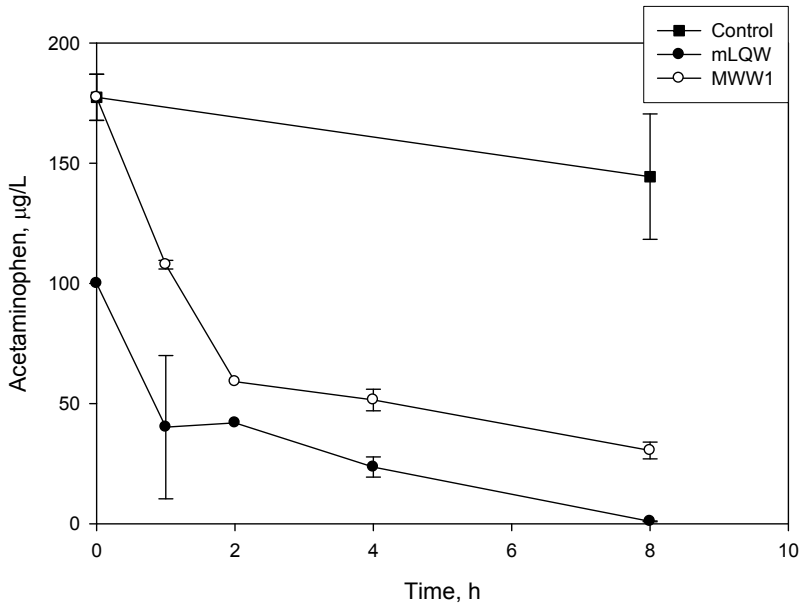
Acetaminophen was detected in both municipal and hospital wastewaters and was significantly transformed by the biocatalysts as shown in Figure VIII.4 and summarized in Table VIII.3.

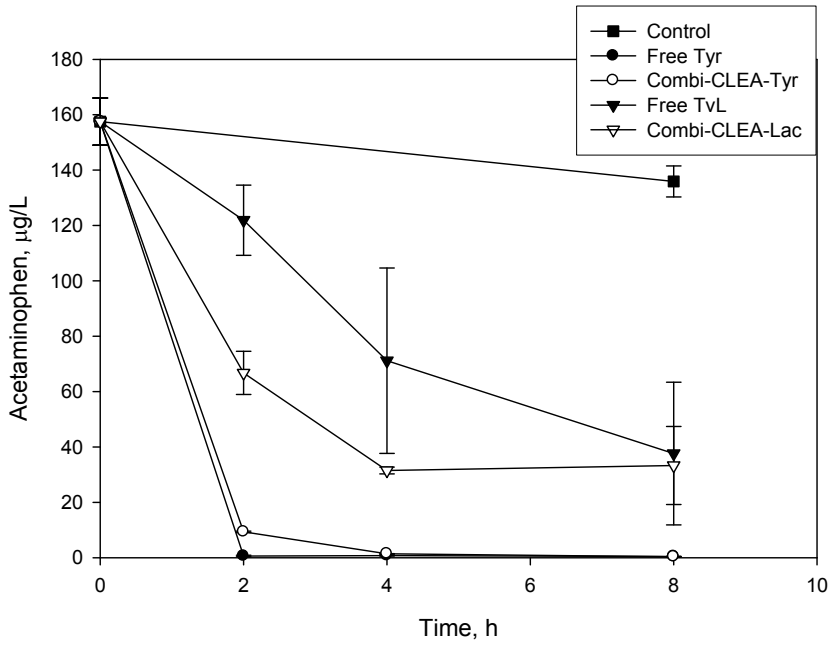
Table VIII.3. Transformation of acetaminophen from water matrices after 8 h treatment with biocatalysts at 20 °C in batch mode

Water matrix	pH	Acetaminophen concentration (µg/L)	Biocatalyst applied	Transformation (%)
mLQW	7.0	100	Combi-CLEA	98.7
MWW1	7.3	177.4	Combi-CLEA	82.8
MWW2	7.3 - 4.0 -	157.5	Free Tyr Combi-CLEA-Tyr Free TvL Combi-CLEA-Lac	99.7 99.8 76.2 78.9
HWW	7.7	90.2	Free Tyr Free TvL Combi-CLEA	98.7 98.4 92.9

Results from the analyses of both mLQW and MWW1 spiked with 100 µg/L of acetaminophen are provided in Figure VIII.4a. After spiking, concentration of acetaminophen in the MWW1 (177.4 µg/L) shows that the wastewater initially contains 77.4 µg/L. The application of combi-CLEA reduced acetaminophen content to 0.99 µg/L and 30.5 µg/L after 8 h from mLQW and MWW1, respectively, corresponding roughly to about 99% and 80% transformations. However, results from the control test also show reduction of acetaminophen content in the MWW1 from 177.4 µg/L to 144.4 µg/L more likely due to other phenomena (cross-coupling reactions, microbial biodegradation, photocatalysis, etc.) rather than the biocatalyst effect only. Nonetheless, the main reduction of acetaminophen is attributed to the combi-CLEA

which demonstrates, as hoped, the efficacy of the biocatalyst for transforming acetaminophen from wastewater.





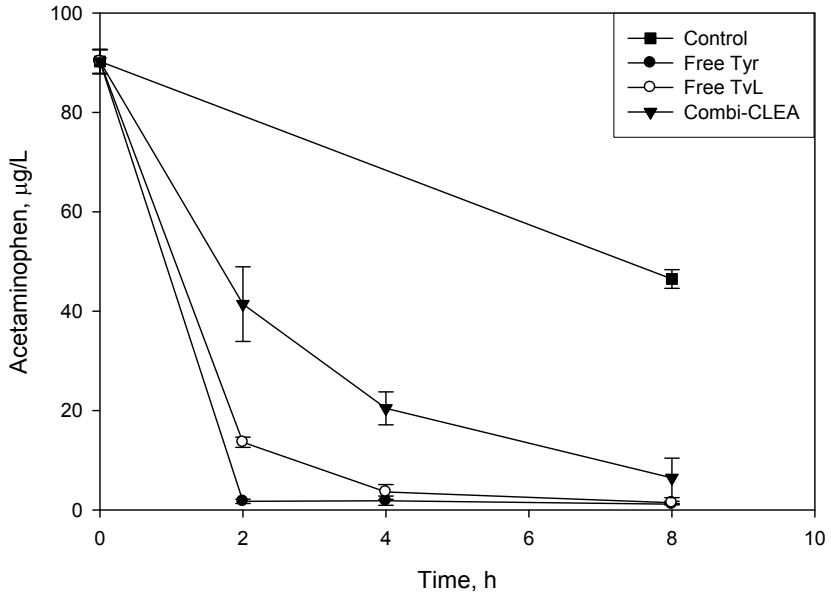


Figure VIII.4. Removals of acetaminophen from mLQW and MWW1 (a); MWW2 (b); and HWW (c) with free and insoluble TvL and free and insoluble Tyr in batch mode and agitation at 20 °C. Each datum point represents the mean of duplicate values ± std dev.

The application of the free and insolubilized biocatalysts to samples of the MWW2 in which the spike concentration was kept at 100 µg/L showed 57.5 µg/L of acetaminophen content in the wastewater sample (Figure VIII.4b). The difference between this value and the previous result in MWW1 is likely because the wastewater samples were taken at different periods of the same summer, indicating short term variability of contamination. These concentrations of acetaminophen detected in the municipal wastewaters (57.5 µg/L and 77.4 µg/L) are similar to the concentrations of this contaminant (25-36 µg/L) found from several STP throughout Quebec (Robert et al. 2011) using LC-MS/MS. The effect of free Tyr and combi-CLEA showed nearly 100% reductions for both biocatalysts after 2 h and 4 h of application and agitation with the MWW2, respectively. The application effect of free TvL and combi-CLEA to the wastewater with pH adjusted from 7.3 down to 4 shows about 80% reductions of acetaminophen similar to the transformation efficiency seen earlier for MWW1 in the control test accounting for about 14% of the transformation not pertaining to the biocatalysts.

The final experiment carried out with HWW in which neither spiking of acetaminophen nor pH adjustment was performed showed that the wastewater contained 90.2 µg/L of acetaminophen. Application of biocatalysts exhibited transformation rates of acetaminophen of about 93% for combi-CLEA and 99% for free Tyr and TvL (Figure VIII.4c). The control test with no biocatalyst exhibited considerable reduction (from 90.2 µg/L to 46.5 µg/L) of acetaminophen from the HWW. It could be assumed that acetaminophen was transformed as a result of its cross-coupling with dissolved natural organic matter (NOM) present in the HWW as demonstrated elsewhere (Lu and Huang. 2009) or by degradation of biomass present in the samples.

In all figures, the transformation rates of acetaminophen were rapid in the first 2 to 4 h of application followed by a markedly lower rate probably due to accumulation of the initial aromatic by-products generated in the mixture leading to a partial inhibition of the enzymatic activity (D'Annibale et al. 1999). After 8 h of treatment, the removal efficiency of acetaminophen with combi-CLEA from MWW2 when the pH was kept unchanged at 7.3 (favoring tyrosinase activity) was 99.8%. This result is higher than that found to be 78.9% when the pH was adjusted to 4 (favoring laccase activity) and all other conditions being identical. This is somewhat expected given that the specific activity of Tyr in the combi-CLEA is higher than that of TvL (i.e. 167.4 U/g *vs* 12.3 U/g). Also, the fact that there was no meaningful difference between the transformation efficiencies between the free enzymes and their combi-CLEA counterparts is supportive of the results from the kinetic study in which insolublization of the enzymes did not appear to induce diffusion limitation of substrate to the combi-CLEA active site. In the HWW, although the pH was not adjusted the free enzymes and the combi-CLEA exhibited nearly equal transformation rates (93-99%) of acetaminophen. However, the high transformation rate of 98.4% by free TvL at pH 7.7 is surprising but could in part be due to the high transformation of acetaminophen seen in the control test. Indeed, it has been proven in other studies that the transformation of aromatic and phenolic pollutants by laccase could be promoted by natural mediators such as NOM, colloids, humic substances, etc. present in the reaction mixture (Cañas et al. 2007; Feng et al. 2013).

XII.3.7. Transformation products of acetaminophen by laccase and tyrosinase

The ESI+ MS spectra of acetaminophen transformation products with laccase showed m/z values of 152, 301, 450, and 599 corresponding to molecular masses of the residual parent compound and its dimer, trimer and tetramer, respectively. These oligomers are identical to metabolites found in laccase-catalyzed reaction of acetaminophen in a previous study (Lu et al. 2009).

The tyrosinase-catalyzed reaction of acetaminophen provided m/z values of 152 and 168 for the residual parent compound and its metabolite 3-hydroxyacetaminophen, respectively. In another study of tyrosinase-mediated oxidative coupling of acetaminophen, the catechol (3-hydroxyacetaminophen) was detected but only as intermediate to the formation of the final metabolite, 4-acetamido-*o*-benzoquinone (MW 165), which was not detected in our study (Valero et al. 2002). However, in agreement with our result, several other studies have proved 3-hydroxyacetaminophen to be a final metabolite in microsomal-mediated oxidation of acetaminophen rather than a common intermediate (Forte et al. 1984; Hinson et al. 1980). The blank solutions, as anticipated, did not contain any of the metabolites found above.

The mixture of both laccase and tyrosinase reactions with acetaminophen showed an initial presence of both the dimer of acetaminophen and its metabolite 3-hydroxyacetaminophen found in the separate oxidative reactions of laccase and tyrosinase with the drug, respectively. Yet, as the reaction proceeded, the 3-hydroxyacetaminophen disappeared from the reaction products unlike the dimer of acetaminophen. We suspect that additional catalytic reactions due to residual activity of either (or both) enzyme(s) or any of their respective metabolites may have further transformed the 3-hyroxyacetaminophen. In order to elucidate the numerous and more complex possible pathways of producible metabolites from such transformation further analytical investigation is needed.

XII.4. Conclusions

To our knowledge, for the first time laccase and tyrosinase are insolubilized as combination of crosslinked enzyme aggregates. The combi-CLEA exhibited high stability under harsh conditions of temperature and chemical denaturation. Application of the biocatalyst to the treatment of acetaminophen in real wastewaters showed high transformation of the drug. The results demonstrated the potential of the biocatalyst in the treatment of phenolic micropollutants in wastewaters known to be unsatisfactorily eliminated from conventional STP. More importantly, these findings also paved the way for the use of the combi-CLEA within bioreactor in a continuous treatment process due to the insoluble character of the biocatalyst.

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Chapter IX:

Degradation of pharmaceuticals in non-sterile urban wastewater by *Trametes versicolor* in a fluidized bed bioreactor.

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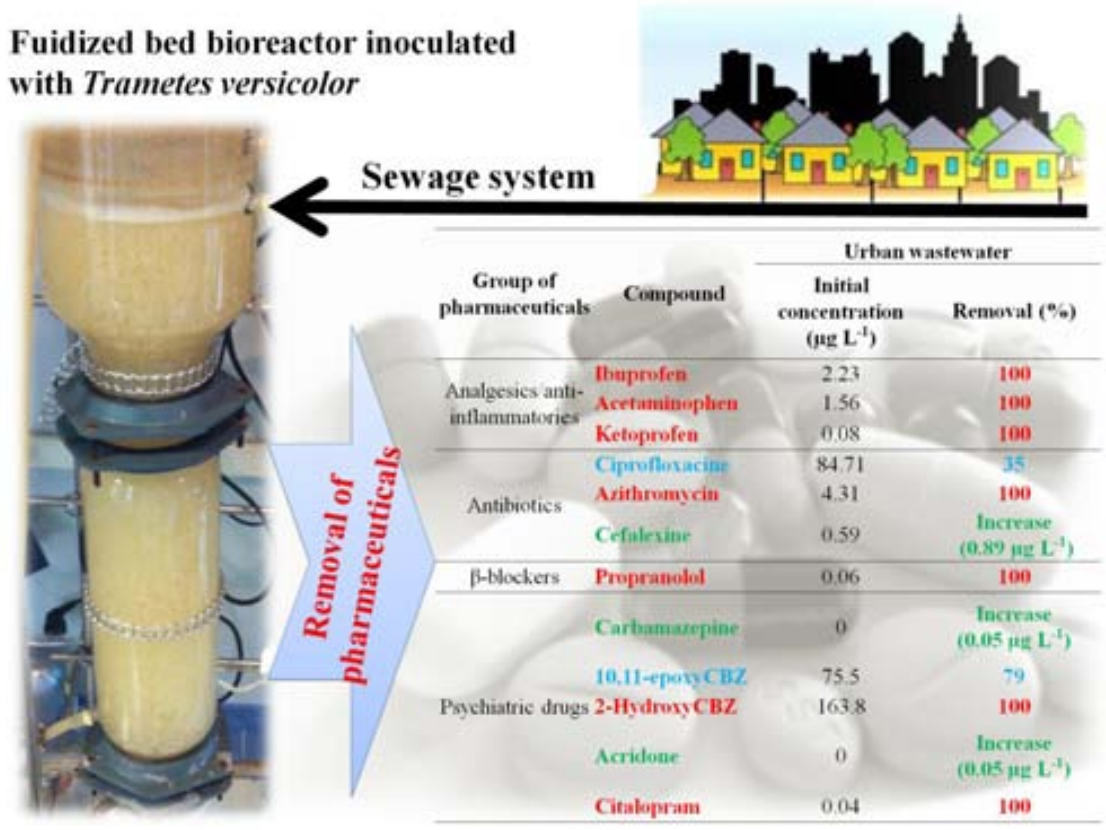

Group of pharmaceuticals	Compound	Urban wastewater: Initial concentration (μg L^{-1})	Urban wastewater: Removal (%)
Analgesics anti-inflammatories	Ibuprofen	2.23	100
	Acetaminophen	1.56	100
	Ketoprofen	0.08	100
Antibiotics	Ciprofloxacine	84.71	35
	Azithromycin	4.31	100
	Cefalexine	0.59	Increase (0.89 μg L^{-1})
β-blockers	Propranolol	0.06	100
Psychiatric drugs	Carbamazepine	0	Increase (0.05 μg L^{-1})
	10,11-epoxyCBZ	75.5	79
	2-HydroxyCBZ	163.8	100
	Acridone	0	Increase (0.05 μg L^{-1})
	Citalopram	0.04	100

Abstract

The constant detection of pharmaceuticals (PhACs) in the environment demonstrates the inefficiency of conventional wastewater treatment plants to completely remove them from wastewaters. So far, many studies have shown the feasibility of using white rot fungi to remove these contaminants. However, none of them have studied the degradation of several PhACs in real urban wastewater under non-sterile conditions, where mixtures of contaminants presents at low concentrations (ng L^{-1} to µg L^{-1}) as well as other active microorganisms are present. In this work, a batch fluidized bed bioreactor was used to study, for the first time, the degradation of PhACs present in urban wastewaters at their pre-existent concentrations under non-sterile conditions. Glucose and ammonium tartrate were continuously supplied as carbon and nitrogen source, respectively, and pH was maintained at 4.5. Complete removal of 7 out of the 10 initially detected PhACs was achieved in non-sterile treatment, while only 2 were partially removed and 1 of the PhACs analyzed increased its concentration. In addition, Microtox test showed an important reduction of toxicity in the wastewater after the treatment.

Keywords: Pharmaceuticals, real urban wastewater, *Trametes versicolor*, degradation, bioreactor.

IX.1. Introduction

Pharmaceutical active compounds (PhACs) are emerging contaminants that have received much attention from the scientific community during the last 10 years due to their wide presence in the environment (Caliman and Gavrilescu, 2009; Mompelat et al. 2009). It is well known that their main route of entrance into the environment is via ingestion, following excretion and direct disposal via wastewater treatment plants (WWTP) and manufacturing (Daughton and Ternes, 1999). Conventional activated sludge technologies applied in WWTPs are not designed to remove these micropollutants, which are present at low concentrations (between ng L^{-1} and μg L^{-1}), and therefore they can pass through unchanged or partially transformed to the receiving environmental compartments (Verlicchi et al., 2012; Ratola et al., 2012). The possible negative ecotoxicological effect provoked by the presence of PhACs in the environment is an issue of environmental concern. Consequently, several investigators have focused on the potential risk of the presence of PhACs in different water compartments, which were recently reviewed by Santos et al., 2010, de Jong et al., 2012 and Stuart et al., 2012. Although, chronic ecotoxicity data are scarce compared to acute studies, accumulative effects have been shown to damage some ecosystems (Daughton and Ternes, 1999).

Physico-chemical technologies such as advanced oxidation and photodegradation have been proposed as alternative approaches to achieve complete removal of some recalcitrant PhACs as carbamazepine and clofibric acid (Doll and Frimmel, 2004; Sirés et al., 2007; Esplugas et al., 2007). However, their main limitation is the formation of undesirable and sometimes toxic transformation products (Negrón-Encarnación and Arce, 2007). Alternatively, white rot fungi (WRF) have shown to be attractive candidates for designing effective bioremediation strategies of PhACs due to their unspecific oxidative enzymatic system, that includes lignin-modifiers enzymes, especially laccase and peroxidases, but also intracellular enzymatic complexes (e.g., cytochrome P450) (Asgher et al., 2008). Regarding the PhACs removal by WRF, fast degradation, from minutes to few days, has been demonstrated for β-blockers (Marco-Urrea et al., 2010a), some anti-inflammatory drugs (Marco-Urrea et al., 2010b, c and

d), antibiotics (Rodríguez-Rodríguez et al., 2012; Prieto et al., 2011) and psychiatric drugs (Jelić et al., 2012), while other anti-microbial agents, estrogens (Cajthalm et al., 2009) and iodinated contrast agents (Rode and Müller, 1998) were removed at slower rates (more than a week). The main reactions involved in the transformation of pharmaceuticals by WRF include hydroxylation, formylation, deamination and dehalogenation (Cruz-Morato et al., 2012; Harms et al., 2011). Mineralization has been barely demonstrated, only suggested for some anti-inflammatory drugs (diclofenac and ketoprofen) (Marco-Urrea et al., 2010b and c). Ecotoxicological assessment of the treated effluents has to be performed, since the transformation products formed from the target contaminants during treatment may exhibit higher toxicity than the parent compound.

To date, most of the published studies on removal of PhACs by WRF were carried out in sterilized synthetic liquid media under controlled conditions of pH and temperature, with absence of competitors or spiking PhACs at concentrations higher than those found in real wastewaters (mg L^{-1}). As far as we know, the only work attempting the elimination of PhACs in non-sterile conditions was reported by Zhang and Geiβen (2012). They observed 60 to 80 % of removal in the elimination of carbamazepine (spiked at 5 mg L^{-1}) in a bioreactor containing the WRF *Phanerochaete chrysosporium* immobilized in polyether foam and achieving a stable continuous operation during 100 days. However, degradation by WRF of PhAC mixtures at real concentrations in non-sterile wastewaters containing is still unproved.

The aim of this study is the use of a fluidized bed bioreactor inoculated with the WRF *Trametes versicolor* to degrade PhACs contained in urban wastewater at both sterilized and non-sterilized conditions. Previously to the batch reactor treatment at non-sterilized conditions, the requirements of nutrients in the real wastewater were studied.

IX.2. Materials and methods

IX.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on 2 % malt extract agar slants (pH 4.5) at 25ºC. Subcultures were routinely made every 30 days.

Pellet production was done as previously described by Font et al. (2003). Pellets obtained by this process were washed with sterile deionized water.

All pharmaceutical standards were of high purity grade (>90 %) and they were purchased from Sigma–Aldrich (Barcelona, Spain), European Pharmacopeia (EP) and Toronto research chemicals (Ontario, Canada).

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA). Glass fiber filters (1 µm) and nylon membrane filters (0.45 µm) were purchased from Whatman (U.K.). HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98 % were supplied by Merck (Darmstadt, Germany). Ammonium hydroxide and Ethylenediaminetetraacetic acid disodium salt solution (Na2EDTA) at 0.1 mol L^{-1} were from Panreac (Barcelona). Glucose, ammonium tartrate dibasic and malt extract were purchased from Sigma-Aldrich (Barcelona, Spain).

IX.2.2. Urban wastewater samples

Urban wastewater samples were collected from the student's village of Universitat Autónoma de Barcelona (Spain). Table IX.1 shows the characteristics of the wastewater. Sample 1 was sterilized at 121 ºC during 30 min. Samples 2 and 3 were directly used at non-sterile conditions.

Table IX.1: Characteristics of the urban wastewater samples from the university village.

Environmental parameter	Sample 1	Sample 2	Sample3
COD (mg L^{-1})	480	420	398
TOC (mg L^{-1})	105.8	135.3	116.02
N-NH_4^+ (mg L^{-1})	14.1	33.3	42
TS (mg L^{-1})	194	201	220
VS (mg L^{-1})	176	181	190.4
Conductivity (µg L^{-1})	287	731	552
pH	8.52	8.2	8.64

IX.2.3. Experimental procedures

IX.2.3.1. Batch bioreactor treatment.

A glass fluidized bed bioreactor with a useful volume of 10 L (Blánquez et al., 2008) was used to carry out both sterile and non-sterile urban wastewater treatments (wastewater samples 1 and 3, respectively). Approximately, 2.5 g dry weight (d.w.) pellets L^{-1} and 1.5 g d.w pellets L^{-1} were inoculated in sterile and non-sterile treatments respectively. Fungal biomass was maintained fluidized by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 second open, 5 seconds close) and the air flow was 12 L h^{-1}. The bioreactor was equipped with a pH controller in order to maintain pH at 4.5 and the temperature was maintained at 25 ºC. Glucose and ammonium tartrate were fed continuously from their stock solution (300 g L^{-1} and 675 mg L^{-1}, respectively) at a flow rate of 0.96 mL h^{-1} to ensure an uptake rate of 0.439 g glucose g^{-1} d.w. pellets d^{-1} and 1.98 mg ammonium tartrate g^{-1} d.w. pellets d^{-1} (Casas et al., 2013). For sterile conditions the bioreactor and the wastewater (sample 1) were autoclaved at 121 ºC for 30 min. Samples of 250 mL were taken periodically. All the samples were filtrated with 0.45 µm filters. 200 mL were stored at -20ºC to be further analyzed by UPLC-QTRAP. 50 mL from each sample were used to measure glucose concentration, COD, N-NH_4^+ and laccase.

IX.2.3.2. Effect of non-sterile urban wastewater on *T. versicolor.*

In order to observe the *T.versicolor* behavior in non-sterile urban wastewater, an experiment was performed applying different conditions of pH and nutrients. The experiment was carried out in Erlenmeyer flasks of 100 mL. In each flask, 170 mg d.w. pellets were inoculated in 30 mL of wastewater (sample 2 from table IX.1).

In table IX.2 the experiment design is shown. In the cases where glucose and ammonium tartrate were added the concentration was 6 g L^{-1} and 3.3 g L^{-1} respectively. Briefly, in experiment named A no nutrients where added, whereas only glucose was added in experiment B and glucose and ammonium tartrate were added in experiment C. In experiment 1 (A1, B1 and C1) the pH was not adjusted, while in experiments 2 (A2, B2 and C2) the pH was adjusted at 4.5 with HCL. All flasks were incubated under orbital shaking (135 rpm) at 25 ºC. Unitary samples by triplicate were collected at times 5 min, 1h, 2 d, 4 d and 7 d. The whole content of each flask was filtered through 0.45 µm glass fiber filter GF/A from (Whatman, Spain).

Table IX.2: Experimental design to study the effect of some parameters on *T. versicolor* under non-sterilized urban wastewater. X indicates that pH was adjusted at 4.5 and the nutrient was supplied.

Sample	pH	Glucose	Ammonium tartrate
A1	-	-	-
A2	x	-	-
B1	-	x	-
B2	x	x	-
C1	-	x	x
C2	x	x	x

IX.2.4. Analytical procedures

IX.2.4.1. Pharmaceuticals analysis in urban wastewater.

The analytical method for 80 different PhACs was carried out following the analytical methodology previously developed by Gros et al. (2012). Briefly, samples were filtered through 0.45 µm nylon membrane filters (Whatman, U.K.). A suitable volume of the chelating agent EDTA was added to all of them to a final concentration of 0.1 % (g solute g^{-1} solution), as it is well known that it improves the extraction of some antibiotics. Pre-concentration of samples was performed by SPE by the automatically extract system GX-271 ASPECTM (Gilson, Villiers le Bel, France). 50 mL of each sample was loaded at 1 mL min^{-1} in the cartridge previously conditioned using 5 mL of methanol followed by 5 mL H_2O-HPLC grade at 2 mL min^{-1}. Elution was performed with 6 mL of pure methanol at a flow rate of 2 mL min^{-1}. The extract was evaporated under gentle nitrogen stream using a Reacti-Therm 18824 System (Thermo Scientific) and reconstituted with 1 mL of methanol-water (10:90, v/v). Finally, 10 µL of standard of internal standard mix at 10 ng μL^{-1} was added in the extract for internal standard calibration and to compensate possible matrix effect.

Chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3 column (50 mm x 2,1 mm i.d. 1,7 µm particle size; Waters Corp. Mildford, MA, USA) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 mm×2.1 mmi.d., 1.7 µm particle size) for the ones analyzed under negative electrospray ionization (NI), both purchased from Waters Corporation. The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All transitions were recorded by using the Scheduled MRM™ algorithm and the data were acquired and processed using Analyst 2.1 software. Analitical parameters as limits of detection and quantification are showed in previous published article (Gros et al., 2012).

IX.2.4.2. Vibrio fischeri luminescence test (Microtox® test)

A Microtox bioassay was used to perform toxicity test. This method is based on the percent decrease in the amount of light emitted by the bioluminescent bacterium *V. fischeri* upon contact with a filtered sample at pH 7. The effective concentration, EC_{50}, was measured after 15 min. Effluent toxicity was expressed in percentages of EC_{50}. The experimental sample tested was collected from both sterile and non-sterile reactor treatments.

IX.2.4.3. Other analyses

Laccase activity was assayed using a modified version of the method for the determination of manganese peroxidase (MnP) as described elsewhere (Kaal et al., 1993). The reaction mixture used consisted in 200 µL of 250 mM sodium malonate at pH 4.5, 50 µL of 20 mM 2,6-dimethoxiphenol (DMP) and 600 µL of sample. DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV-vis spectrophotometer at 30ºC. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

Biomass pellets dry weight was determined after vacuum-filtering the cultures through pre-weighed glass-fiber filters (Whatman GF/A, Barcelona, Spain). The filters containing the biomass pellets were placed on glass plates and dried at 100 ºC to constant weight.

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instrument and Co. (Yellow Springs, OH, USA).

Total organic carbon (TOC), total solids (TS) and volatile solids (VS) were analyzed according to APHA (1995). The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analyzed by using commercial kits (LCH302 and LCK114 respectively, Hach Lange, Düsseldorf, Germany).

IX.3. Results and discussion

IX.3.1. Sterile batch bioreactor treatment.

Removal experiments were performed to study the ability of *T. versicolor* to degrade PhACs, as an alternative treatment to conventional WWTP, which are not designed for their complete removal (Verlicchi et al., 2012).

First of all, the wastewater treatment was carried out in sterile conditions in order to measure the removal of the PhACs by the fungus without the interference of any other microorganisms present in the wastewater. From the 80 PhACs analyzed, 13 were detected in the sterile urban wastewater effluent from the university village (Table IX.3). The most abundant PhACs belonged to the group of analgesic/anti-inflammatory compounds: Naproxen (35.58 ± 4.8 µg L^{-1}) and Ibuprofen (12.61 ± 1.79 µg L^{-1},). The PhACs concentration profile during the batch treatment is shown in figure IX.1.

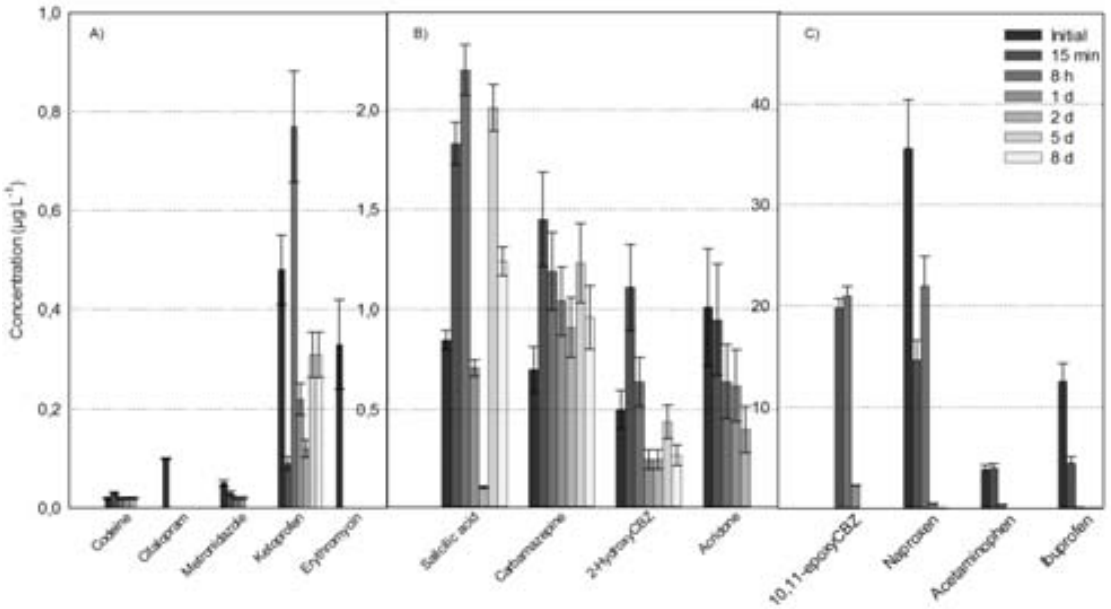

Figure IX.1: Pharmaceutical removal by *T. versicolor* during the batch fluidized bed bioreactor treatment at sterile conditions. A, B and C at different scales to the proper observation of their degradation profiles.

Complete removal of both analgesics, ibuprofen and naproxen, occurred within 24 h of the fungal treatment. The ability of *T. versicolor* to degrade these compounds

in sterile defined liquid medium was previously reported by Marco-Urrea et al. (2009, 2010d). Other analgesics as acetaminophen and codeine were initially detected at concentrations of 3.87 ± 0.41 μg L^{-1} and 0.02 ± 0.001 μg L^{-1} and were completely removed after 8 h and 2 d, respectively. Until now, the degradation of these latter compounds by WRF and its degradation products had not already been reported.

The analgesics ketoprofen and salicilyc acid were also initially detected in the wastewater at 0.48 ± 0.07 μg L^{-1} and 0.85 ± 0.11 μg L^{-1}, respectively. The concentration evolution during the treatment shows unexpected behavior with increase and decrease, but after 8 d their concentration were 0.31 ± 0.04 μg ketoprofen L^{-1} and 1.24 ± 0.07 μg salicylic acid L^{-1}, corresponding with a ketoprofen elimination of 35 % and an increase in the salycilic acid concentration of 46 %. Possible release of these compounds can be explained by deconjugation of glucuronides during biological treatment (Lishman et al., 2006). Previous experiments in sterile conditions showed the degradation of ketoprofen and their byproducts by *T. versicolor* (Marco-Urrea et al., 2010c). However, the presence of other contaminants, even the conjugation with other molecules, and the lower ketoprofen concentration could explain the increased concentration by the time and the consequent lower removal observed at the end of that study.

Table IX.3: Initial concentration and final removal of pharmaceuticals during both sterilized and non-sterilized batch bioreactor treatment

Group of pharmaceuticals	Compound	Sterilized urban wastewater		Non-sterilized urban wastewater	
		Concentration (µg L^{-1})	Removal (%)	Concentration (µg L^{-1})	Removal (%)
Analgesics/anti-inflammatories	Naproxen	35.58	100	N.D.	-
	Ibuprofen	12.61	100	2.23	100
	Acetaminophen	3.87	100	1.56	100
	Salicylic acid[a]	0.85	-46 (1.24 µg L^{-1})	N.D.	-
	Ketoprofen	0.48	35	0.08	100
	Codeine	0.02	100	N.D.	-
Antibiotic	Eryhromycin	0.33	100	N.D.	-
	Metronidazole	0.05	100	N.D.	-
	Ciprofloxacine	N.D.	-	84.71	35
	Azithromycin	N.D.	-	4.31	100
	Cefalexine[a]	N.D.	-	0.59	-51 (0.89 µg L^{-1})
β-blockers	Propranolol	N.D.	-	0.06	100
Psychiatric drugs	Carbamazepine[a]	0.7	-37 (0.96 µg L^{-1})	0	Increase (0.05 µg L^{-1})
	10,11-epoxyCBZ	19.82 (after 15min)	100	75.5	79
	2-HydroxyCBZ	0.5	46	163.8	100
	Acridone	1.01	100	0	Increase (0.05 µg L^{-1})
	Citalopram	0.1	100	0.04	100

a. Negative results mean increase of PhACs concentration. In brackets final concentration.

b. N.D. *means not detected.*

Psychiatric drugs were also detected in the wastewater. Carbamazepine, detected at 0.7 μg L^{-1}, is a known recalcitrant compound in activated sludge and MBR treatments (Verlicchi et al., 2012). To date, white rot fungi are the only microorganisms capable to remove this contaminant (Zhang and Geiβen, 2012). Several bioreactor types such as fluidized bed bioreactors, fixed-bed and stirred tank reactors have been used in sterile defined mediums at a range of concentrations from 100 μg L^{-1} to 1 g L^{-1}. The percentages of carbamazepine degradation vary from partially (47 %) to complete removal in few days (Jelić et al., 2012; Rodarte-Morales et al., 2012). However, the results of our study shown no removal of this compound in the real wastewater used in the sterile treatment, an increase in its concentration (37 %) was even observed after 8 d. The main metabolites of carbamazepine degradation in mammalians are also detected in the wastewater and consequently, they are not degradation products of this fungal treatment. Although 10,11-epoxycarbamazepine was detected at 15 min, it was completely removed at the end. Previous studies evidenced that it was also the main transformation product in carbamazepine degradation by *T. versicolor* (Jelić et al., 2012). However, the fast increasing concentration (15 min) and the non-elimination of carbamazepine observed in this study, leads us to deduce that 10,11-epoxycarbamazepine may appear by the desconjugation of glucuronides (Jelić et al., 2011). With regards to the two other metabolites, acridone was completely removed and 2-hydroxycarbamazepine was partially removed (46 %) after 8 d. Previous studies about the carbamazepine degradation by *T. versicolor* in synthetic medium showed the appearance and the subsequently possible degradation of all carbamazepine metabolites detected in this study (Jelić et al., 2012).

Antibiotics were successfully removed. Erythromycin and metronidazole were the only antibiotics detected in sterile wastewater, although at low concentrations (0.3 ± 0.089 μg L^{-1} and 0.05 ± 0.0064 μg L^{-1}, respectively). Erythromycin totally disappeared in 15 min, while metronidazole removal was achieved after 2 d.

Figure IX.2 shows the evolution of some environmental parameters during the batch bioreactor treatment. The highest activity of laccase was reached at the end of the experiment (around 100 U L^{-1}). Although glucose was accumulated during the first

hours, it was almost completely consumed later. The glucose and nitrogen consumption and the laccase production observed indicate that *T. versicolor* was active through the experiment. Biomass concentration was constant during the treatment (2.3 g d.w. L^{-1} at the end) because the process was carried out at maintenance conditions. During the first hours of the experiment COD increased from 0.4 g L^{-1} to 3 g L^{-1} due to the glucose addition, but after 1 day COD was maintained constant until the end.

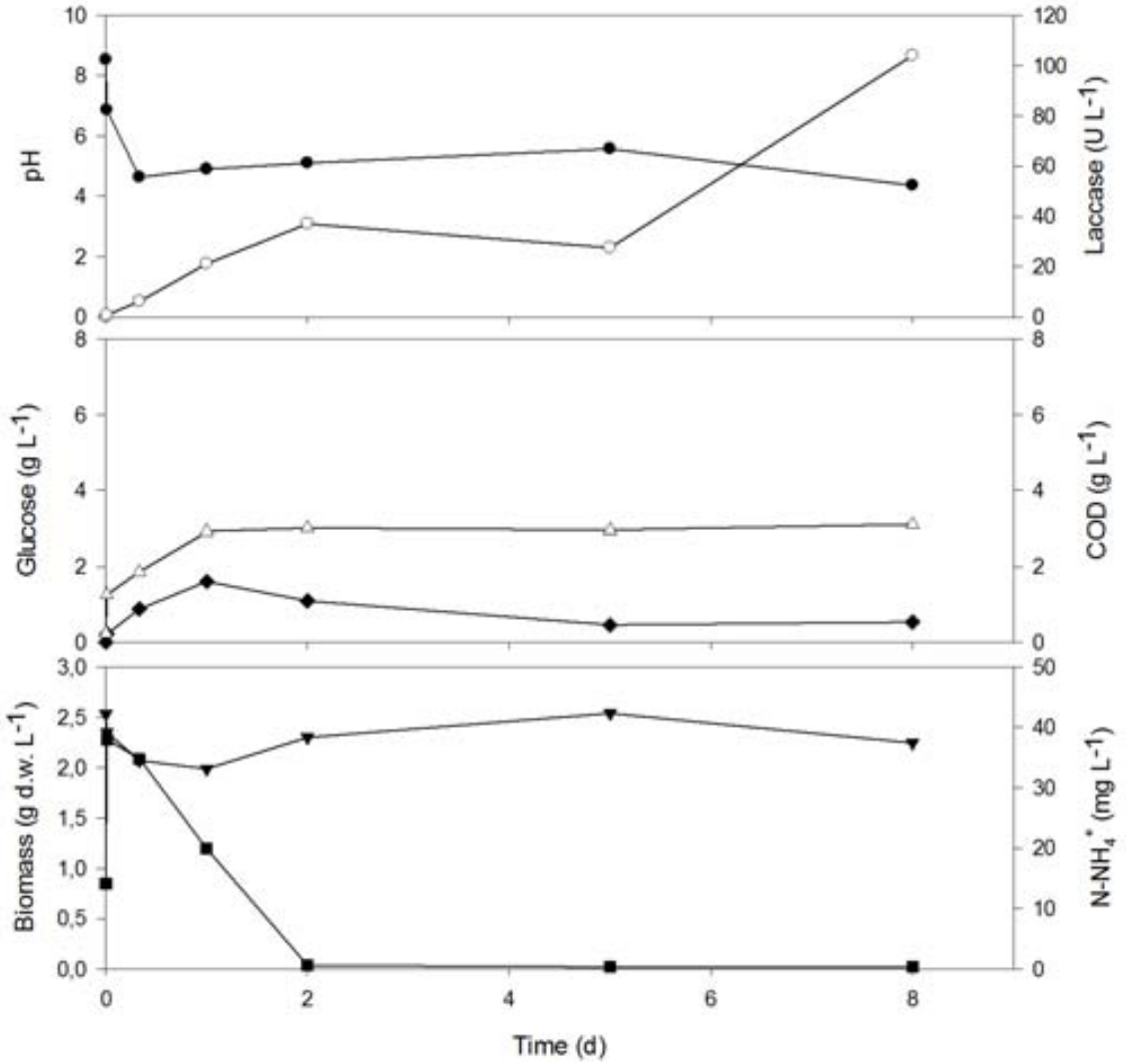

Figure IX.2: Environmental parameters profile during degradation of pharmaceuticals in sterile urban wastewater in a bioreactor. Simbols: pH (●), laccase (○), glucose (◆), COD (△), biomass concentration (▼) and $N\text{-}NH_4^+$ (■);

IX.3.2. Effect of non-sterile urban wastewater on *T. versicolor*

The fungus was inoculated into the non-sterile wastewater (sample 2 in Table 1) in order to evaluate its behavior and nutrient requirements. Different conditions of pH and nutrients addition (Table 2) were applied in order to find the optimal conditions for the fungus with regards to the enzymatic activity.

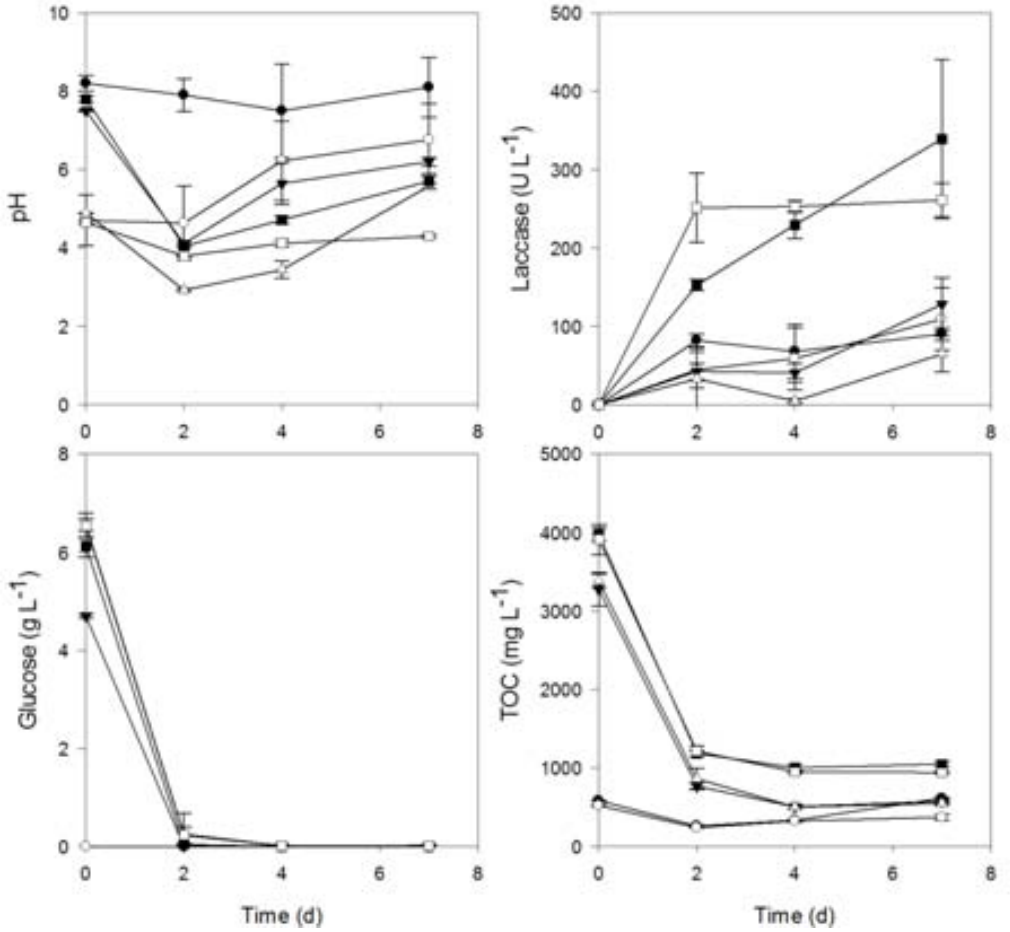

Figure IX.3: pH, glucose concentration, laccase activity and TOC during the fungus behavior experiments in non-sterile urban wastewater. Simbols: A1(●), A2(○), B1(▼), B2(△), C1(■) and C2(□).

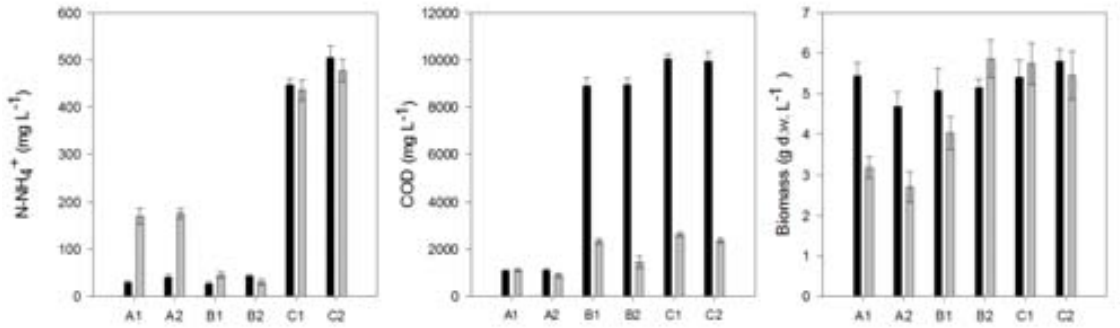

Figure IX.4: N-NH_4^+, COD and biomass concentration during fungus behavior experiments in non-sterile urban wastewater. Black bars correspond to initial values and grey bars refers to values after 7 d of the experiment.

Figure IX.3 shows the variation along the time of glucose concentration, laccase activity, pH and TOC. Figure IX.4 shows the initial and final (7d) level of COD, N-NH_4^+ and biomass concentration. It is known that the optimal pH for *T. versicolor* activity is acidic (pH 4.5) (Tavares et al., 2005) and nutrients (carbon and nitrogen) are needed to produce laccase (Tavares et al. 2006). As expected, in this study laccase production was higher when glucose and ammonium tartrate were provided as a source of carbon and nitrogen (experiments C1 and C2 in Table 2). Regarding the pH, it remained constant arround 8 when pH wastewater was not modified (A1). However, pH decreased from 8 to 4 when glucose was supplied (B1 and C1), but subsequently pH values increased again (arround 6) after 2 d, corresponding with the total glucose depletion. In concordance, in sample A2 (pH adjusted to 4.5 and no glucose supplied) and also after the total consumption of the glucose (2 d) in sample B2 the pH increased arround 6-7. In experiment C2, with addition of both nitrogen (ammonium tartrate) and carbon (glucose) sources, pH remained contant at 4.5 up to the end of the experiment. Our pH variations agree with previous results obtained by Zhang and Geiβen (2012) at non-sterile conditions. These authors linked the pH decrease to the activity of acidogenic bacteria present in the non sterile wastewater. On the other hand Borràs et al. (2008) described the same behavior for pH during the glucose consumption period of *T. versicolor* growing in sterile conditions and they related the drooping down of the pH to the synthesis of organic acids, such as oxalic and glycoxalic acids. Roy and Archibald (1993) associated those acids production with the fungal

primary metabolism when glucose is consumed. Nevertheless, after the glucose depletion a gradually pH increase is observed.

On the other hand, *T. versicolor* only removed the COD and TOC corresponding to the amount of added glucose and it was not able to remove COD and TOC from the wastewater as could be observed in samples A1 and A2 (Figure IX.4). In contrast, Zhang and Geißen (2012) reported complete removal of disolved organic carbon after 5 d in the treatment of a WWTP efluent by *Phanerochaete chrysosporium*.

The biomass concentration remained constant when both nutrients (C1 and C2) were supplied. However, it decreased (50 %) when no nutrients were provided (samples A1 and A2). When only glucose was added (B1), the biomass decreased around 20 %, while samples also adjusted at pH 4.5 (B2) it remained constant. A decrease in the biomass concentration could be explained because the fungus needs nutrients for its maintenance. The lack of nutrients causes the lysis of the mycelia and consequently the decrease in the biomass concentration measured. It seems that higher pH may promote earlier lysis than adjusted mediums at pH 4.5 which it is the optimum for *T. versicolor*.

In samples with nutrients scarcity (A1 and A2), nitrogen concentration in form of ammonium increased. It is in accordance with previous studies where observed nitrogen accumulation under starvation conditions for the degradation of carbamazepine by *P. chrysosporium* produced by the lysis of fungal mycelia (Zhang and Geiβen, 2012).

All the results presented above demonstrate that *T. versicolor* may be active in real wastewater where bacteria and contaminants are present. We conclude that the fungus needs a source of nutrients (glucose and nitrogen) to maintain the biological activity and consequently the enzymatic production. Therefore, next degradation experiments of pharmaceuticals in urban wastewater in bioreactor at non-sterile conditions were carried out supplying glucose and ammonium tartrate as a source of carbon and nitrogen, respectively. In addition, pH was controlled at 4.5. These are the optimal conditions to guarantee the fungal activity to degrade pharmaceuticals contaminants in real wastewater.

IX.3.3. Non-sterile batch bioreactor treatment.

Once the fungus ability to degrade PhACs in sterile conditions has been demonstrated, it was performed the same treatment in non-sterile conditions, where many other active microorganism may be present. The general characteristics of the urban wastewater are presented in Table 1 (Sample 3). In addition, Table 3 shows the initial concentration of the detected PhACs and their removal percentage after 7 days of fungal treatment. From 80 PhACs analysed, only 10 were initially detected in wastewater (sample 3) belonging to 4 different drug groups: analgesics, β-blockers, antibiotics and psychiatrics, being the latter the highest concentration (up to 163.8 ± 32.9 μg L^{-1}).

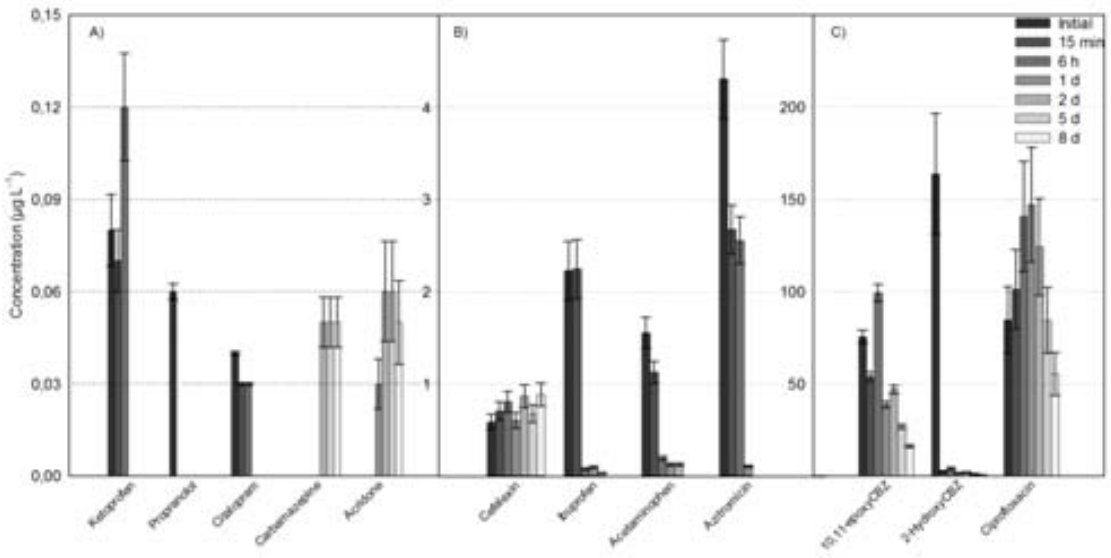

Figure IX.5: Pharmaceutical removal by *T. versicolor* during the batch fluidized bed bioreactor treatment at non-sterile conditions. A, B and C at different scales to the proper observation of their degradation profiles.

In figure IX.5 it can be observed the profile of all PhACs detected during the non-sterile treatment. Analgesics/anti-inflammatories exhibited a highly efficient elimination in non-sterile as well as in sterile condition. Ibuprofen and acetaminophen were completely removed in both, sterile and non-sterile conditions, despite of their high concentration. In activated sludge WWTP as well as membrane bioreactors (MBR) removal efficiencies from 82.5 % to 99 % have been reported for these two analgesics (Verlicchi et al., 2012). As consequence, their removal by biological processes appears

feasible. Ketoprofen found at 0.08 ± 0.012 μg L^{-1} was completely removed after 2 d, but seems to follow the same profile as the sterile treatment caused an increase of its concentration (0.12 ± 0.017 μg L^{-1}) during the first hours. The total elimination of ketoprofen is quite promising since only partial degradability (around 50 %) has been demonstrated in activated sludge of a WWTPs (Santos et al., 2009).

Three antibiotics were detected in the non-sterile urban wastewater and they showed different removal yields after the fungal treatment. Ciprofloxacin, detected at 84.71 ± 0.017 μg L^{-1} corresponding with the second highest concentration of the detected PhACs, was 35 % removed after 8 d. However, during the first day its concentration increased, maybe as in above cases, by desconjugation of glucuronides. Removal efficiencies of this pollutant from 50 % to 96 % were reported in different conventional WWTPs (Verlicchi et al., 2012). However, more than 80 % of the removal is attributed to the sorption in the sludge (Jia et al., 2012). Almost complete removal of ciprofloxacin was previously demonstrated by this fungus in sterile conditions (Prieto et al., 2011). Azithromycin, detected at 4.31 ± 0.42 μg L^{-1}, was almost completely removed after 1 d. Nevertheless, from no elimination to low partial removal (40 %) was observed in MBR (Göbel et al., 2007). On the other hand, cefalexine concentration was very low (0.59 μg L^{-1}) and slightly rose during the treatment maintaining at insignificant levels. Cefalexine was almost completely removed (96 %) in different configurations of WWTPs (Verlicchi et al., 2012). So far, the removal of the latter antibiotics by WRF had not been demonstrated.

Propanolol, a β-blocker drug, was detected at the lowest concentration (0.06 ± 0.003 μg L^{-1}) in the wastewater but it was not detected in the next samples. Its biological removal has been previously reported with elimination yields up to 59 % or higher than 70 % in conventional activated sludge and MBR, respectively (Radjenović et al. 2009).

In the case of psychiatric drugs, none of the compounds used for the human intake were detected (for example carbamazepine, diazepam, etc…) in the non-sterile wastewater. However, two of the carbamazepine metabolites produced in mammalians, 10,11-epoxycarbamazepine and 2-hydroxycarbamazepine, where found

at high concentrations, 75.5 ± 3.54 μg L^{-1} and 163.8 ± 32.9 μg L^{-1}, respectively. Despite of the higher concentrations and the non-sterile conditions, the removal yields achieved were very high (80 and 100 %). Carbamazepine and acridone were not initially detected, but after 1 d they were detected at very low concentration, approximately 0.05 ± 0.013 μg L^{-1} (Figure IX.5). In conventional WWTP, also higher concentration of carbamazepine was detected in the effluent compared with the influent (Jelić et al., 2011). This increase could be attributed to the fact that the pollutant could deconjugate from other compounds like glucuronides, or maybe by the fact that some metabolites presents in the initial wastewater could be transformed in their parent compound during the treatment.

Regarding the performance of the non-sterile batch bioreactor treatment (Figure IX.6), glucose was totally consumed through the experiment. Laccase production reached its highest value (around 70 U L^{-1}) on day 5. Biomass concentration was maintained constant up to the day 5. After that an increase of the free mycelia and the turbidity of the broth were observed. At day 8 the biomass level was 0.98 g d.w. L^{-1}. The COD measured was constant along the treatment and corresponds to the initial wastewater COD because the glucose was completely consumed.

The results obtained in the PhACs fungal degradation at non-sterile conditions evidenced that the treatment of a real wastewater is feasible with similar results to the sterile conditions.

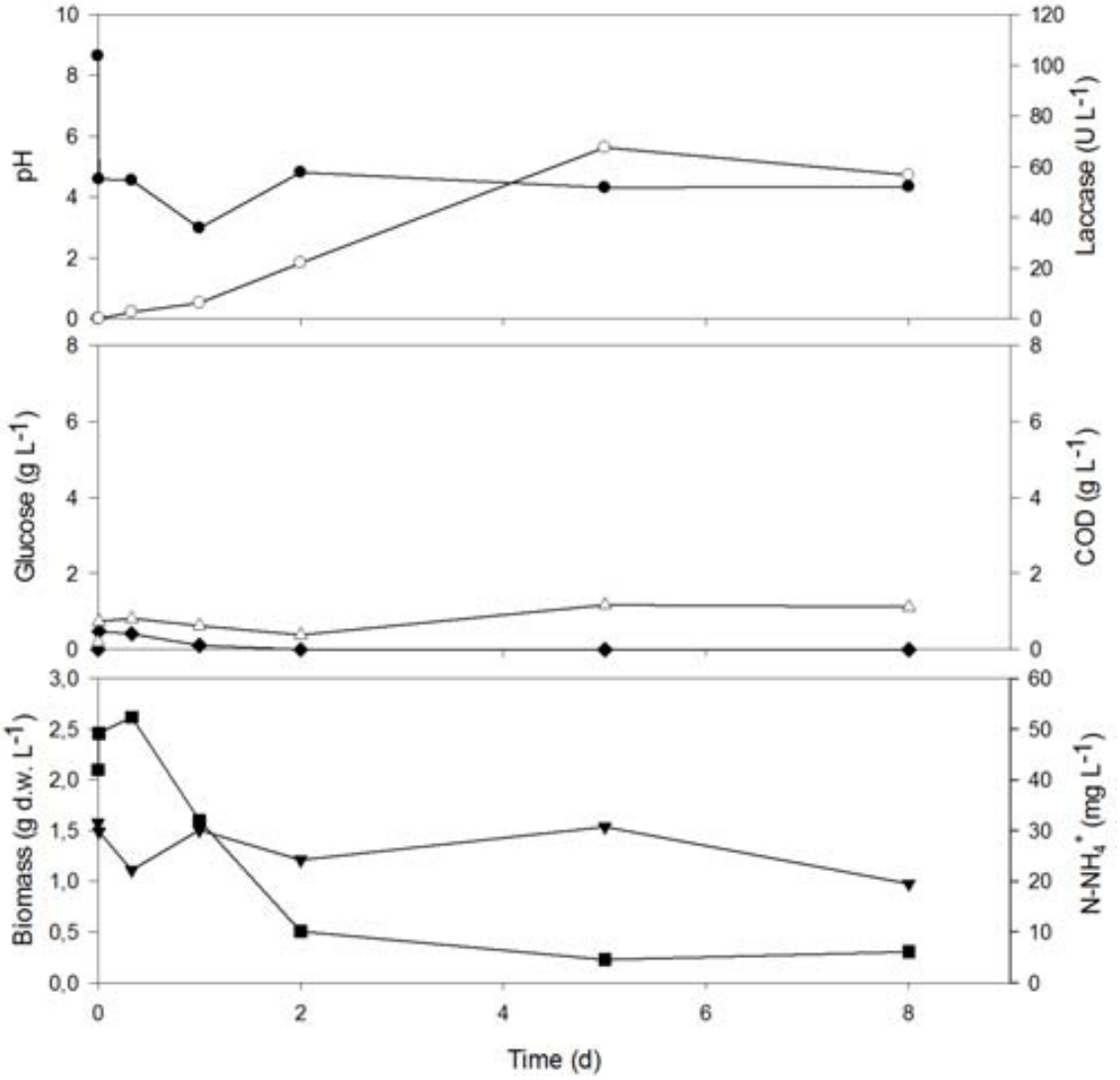

Figure IX.6: Environmental parameters profile during degradation of pharmaceuticals in non-sterile urban wastewater in a bioreactor. Simbols: pH (●), laccase (○), glucose (◆), COD (△), biomass concentration (▼) and N-NH_4^+ (■).

IX.3.4. Toxicity assessment (Microtox® test)

The degradation of some PhACs by *T. versicolor* can produce transformation products that can present higher toxicity than its parent compound (Marco-Urrea et al., 2009). Therefore, eco-toxicity estimation by Microtox test (bacterium *V. ficheri*) was performed to determine the change in the toxicity during the PhACs treatment from urban wastewater.

Raw urban wastewater presented non-toxic values (EC_{50} around 20-30 %). After the first 24 h of fungal treatment a decrement in toxicity was observed in both sterile and non-sterile conditions with EC_{50} about 100 % and 40 %, respectively. These values correspond with the almost complete removal of the principal PhACs detected at the highest concentration (10,11-epoxycarbamazepine, naproxen, acetaminophen and ibuprofen). At the end of treatments the eco-toxicity remained at non-toxic values (EC_{50} in the range of 75-100 %). It must be said that the decrease in the toxicity may be attributed not only to the degradation of the PhACs detected but also to the likely degradation of other pollutants by the fungus. These pollutants, not analyzed in this study, can be toxic for the environment and can contribute thus the toxicity of the effluent measured by the microtox test.

IX.4. Conclusion

It has been demonstrated the possibility of using a fluidized bed bioreactor for the elimination of PhACs at environmentally relevant concentrations at non-sterile conditions by *T. versicolor*. Complete removal for around 50 % of the detected PhACs was achieved, while only 25 % were partially removed (25 %). In addition, high removal percentages are obtained in the degradation of the mammalian metabolites from some PhACs such as carbamazepine, present in the WWTP influent. The treatment of real wastewater evidenced that the fungus is able to degrade emerging pollutants such as PhACs, which are present at low concentrations in real and complex matrices. Despite the few reported studies of WRF applied to real wastewater, our results encourage to continue the study of depuration of such effluents in order to analyze other operational strategies and optimize the process to extent and improve the treatment.

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Chapter X:

Degradation of the X-Ray constrast agent iopromide and the fluoroquinolone antibiotic ofloxacin in non-sterile hospital wastewater by *Trametes versicolor* in a fluidized bed bioreactor.

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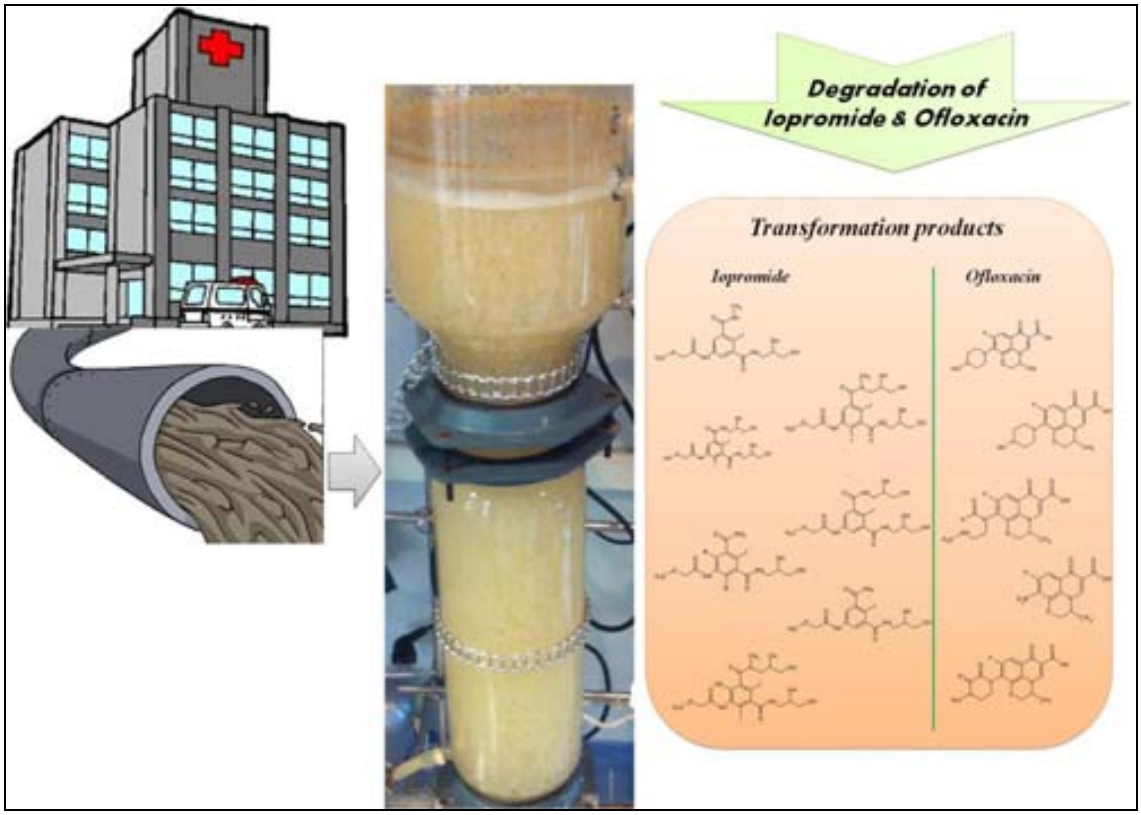


Abstract

This chapter describes the degradation of the X-ray contrast agent iopromide (IOP) and the antibiotic ofloxacin (OFLOX) by the white-rot-fungus *Trametes versicolor*. Batch studies in synthetic medium revealed that between 60 and 80% of IOP and OFLOX were removed when spiked at approximately 12 mg L^{-1} and 10 mg L^{-1}, respectively. A significant number of transformation products (TPs) were identified for both pharmaceuticals, confirming their degradation. IOP TPs were attributed to two principal reactions: (i) sequential deiodination of the aromatic ring and (ii) N-dealkylation of the amide at the hydroxylated side chain of the molecule. On the other hand, OFLOX transformation products were attributed mainly to the oxidation, hydroxylation and cleavage of the piperazine ring.

Experiments in 10 L-bioreactor with fungal biomass fluidized by air pulses operated in batch achieved high percentage of degradation of IOP and OFLOX when load with sterile (87% IOP, 98.5 % OFLOX) and unsterile (65.4% IOP, 99% OFLOX) hospital wastewater (HWW) at their real concentration (μg L^{-1} level). Some of the most relevant IOP and OFLOX TPs identified in synthetic medium were also detected in bioreactor samples. Acute toxicity tests indicated a reduction of the toxicity in the final culture broth from both experiments in synthetic medium and in batch bioreactor.

Keywords: White-rot fungi, pharmaceutical compounds, bioreactor, hospital wastewater.

X.1. Introduction

Large amounts of different pharmaceuticals, belonging to several therapeutic groups, are used worldwide and their sales have been continuously increasing in the last decade (Verlicchi et al. 2012;Kummerer 2001). Wastewaters are the primary route of entry of pharmaceuticals in the environment and hospitals are considered important sources and significant contributors of pharmaceutical residues in influent municipal wastewater treatment plants (WWTP) (Hawkshead 2008;Nagarnaik et al. 2012). Despite their specific nature, in many countries, hospital effluents are discharged into public sewer networks and collected to WWTP where they are co-treated with urban wastewater (Verlicchi et al. 2012). Several scientists have questioned this common practice of co-treating hospital and urban wastewaters (Pauwels and Verstraete 2006;Kovalova et al. 2012), and have recommended a pre-treatment of hospital effluents before being discharged into public wastewaters. Treatment of the wastewater at the source has advantages of avoiding dilution due to mixing with the urban sewage and avoiding losses into the environment due to sewer leakage and combined sewer overflows (Kovalova et al. 2012). In the case of HWW, specific concerns are to avoid spread of multi-resistant or pathogenic bacteria, viruses and parasite eggs as well as to avoid input of large quantities of pharmaceuticals, diagnostic agents and disinfectants (Kovalova et al. 2012).

Some studies published have reported on the efficiency of several advanced wastewater treatment technologies to remove pharmaceutical residues from HWW (Kovalova et al. 2012;Beier et al. 2012;Beier et al. 2011;Beier et al. 2010;Nielsen et al. 2013). Even though these techniques proved to be very efficient in the removal of recalcitrant pharmaceuticals one of their main limitations may be the formation of undesirable and sometimes toxic by-products (del Mar Gomez-Ramos et al. 2011;Trovo et al. 2011).

The ability of white-rot fungi (WRF) to oxidize a large number of organic contaminants from liquid medium has been widely proven (Pointing 2001). This high degradation capability is attributed to the non-specific nature of their ligninolytic

enzymes, which include high redox potential peroxidases and laccases (Harms et al. 2011;Cerniglia 1997;Asgher et al. 2008). In addition, WRF have the potential to metabolize xenobiotics intracellularly by means of the cytochrome P450 system in a similar way to mammals (Cerniglia 1997;Doddapaneni and Yadav 2004). Another advantage of WRF is that they do not require preconditioning to particular pollutants because a number of their ligninolytic isoenzymes are produced constitutively and others after induction by nutrient deprivation (Harms et al. 2011;Munoz et al. 1997). The high unspecificity of WRF in the removal of organic pollutants from environmental matrices makes these organisms an interesting option to be taken into account for remediation processes. Among the WRF, *Trametes versicolor* has the advantage to produce pellets when it grows in submerged cultures. In addition it has been proved to be a powerful decontaminant of different types of pollutants such as dyes, UV-filters, chlorobenzenes, polybrominated flame retardants and pharmaceuticals (Blanquez et al. 2004;Badia-Fabregat et al. 2012;Rodriguez-Rodriguez et al. 2012;Marco-Urrea et al. 2009;Cruz-Morató C. 2012). However, studies applying *T. versicolor* in lab-scale bioreactors are still quite scarce (Blanquez et al. 2008;Libra et al. 2003;Jelic et al. 2012;Cruz-Morato et al. 2013;Yang et al. 2013) and few of them working in non-sterile conditions (Blanquez et al. 2008;Libra et al. 2003;Cruz-Morato et al. 2013;Yang et al. 2013). This is the first study showing degradation of pharmaceuticals by WRF in batch bioreactors loaded with real hospital wastewater and at their realistic concentration.

Here, we evaluate the capability of the WRF *Trametes versicolor* to degrade the X-ray contrast agent iopromide (IOP) and the fluoroquinolone antibiotic ofloxacin (OFLOX) by two different approaches: (i) experiments performed in Erlenmeyer flasks containing defined liquid medium spiked with the target compounds at high concentrations, in order to identify possible transformation products (TPs) and (ii) experiments in a pilot scale bioreactor, operating in batch mode and load with real unspiked hospital wastewater under sterile and non-sterile conditions. IOP and OFLOX were selected because they are ubiquitous pharmaceuticals in hospital wastewaters (Chang et al. 2010;Brown et al. 2006;Perez and Barcelo 2007), they were detected in the HWW used in the present study (approximately 190 $\mu g\ L^{-1}$ for IOP and 24 $\mu g\ L^{-1}$ for OFLOX) and show moderate to low removal efficiency under conventional wastewater

treatment processes (Perez and Barcelo 2007;Joss et al. 2005). TPs formed during the time-course experiments were analyzed and identified. Acute toxicity was tested in order to evaluate the toxicity of the treated aqueous medium.

X.2. Materials and Methods

X.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at 25ºC. Subcultures were routinely prepared every 30 days. Pellet production was done as previously described (Blanquez et al. 2004).

Glucose, ammonium tartrate dibasic and malt extract were purchased from Sigma-Aldrich (Barcelona, Spain).

All pharmaceutical standards were of high purity grade (>90%). IOP was purchased from United States Pharmacopeia (USP) whereas OFLOX was acquired from Sigma-Aldrich (Barcelona, Spain). Isotopically labeled compounds iopromide-d_3 and ofloxacin-d_3 were purchased from Toronto Research Chemicals (Canada) and Sigma-Aldrich, respectively.

Solid phase extraction cartridges were Oasis HLB (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA). Glass fiber filters (1 µm) and nylon membrane filters (0.45 µm) were purchased from Whatman (Spain) while regenerated cellulose syringe filters (13 mm, 0.45 µm) were acquired from Cronus SMI-Labhut (UK). HPLC grade methanol and water (Lichrosolv) were supplied by Thermo Fisher Scientific. A Barnstead Nanopure system from Thermo Scientific was used to obtain HPLC grade water. Ethylenediaminetetraacetic acid disodium salt solution (Na_2EDTA) at 0.1 mol L^{-1} was from Panreac, formic acid 98% was from Scharlau, ethanol and methanol were from Merck (Darmstadt, Germany). Nitrogen for extract drying was from Abelló Linde S.A (Spain) and it was of 99.999% purity.

X.2.2. Biodegradation experiments

X.2.2.1. Degradation in Erlenmeyer flasks

Degradation experiments were performed in 500 mL Erlenmeyer flasks containing appropriate amounts of mycelial pellets (0.60 g dry weight) in a total volume of 100 mL of defined medium, consisting of 8 g L^{-1} of glucose, 3.3 g L^{-1} of ammonium tartrate, 1.168 g L^{-1} of 2,2-dimethylsuccinate buffer, 1 and 10 mL of a micro and macronutrient solution from Kirk medium (Kirk 1978). The pH of the medium was adjusted to 4.5. Appropriate volumes from stock solutions of IOP (5000 mg L^{-1}) in water and OFLOX (1600 mg L^{-1}) in ethanol were added into the flasks to give the desired final concentration (approximately 12 and 10 mg L^{-1}, respectively). After pharmaceutical addition, the flasks were incubated under orbital shaking (135 rpm) at 25 ºC for twelve days. To avoid the possible influence of light on compound stability, all the experiments were carried out in the dark. Experiments were performed under sterile conditions (121ºC for 30 min).

The whole content of the flasks was sacrificed at times 15 min, 2 h, 8 h, 1 d, 2 d, 4 d, 7 d and 12 d. All samples were filtered through a 0.45 µm glass fiber Whatman filter GF/A, after which 1 mL aliquot was used to analyze target compounds, by high performance liquid chromatography (HPLC), glucose and laccase . The remaining sample was used to identify the TPs of target compounds.

Heat-killed and non-inoculated controls are included to assess the adsorption onto the fungi and non-biotic processes, respectively. Heat-killed controls consisted of autoclaved cultures (121 ºC for 30 min), which were set-up under identical conditions to those of the experimental cultures, whereas non-inoculated controls consisted on the addition of target compounds without fungi.

X.2.2.2. Batch bioreactor treatment

A 10L glass air fluidized bed bioreactor (Blanquez et al. 2008) was used to carry out the treatment of HWW under sterile and unsterile conditions. HWW used for the sterile and unsterile treatment were collected at different days. The characteristics of the wastewaters are described in Table 1. Fungal biomass was maintained fluidized by air pulses generated by an electrovalve, which was controlled by a cyclic timer (1 second open, 5 seconds close) and the air flow was 12 L h^{-1}. The bioreactor was equipped with a pH controller in order to maintain pH at 4.5 by the addition of NaOH and the temperature was maintained at 25 ºC. Nutrients solution containing glucose and ammonium tartrate was fed continuously from their stock solution (300 g L^{-1} and 675 mg L^{-1}, respectively) at a flow rate of 0.96 mL h^{-1} to ensure an uptake rate of 0.439 g glucose g^{-1} dry weight (d.w) pellets d^{-1} and 1.98 mg ammonium tartrate g^{-1} d.w. pellets d^{-1} (Casas et al. 2013). Approximately, 1.4 g d.w. biomass L^{-1} was inoculated into the bioreactor. For the sterile treatment, the bioreactor and the wastewater were autoclaved at 121 ºC for 30 min. In both unsterile and sterile treatments, 200 mL samples were taken from the middle of the bioreactor periodically and were filtered previously to analyse.

Table X.1: Characteristics of the hospital wastewater samples used in the sterile and non-sterile treatment.

Environmental parameter	Wastewater treatment	
	Sterile	Non-sterile
COD (mg L^{-1})	490	357
TOC (mg L^{-1})	261.2	153.3
N-NH_4^+ (mg L^{-1})	68.1	49.5
TSS (mg L^{-1})	11.15	6.08
Conductivity (µg cm^{-1})	1455	1194
pH	8.33	8.10

X.2.3. Analytical procedures

X.2.3.1. Analysis of IOP and OFLOX to monitor their removal in Erlenmeyer scale experiments.

Analysis of IOP and OFLOX was performed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) using GraceSmart RP C18 column (250 mm x 4 mm, particle size 5 µm) and equipped with a UV detector at 238 nm and 280 nm, respectively. The column temperature was 30 ºC and a sample volume of 20 µL was injected from a Dionex autosampler (Barcelona, Spain). The mobile phase consisted of acetonitrile (A) and 0.1 % phosphoric acid in water (B). IOP analysis was performed isocratically (85 % B) at 0.7 mL min^{-1}, while OFLOX was analyzed by gradient, at a flow rate of 1 mL min^{-1}, starting at 10 % A, increasing to 90 % A in 15 min and then coming back to initial conditions in 5 min. The retention time of both IOP and OFLOX was 5.52 min and 6.59 min respectively. The detection limit for both compounds was 0.1 mg L^{-1}.

X.2.3.2. Analytical methodology for the identification of IOP and OFLOX transformation products in Erlenmeyer flasks.

Degradation products were identified by on-line solid phase extraction (SPE) coupled to liquid chromatography-high resolution mass spectrometry (HRMS). The on-line SPE-HPLC method used was adapted from one previously developed by Kovalova et al. (Kovalova et al. 2012). Briefly, samples were filtered through regenerated cellulose syringe filters (0.45 µm). Since IOP and OFLOX were spiked at high concentrations in the degradation experiment (approximately 10 mg L^{-1}), 0.5 mL of these filtered aliquots were diluted in 100 mL of MilliQ water prior to the on-line SPE analysis. Samples were also spiked with a solution containing iopromide-d_3 and ofloxacin-d_3 at 2 µg L^{-1} as surrogate standards. 20 mL were then extracted by on-line SPE, as described in Kovalova et al. (Kovalova et al. 2012).

The MS analysis was performed with an electrospray ionization (ESI) interface coupled to a quadrupole-orbitrap mass spectrometer (Qexactive Orbitrap™, Thermo Scientific). Mass calibration and mass accuracy checks were performed prior to every sample sequence. The instrument was calibrated using the manufacturer's calibration

solution (containing caffeine, the tetrapeptide MRFA, and a mixture of fluorinated phosphazines ultramark 1621). Mass accuracy was always within ±5 ppm. Samples were injected twice; one run was acquired under positive electrospray ionization mode while the other run was performed under negative ionization mode, using the same chromatographic conditions. Data-dependent MS/MS acquisition was triggered using an inclusion list of accurate masses of (i) plausible TPs predicted by the University of Minnesota Pathway Prediction System (UM-PPS) software and (ii) known TPs of target compounds reported in the scientific literature. These known TPs correspond mainly to biodegradation products of the target pharmaceuticals after their exposure to diverse microorganisms (more detailed information can be found in section 3.2). In all data-dependent experiments, the mass spectrometer acquired full scan data within a mass-to-charge (m/z) range of 100 to 1000 m/z at a resolving power of 70.000 FWHM. Additionally, a MS/MS spectrum showing the fragmentation of the detected ions present in the inclusion list was acquired, using normalized collision energies (NCE) of 25 for IOP and 35 for OFLOX and a resolution of 35000 FWHM. In all MS/MS modes, the isolation window of the quadrupole was set to 1.0 Da. Data-dependent analysis was triggered by using an underfill ratio of 5% and a dynamic exclusion of 5 seconds, the intensity threshold was set to $8.3x10^4$ and the appex trigger was activated. XCalibur™ 2.2 (Thermo Scientific) software was used for chromatographic analysis and data interpretation.

X.2.3.3. Analytical procedure followed to monitor IOP, OFLOX and their TPs in a fluidized bed bioreactor fed with hospital wastewater.

The method used to monitor IOP and OFLOX degradation, as well as the evolution of the TPs (identified in Erlenmeyer experiments) in HWW, was based on off-line solid phase extraction followed by ultra-high-performance liquid chromatography coupled to a hybrid linear ion trap-orbitrap mass spectrometer (Orbitrap Velos™, Thermo Scientific). The solid phase extraction method used was the one described by Gros et al. (Gros et al. 2012) (for more information see SI.2).

Chromatographic separation was achieved with an Acquity HSS T_3 colum (50 mm x 2.1 mm i.d., 1.7 µm particle size), using a Turboflow™ system coupled to an Accela™ UHPLC system (Thermo Scientific). Mobile phases used were methanol and 10 mM formic acid/ammonium formiate (pH 3.2) at a flow rate of 0.5 mL/min and the injection volume was 10 µL. The MS analysis was performed with an ESI interface coupled to a linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap Velos™, Thermo Scientific). Samples were analyzed under positive ionization conditions.

Data-dependent MS/MS acquisition was triggered, using an inclusion list of accurate masses. This time, masses fitted in the list corresponded to IOP and OFLOX, and the exact masses of the TPs identified in the biodegradation experiments in Erlenmeyer flasks.

For this experiment, the mass spectrometer acquired full scan data within a mass-to-charge (m/z) range of 150 to 1500 m/z at a resolving power of 60.000 FWHM. Additionally, a full-scan showing the fragmentation of all ions detected (the ones present in the inclusion list) was acquired at a resolution of 30.000 FWHM, using a NCE of 25. Data-dependent analysis was triggered by using an isolation width of 2.00. XCalibur™ 2.2 (Thermo Scientific) software was used for chromatographic analysis and data interpretation.

X.2.3.4. Other analyses and toxicity evaluation

The methods used to measure glucose concentration, laccase activity and toxicity are described in previous chapters.

A Microtox® bioassay was used to perform toxicity test of the samples along the time of degradation experiments. This method is based on the percent decrease in the amount of light emitted by the bioluminescent bacterium *V. fischeri* upon contact with a filtered sample at pH 7. The effective concentration, EC_{50}, was measured after 15 min. Effluent toxicity was expressed in percentages of EC_{50}. The experimental sample tested was collected from both Erlenmeyer and bioreactor treatments.

X.3. Results and discussion

X.3.1. Degradation of IOP and OFLOX by *T. versicolor* in Erlenmeyer flasks

Time-course degradation experiments performed in Erlenmeyer flasks showed that approximately 62% of the IOP was degraded by *T. versicolor* after 7 days of incubation (Figure X.1A), while for OFLOX almost 80% was degraded within the same period of incubation (Figure X.1B). IOP and OFLOX removal was attributed to the degradation by *T. versicolor*, since no IOP and OFLOX removal were observed neither in the heat-killed controls nor in the abiotic controls.

The degradation of IOP by *T. versicolor* was previously described by Engels-Matena and co-workers (Engels-Matena 1996), who reported removal of 80% after 12 d of incubation, when IOP was added at 1 mM. However, to the author's knowledge, our work is the first one to report on the degradation of OFLOX by *T. versicolor* and identifying IOP and OFLOX TPs.

It can also be observed from Figure ´X.1A and X.1B that laccase was excreted in the medium by *T. versicolor* during the degradation process. Laccase activity is of interest because it plays a role in the degradation of many pharmaceuticals (Cruz-Morató C. 2012). In addition the laccase production, together with glucose consumption, was used to evidence fungal metabolic activity through the experiment.

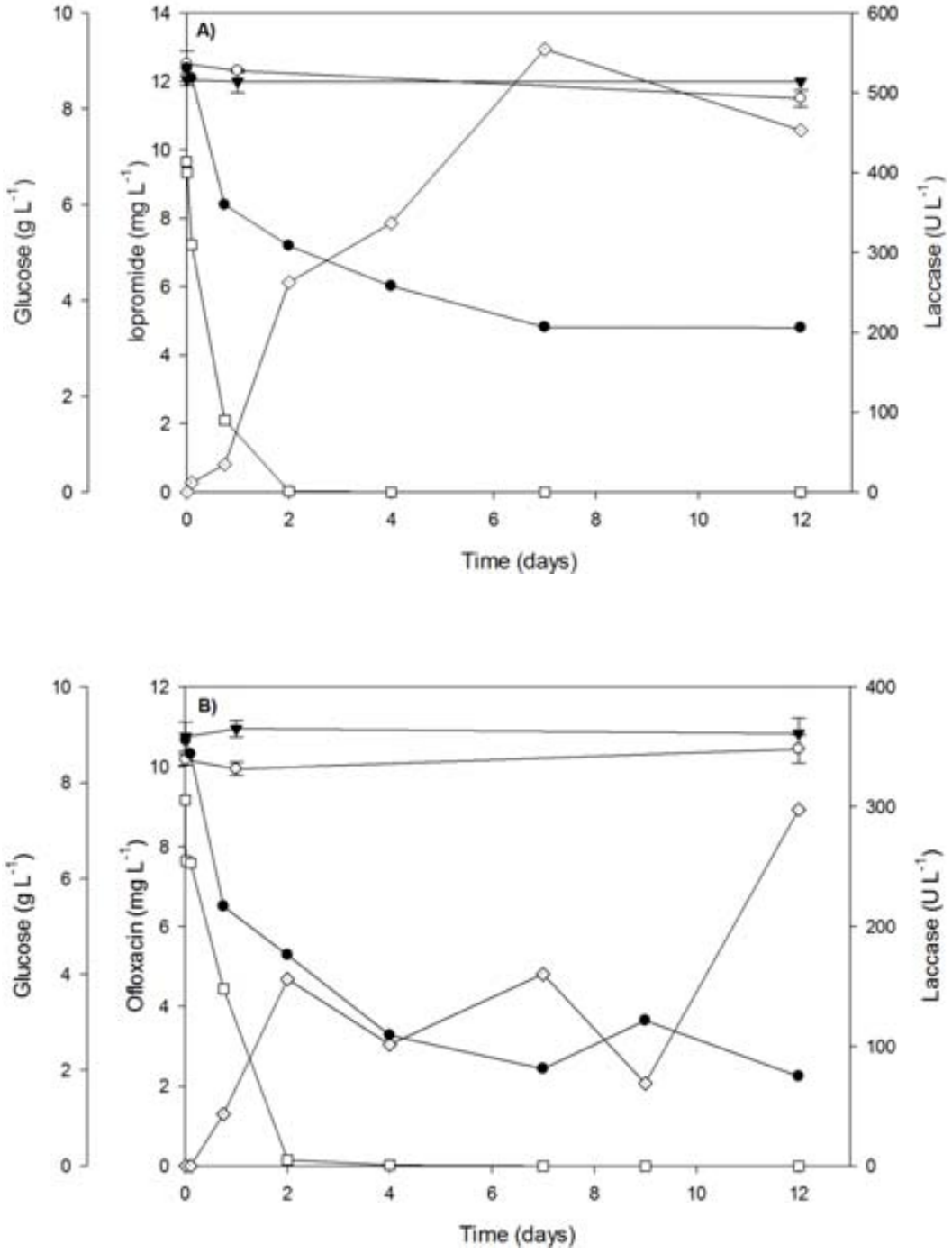


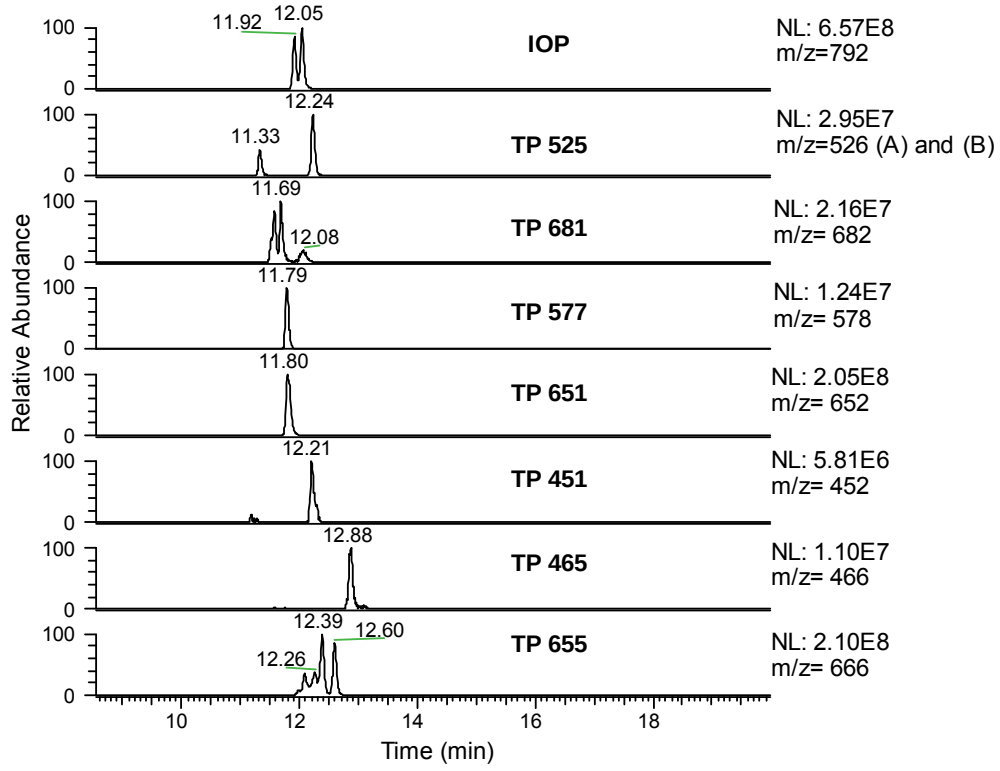
Figure X.1: Time course degradation of iopromide (A) and ofloxacin (B) (10 mg L^{-1}) by *T. versicolor* pellets in Erlenmeyer flasks. Symbols: experimental cultures (●), uninoculated controls (▼), heat-killed controls (○), glucose (□) and laccase activity (◊).

X.3.2. Identification of transformation products in Erlenmeyer scale experiments.

Data-dependent experiments, combining full-scan MS data with product ion spectra were acquired using a quadrupole-orbitrap MS instrument, in order to identify the molecular ions of the TPs of IOP and OFLOX, to propose empirical formulae and to elucidate their chemical structure. Two different strategies were followed to identify TPs, as described by Helbling et al. and Kern et al. (Helbling et al. 2010;Kern et al. 2009): (a) a suspect exact mass screening of molecular ions of plausible TPs predicted by the UM-PPS software, and accurate masses of known TPs of target compounds, identified in previous biodegradation studies (Engels-Matena 1996;Perez et al. 2006;Schulz et al. 2008;Kormos et al. 2011) and metabolites (Zivanovic et al. 2006). These molecular ions were fitted in an inclusion list within the data dependent experiments. Two procedures were followed to identify TPs by the (a) target approach and (b) a non-target screening, where full-scan MS data of treated samples (t>0) are compared with a sample at t=0 h and controls, to identify compound masses formed during the biodegradation experiment. These masses identified were afterwards added in the inclusion list, and samples were re-injected, in order to achieve information about their MS/MS spectra. For both approaches, the only masses considered as TPs candidates where the ones whose intensity changed over the time course of the experiment, and which were not present in the control samples.

Using these approaches, seven and six major TPs were identified for IOP and for OFLOX, respectively. Detailed information about the TPs identified for each compound is given in the following sections. Chromatograms showing parent compounds and identified TPs are included in Figure X.2.

(A)



(B)

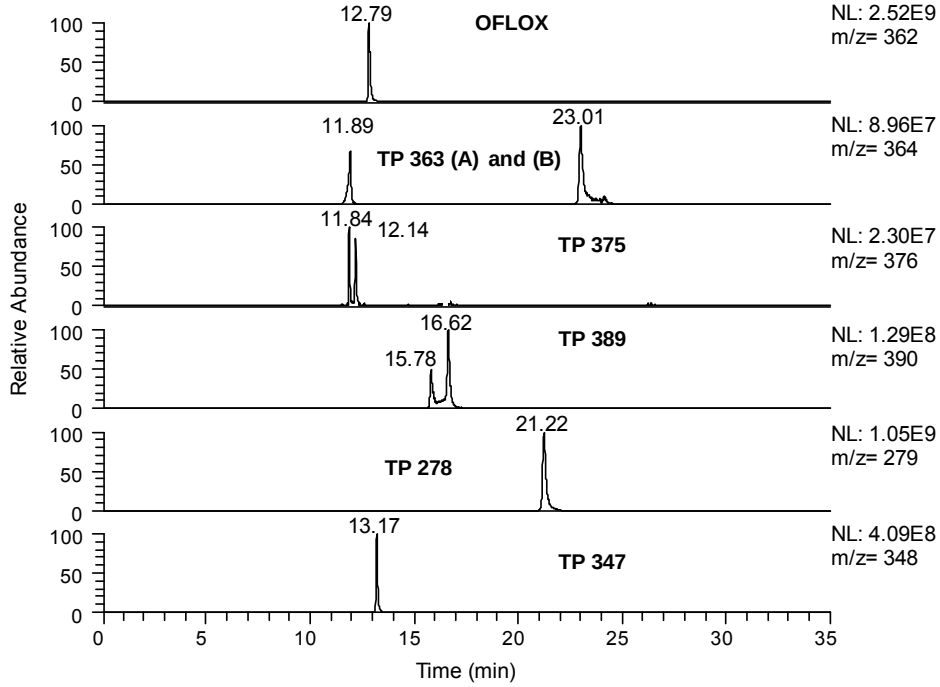


Figure X.2. Extracted ion chromatograms of iopromide (A) and ofloxacin (B) and their corresponding degradation products.

X.3.2.1. IOP degradation products

Table X.2 shows the results of the HRMS measurements of the identified IOP TPs in positive ionization mode, as well as their proposed chemical structures.

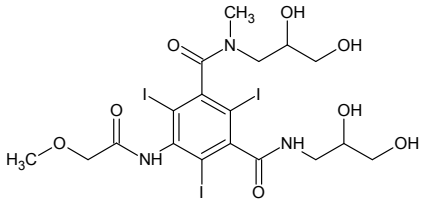
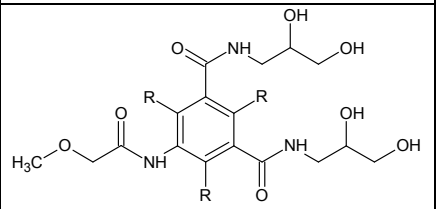
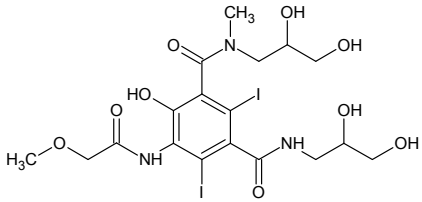
IOP (11.92-12.05 min) formed a protonated molecule at m/z 791.87704 under (+) ESI conditions. Upon fragmentation of the protonated IOP molecule, several fragments were detected, corresponding to the loss of water (m/z 773.86670) followed by loss of HI (m/z 645.95441), the cleavage of the amide bond in side chain A (see SI) (loss of 105 Da, giving rise to m/z 686.79846) or side chain B (-91Da, giving rise to fragment 700.81415) and the subsequent loss of HI (128 Da) to yield m/z 558.88605 and 572.90173, respectively.

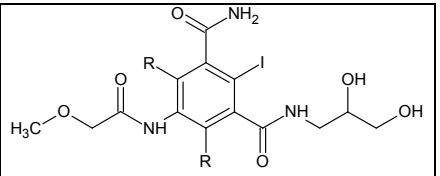
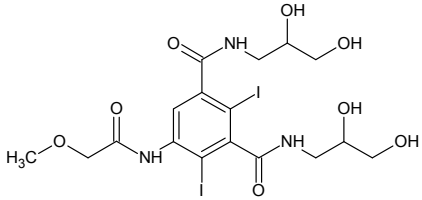
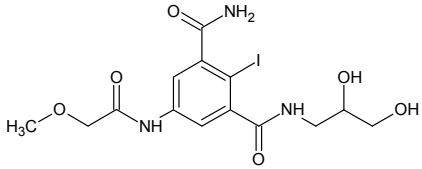
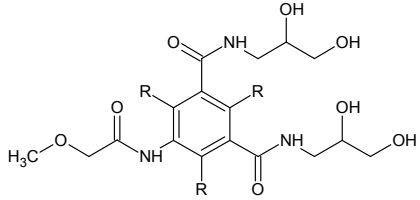
For the TPs, three of them (m/z 466, m/z 526 and m/z 666) were identified by exact mass screening approach, whereas four TPs (m/z 578, m/z 651, m/z 682 and m/z 452) were identified by non-target screening.

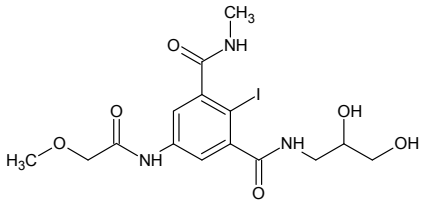
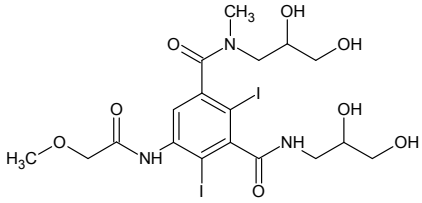
TPs found using the exact mass screening were tentatively identified based on literature search. None of the TPs predicted by the UM-PPS software was identified in the samples. TPs with m/z 466, 526 and 666 have already been previously identified by Engels-Matena (Engels-Matena 1996), when they studied the degradation of IOP by *T. versicolor* in mycological broth cultures. Concerning TP465, the suggested elemental composition is $C_{15}H_{20}O_6N_3I$. The loss of 91 Da, (fragment at m/z 375), together with the fact that no fragments originating from the loss of 105 Da (cleavage of side chain A of the molecule) were observed, indicates that this TP is generated by the scission of the CON (CH_3)-CH_2 bond in side chain A and the loss of two iodine atoms.

For TP525, two chromatographic peaks appeared in ESI (+), sharing the molecular formula of $C_{17}H_{24}O_8N_3I$. The fact that the MS/MS spectra of both closely neighboring peaks is very similar, (they have as a major fragment the one at m/z 435) and that they share the same elemental composition, makes us think that these compounds are isomers. The structure proposed for this compound/s results from the loss of CH_3 from the tertiary amide in side chain A, (fragment at m/z 435, due to the loss of 91 Da, which means that side chain B of the molecule is unchanged).

Table X.2. Accurate mass measurements of Iopromide (IOP) and its transformation products as determined by HPLC-ESI (+)-Qexactive Orbitrap™ in MS and MS/MS mode.

Retention time (min)	Compound	Ion	Measured mass [m/z]	Elemental composition	Calculated mass [m/z]	Relative error [ppm]	Losses to give fragments	RDB	Structure proposed
12.00*	IOP	$[M+H]^+$							
		792	791.87712	$C_{18}H_{25}O_8N_3I_3$	791.87702	-0.114		6.5	
		773	773.86761	$C_{18}H_{23}O_7N_3I_3$	773.86646	1.492	-H_2O	7.5	
		700	700.81329	$C_{15}H_{16}O_6N_2I_3$	700.81369	-0.573	-Amide bond (B)	7.5	
		687	686.79919	$C_{14}H_{14}O_6N_2I_3$	686.79804	1.672	-Amide bond side (A)	7.5	
		646	645.95337	$C_{18}H_{22}O_7N_3I_2$	645.95416	-1.227	-H2O; -HI	8.5	
		573	572.90173	$C_{15}H_{15}O_6N_2I_2$	572.90140	0.578	-Amide bond (B); -HI	8.5	
		559	558.88672	$C_{14}H_{13}O_6N_2I_2$	558.88575	1.738	-Amide bond (A); -HI	8.5	
11.33 •	TP 525 (A)	$[M+H]^+$							 R=2*H, 1*I
		526	526.06818	$C_{17}H_{25}O_8N_3I$	526.06808	0.182		6.5	
		508	508.05811	$C_{17}H_{23}O_7N_3I$	508.05752	1.162	-H_2O	7.5	
		435	435.00510	$C_{14}H_{16}O_6N_2I$	435.00476	0.792	-Amide bond (B)	7.5	
11.58-11.69**	TP 681	$[M+H]^+$							
		682	681.97549	$C_{18}H_{26}O_9N_3I_2$	681.97549	0.291	-	6.5	

11.79	TP 577	$[M+H]^+$ 578 560 487 359 328	577.92781 559.91766 486.86478 358.95227 327.93384	$C_{14}H_{18}O_6N_3I_2$ $C_{14}H_{16}O_5N_3I_2$ $C_{11}H_9O_4N_2I_2$ $C_{11}H_8O_4N_2I$ $C_{10}H_5O_3N_2I$	577.92795 559.91738 486.86462 358.95233 327.93394	-0.238 0.495 0.330 -0.157 -0.294	-H_2O -Amide bond (B) -Amide bond (B); -HI -Amide bond (B); -HI; -CH_3O	6.5 7.5 7.5 8.5 9.0	 R=1*I, 1*H
11.80	TP 651	$[M+H]^+$ 652 634 561 433 402	651.96483 633.95459 560.90173 432.98926 401.97083	$C_{17}H_{24}O_8N_3I_2$ $C_{17}H_{22}O_7N_3I_2$ $C_{14}H_{15}O_6N_2I_2$ $C_{14}H_{14}O_6N_2I$ $C_{13}H_{11}O_5N_2I$	651.96473 633.95416 560.90140 432.98911 401.97072	0.158 0.674 0.591 0.357 0.284	-H_2O -Amide bond (B) -Amide bond (B); -HI -Amide bond (B); -HI; -CH_3O	6.5 7.5 7.5 8.5 9.0	
12.21	TP 451	$[M+H]^+$ 452 434 361 233	452.03124 434.02094 360.96805 233.05580	$C_{14}H_{19}O_6N_3I$ $C_{14}H_{17}O_5N_3I$ $C_{11}H_{10}O_4N_2I$ $C_{11}H_9O_4N_2$	452.03130 434.02094 360.96805 233.05580	-0.143 0.461 0.204 0.576	-H_2O -Amide bond (B) -Amide bond (B); -HI	6.5 7.5 7.5 9.0	
12.24 •	TP 525 (B)	$[M+H]^+$ 526 508 435 307 276	526.06826 508.05795 435.00494 307.09253 276.07419	$C_{17}H_{25}O_8N_3I$ $C_{17}H_{23}O_7N_3I$ $C_{14}H_{16}O_6N_2I$ $C_{14}H_{15}O_6N_2$ $C_{13}H_{12}O_5N_2$	526.06808 508.05752 435.00476 307.09246 276.07407	0.334 0.906 0.424 0.219 0.424	-H_2O -Amide bond (B) -Amide bond (B); -HI -Amide bond (B); -HI; -CH_3O	6.5 7.5 7.5 8.7 9.0	 R=2*H, 1*I

12.88	TP 465	$[M+H]^+$ 466 448 375 247 216	 466.04696 448.03644 374.98355 247.07135 216.05298	 $C_{15}H_{21}O_6N_3I$ $C_{15}H_{19}O_5N_3I$ $C_{12}H_{12}O_4N_2I$ $C_{12}H_{11}O_4N_2$ $C_{11}H_8O_3N_2$	 466.04695 448.03644 374.98355 247.07135 216.05298	 0.011 0.111 -0.204 0.067 0.168	 $-H_2O$ -Amide bond (B) -Amide bond (B); -HI -Amide bond (B); -HI; $-CH_3O$	 6.5 7.5 7.5 8.5 9.0	
12.26-12.60**	TP 665	$[M+H]^+$ 666 648 575 561 447 433	 665.98035 647.97021 574.91748 560.90167 447.00485 432.9832	 $C_{18}H_{26}O_8N_3I_2$ $C_{18}H_{24}O_7N_3I_2$ $C_{15}H_{17}O_6N_2I_2$ $C_{14}H_{15}O_6N_2I_2$ $C_{15}H_{16}O_6N_2I$ $C_{14}H_{14}O_6N_2I$	 665.98038 647.9681 574.91705 560.90140 447.00476 432.98911	 -0.041 0.613 0.750 0.484 0.211 0.495	 $-H_2O$ -Amide bond (B) -Amide bond (A) -Amide bond (B); -HI -Amide bond (A); -HI	 6.5 7.5 7.5 7.5 8.5 8.5	

* For IOP, two chromatographic peaks appear at 11.92 and 12.05minutes, due to isomers

• Two resolved chromatographic peaks at 11.33 and 12.24 with the same elemental composition and MS/MS spectra, attributed to isomers.

** Peak clusters which start at minute 12.26 until 12.60 for TP 665 and at min 11.58 until 11.69 for TP 681 were observed.

RDB: Double bond equivalents.

Amide bond (A): Loss of amide bond from side chain A of the molecule; Amide bond B: Loss of amide bond from side chain B of the molecule.

For TP665, the elemental composition suggested is $C_{18}H_{25}O_8N_3I_2$, where one iodine atom is substituted by one hydrogen atom which can result in three different TPs. The MS/MS spectra of these TP shares many similarities with IOP, such as the loss of 105 Da (cleavage of the amide bond in side chain A), giving rise to fragment at m/z 561, the loss of 91 Da (amide bond cleavage in side chain B), obtaining the fragment at m/z 595 and the fragment at m/z= 447 (loss of one iodine atom).

For the TPs that were only identified by non-target screening, a plausible elemental composition for TP577 is $C_{14}H_{17}O_6N_3I_2$. This compound results from the loss of CH_3 and a cleavage of the N-CH_2 bond from the tertiary amide in side chain A of the molecule, becoming a primary amide. Moreover, when looking at the MS/MS spectra, the fragment at m/z=487 (loss of 91 Da) indicates that side chain B of the molecule remains unchanged.

For TP651 (elemental composition $C_{17}H_{23}O_8N_3I_2$), the net loss of CHI in the elemental composition relative to that of the parent compound, indicates the loss of one iodine atom and also that a N-demethylation occurs. Thus, the tertiary amide in side chain A of the molecule might be converted into a secondary amide. This structure is supported by the fact that in the MS^2 spectrum no fragments corresponding to a 105 Da loss were observed, but instead, a fragment corresponding to the loss of 91Da was detected (m/z 561). For TP451, the suggested elemental composition is $C_{14}H_{18}O_6N_3I$. This TP has a fragmentation pattern very similar to that observed for TP577 (fragment at m/z=361 due to loss of 91 Da).

For TP681, ($C_{18}H_{25}O_9N_3I_2$) the occurrence of two chromatographic peaks could be attributed to isomers. This TP results from the substitution of iodine by one hydroxyl group. Unfortunately, no MS/MS spectrum is available for this structure to prove this hypothesis. TP681, together with TPs 665, 651, 525, 465 and 451, have already been identified by Perez et al. (Perez et al. 2009) who studied the photolysis of IOP under simulated sunlight. For TP525, these authors only detected one compound that eluted later than IOP. TPs 577, 665, 651 and TPs 525 were also identified via ESI (-) analysis.

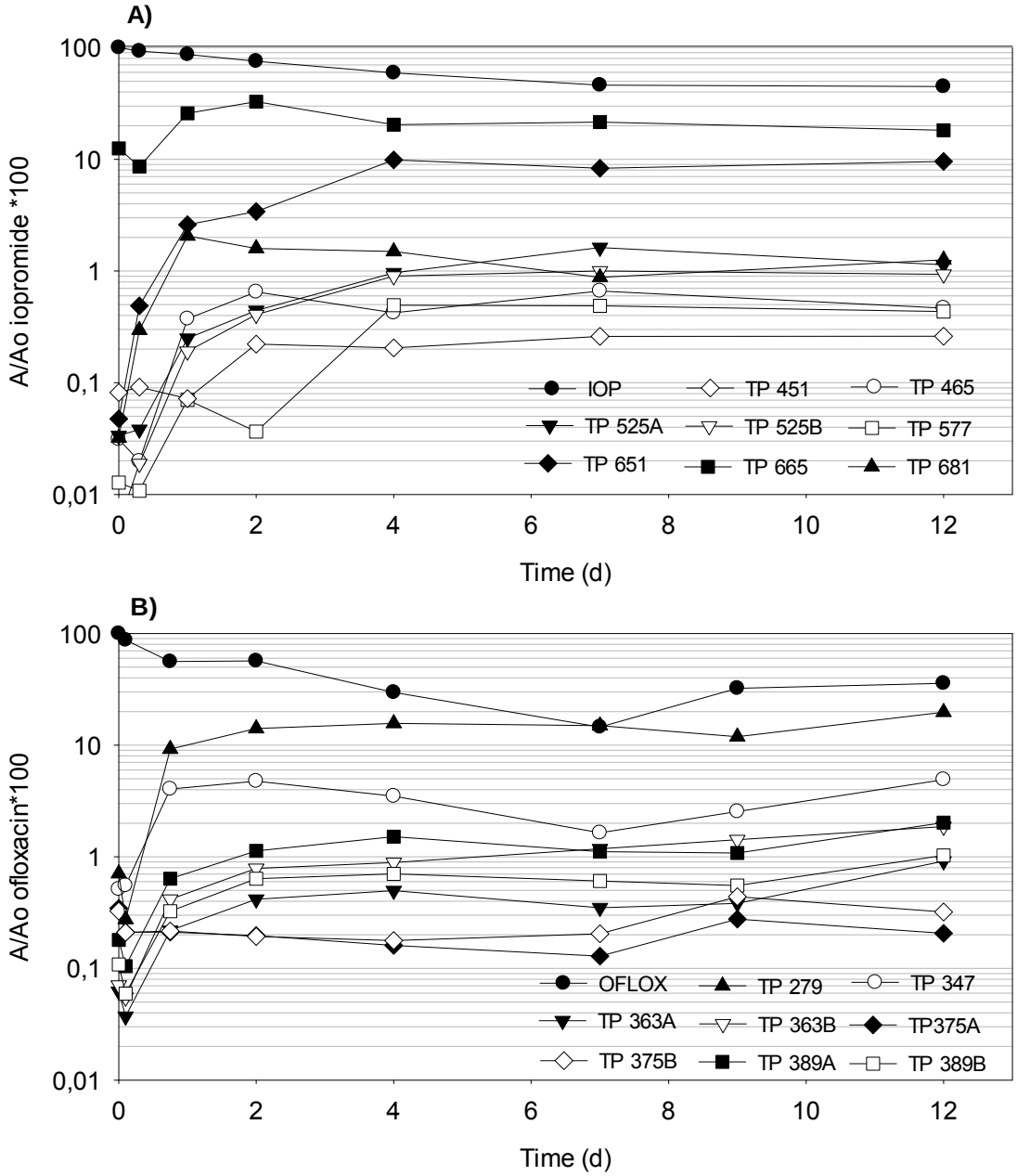


Figure X.3: Semilogarithmic plots for time-course degradation of IOP (A) and OFLOX (B) and evolution of their transformation products in Erlenmeyer flasks using *T. versicolor.* The legend refers to transformation products identified in Figure X.2.

Figure X.3A depicts the decay of IOP and the evolution of its TPs during the experiment in Erlenmeyer flasks. Almost all TPs were detected after 1 day of incubation, although with different rates of production. The concentrations of the TPs generally remained constant after 4 days of incubation. Some exceptions include TP525A, whose concentration increased after seven days and decreased again until 12 days, TP665 which slightly decreased after two days of incubation but remained

constant until the end of the experiment and TP681, whose maximum concentration was found at the first day of incubation, but began to be removed at the end of the experiment. Nevertheless, many of these TPs appear to be persistent given that their concentrations remain constant even after twelve days of incubation.

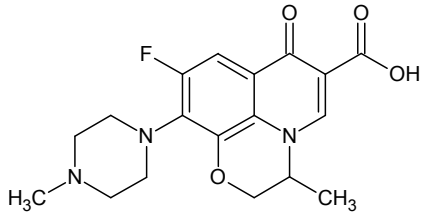
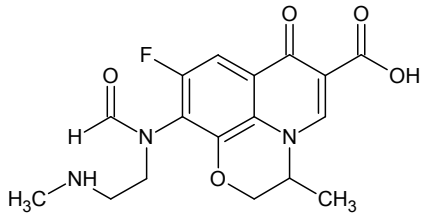
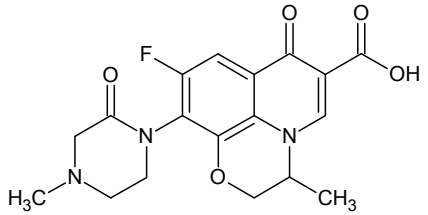
X.3.2.2. OFLOX degradation products

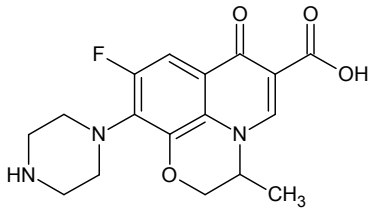
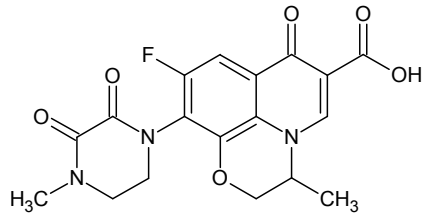
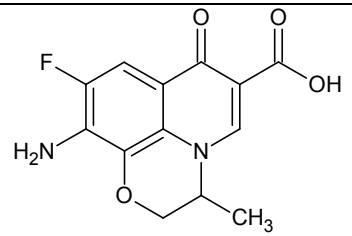
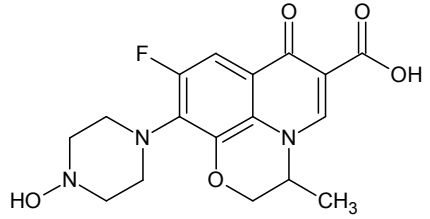
Table X.3. shows the results of the high resolution mass spectrometry measurements of the identified OFLOX TPs and their proposed chemical structures. Unlike IOP, TPs were only detected under positive ionization mode.

Regarding the parent compound, OFLOX (12.79 min) formed a protonated molecule at m/z 362.15115 under ESI (+) conditions. Several fragments are detected, corresponding to the loss of water (m/z 344.14087), loss of CO_2 (m/z 318.16156) and loss of CO_2 and methylaziridine (fragment at m/z 261.10367). For the TPs, three substances (two at m/z 364 and m/z 348) were identified by the exact mass screening approach.

Two chromatographic peaks of m/z 364 share the same elemental composition ($C_{17}H_{18}FN_3O_5$ uncharged molecule). The significant difference in their retention times suggest that these two peaks correspond to two TPs with different chemical structure. The UM-PPS software predicted several structures for this specific elemental composition. The fragments at m/z 333 (loss of $–CH_3NH$) and at m/z 235 (loss of CO_2, -CH_3NH and CHO), suggests that a plausible chemical structure is the one presented in Table X.3. Other important fragments for this TP are the ones observed at m/z 346 (loss of H_2O) and m/z 320 (loss of CO_2). A similar transformation (oxidation and further opening of the pyperazine ring) was reported by Wetzstein and coworkers (Wetzstein et al. 2006) in a study where they describe the patterns of metabolites of the fluoroquinolone antibiotic enrofloxacin produced by *basidiomycetes indogenous.*

Table X.3. Accurate mass measurements of Ofloxacin (OFLOX) and its transformation products as determined by HPLC-ESI (+)-Qexactive Orbitrap™ in MS and MS/MS mode.

Retention time (min)	Compound	Ion	Measured mass [m/z]	Elemental composition	Calculated mass [m/z]	Relative error [ppm]	Losses to give fragments	RDB	Structure proposed
12.79	OFLOX	$[M+H]^+$							
		362	362.15115	$C_{18}H_{21}O_4N_3F$	362.15106	0.089		9.5	
		344	344.10870	$C_{18}H_{19}O_3N_3F$	344.14050	1.086	$-H_2O$	10.5	
		318	318.16156	$C_{17}H_{21}O_2N_3F$	318.16123	1.032	$-CO_2$	8.5	
		261	261.10367	$C_{14}H_{14}O_2N_2F$	261.10338	1.101	$-CO_2$; -Methylaziridine	8.5	
11.89	TP 363 (A)	$[M+H]^+$							
		364	364.13048	$C_{17}H_{19}O_5N_3F$	364.13033	0.425		9.5	
		346	346.12009	$C_{17}H_{17}O_4N_3F$	346.11976	0.951	$-H_2O$	10.5	
		320	320.14096	$C_{16}H_{19}O_3N_3F$	320.14050	1.449	$-CO_2$	8.5	
		333	333.08850	$C_{16}H_{14}O_5N_2F$	333.08813	1.122	$-NHCH_3$	10.5	
		289	289.09863	$C_{15}H_{14}O_3N_2F$	289.09830	1.152	$-CO_2$; $-NHCH_3$	9.5	
		261	261.10373	$C_{14}H_{14}O_2N_2F$	261.10338	1.331	$-CO_2$; $-NHCH_3$; CHO	8.5	
		235	235.08807	$C_{12}H_{12}O_2N_2F$	235.08773	1.436	$-CO_2$; $-NHCH_3$; loss of the cyclohex-2-enone	7.5	
11.84 12.14	TP 375	$[M+H]^+$							
		376	376.13072	$C_{18}H_{19}O_5N_3F$	376.13033	1.049		10.5	
		358	358.11987	$C_{18}H_{17}O_4N_3F$	358.11976	0.305	$-H_2O$	11.5	
		348	348.13593	$C_{17}H_{19}O_4N_3F$	348.13541	1.491	-CO	9.5	
		$[M+H]^+$							
		376	376.13054	$C_{18}H_{19}O_5N_3F$	376.13033	0.571		10.5	
		358	358.11911	$C_{18}H_{17}O_4N_3F$	358.11976	-1.817	$-H_2O$	11.5	
		348	348.13577	$C_{17}H_{19}O_4N_3F$	348.13541	1.032	-CO	9.5	

13.17	TP 347	$[M+H]^+$ 348 330 304 261	 348.13549 330.12497 304.14584 261.10367	 $C_{17}H_{19}O_4N_3F$ $C_{17}H_{17}O_3N_3F$ $C_{16}H_{19}O_2N_3F$ $C_{14}H_{14}O_2N_2F$	 348.13541 330.12485 304.14558 261.10338	 0.228 0.375 0.850 1.101	 -H_2O -CO_2 -CO_2; aziridine	 9.5 10.5 8.5 8.5	
15.78 16.62	TP 389	$[M+H]^+$ 390 372 362 $[M+H]^+$ 390 372 362	 390.10995 372.09933 362.11536 390.10966 372.09930 362.11502	 $C_{18}H_{17}O_6N_3F$ $C_{18}H_{15}O_5N_3F$ $C_{17}H_{17}O_5N_3F$ $C_{18}H_{17}O_6N_3F$ $C_{18}H_{15}O_5N_3F$ $C_{17}H_{17}O_5N_3F$	 390.10959 372.09903 362.11468 390.10959 372.09903 362.11468	 0.923 0.819 1.891 0.180 0.738 0.952	 -H_2O -CO -H_2O -CO	 11.5 12.5 10.5 11.5 12.5 10.5	
21.22	TP 278	$[M+H]^+$ 279 261 238	 279.07775 261.06726 238.03874	 $C_{13}H_{11}O_4N_2F$ $C_{13}H_{10}O_3N_2F$ $C_{10}H_7O_4N_2F$	 279.07756 261.06700 238.03844	 0.675 1.008 1.275	 -H_2O	 8.5 9.5 8.0	
23.01	TP 363 (B)	$[M+H]^+$ 364 346	 364.13054 346.12009	 $C_{17}H_{19}O_5N_3F$ $C_{17}H_{17}O_4N_3F$	 364.13033 346.11976	 0.507 0.951	 -H_2O	 9.5 10.5	

RDB: Double bond equivalents.

For TP363B one tentative structure that we propose (Table X.3) corresponds to an N-hydroxylation of the secondary amine in the piperazine ring. This assumption is based mainly on its HPLC retention time. This TP is expected to be less polar than OFLOX and this is in agreement with the fact that it elutes later than the parent compound. Unfortunately, the identity of this TP cannot be accurately confirmed by MSMS analysis, due to poor fragmentation.

For TP347 (elemental composition of $C_{17}H_{18}FN_3O_4$) the loss of one carbon and two hydrogen atoms suggests that this TP results from the loss of the methyl group bound to the nitrogen in the piperazine ring. This structure was also predicted by the UM-PPS software and confirmed by the fragments observed in the MS/MS spectrum (see Table X.3). Similar transformations were reported by Prieto et al. (Prieto et al. 2011) and by Wetzstein et al. (Wetzstein et al. 1998;Wetzstein et al. 1999) for the antibiotics norfloxacin, ciprofloxacin and enrofloxacin.

For the TPs detected by non-target screening, three substances (without taking into account possible isomers) were identified (m/z 390, m/z 376 and m/z 279). For TPs 389 and 375, two chromatographic peaks eluting very close to each other were found (see Figure X.2), which shared the same exact mass and elemental composition ($C_{18}H_{16}FN_3O_6$ for TP389 and $C_{18}H_{18}FN_3O_5$ for TP375, uncharged molecule). Due to poor fragmentation, it is difficult to assign a plausible chemical structure for both substances with a high degree of certainty. However, since the two compounds shared the same fragments (fragment at m/z 372, for TPs 389, and at m/z 358 for TPs 375, due to the loss of water, and fragments at m/z 362 for TPs 389 and at m/z 348 for TPs 375 due to the loss of CO) these two peaks might correspond to isomers. For TPs 389, the loss of four hydrogen atoms and the presence of two additional oxygen atoms, in comparison with OFLOX elemental composition, indicate the formation of an oxamide, while for TPs 375, the loss of two hydrogen atoms and the presence of one additional oxygen atom points out that this TP originates from the hydroxylation of only one carbon in the piperazine ring and further oxidation. These transformations were also identified by Wetzstein et al. (Wetzstein et al. 2006).

For TP278 (elemental composition $C_{13}H_{11}FN_2O_4$), the structure proposed is confirmed by its retention time (which is presumably less polar than the parent compound) and by the MS/MS spectrum (none of the characteristic fragments of the piperazinyl group are observed). A similar transformation was also observed by Prieto et al.(Prieto et al. 2011) and by Wetzstein et al.(Wetzstein et al. 1997).

Figure X.3B shows the decay of OFLOX and the evolution of its TPs during the experiment in Erlenmeyer flasks. Few TPs were formed during the first hours of exposure, at different rates of production, but the majority of them were generated after 18 hours of incubation. Generally, TPs increased until 4 d, then, their concentration remained constant or decreased up to 9 d (TPs 389 and 363A). A slight increase in TP concentration was observed at the end of the experiment. An exception to this is TP363 (B) whose concentration increased during the entire experiment.

Aside from these TPs, there were three other compounds (at m/z 378, 336 and 318), which were detected in both the samples incubated with the fungi and the control samples. They were identified due to their presence in the inclusion list of exact masses (prediction by UM-PPS software and literature search). The structure and evolution of these compounds throughout the duration of the experiment is discussed in the SI.

X.3.3. HWW treatment in bioreactor

The next step to assess the potential use of this technology is the implementation of treatment by WRF in bioreactors. As a first approach, the degradation capability of WRF was tested using sterile wastewater, in order to evaluate whether the fungus was able to degrade pharmaceuticals in real wastewater matrix without any other competing microorganisms. Secondly, the degradation capability was also tested in non sterile wastewater, in order to assess their performance under more realistic conditions.

For the analysis of samples and monitoring of the occurrence of IOP, OFLOX and their TPs in bioreactor samples, an exact mass screening approach (similar to the one used for the analysis of samples from Erlenmeyer experiments) was followed. For

some TPs the MS/MS spectra was not available, hence, their tentative identification was based only on accurate mass measurements and isotopic profile in the full-scan MS.

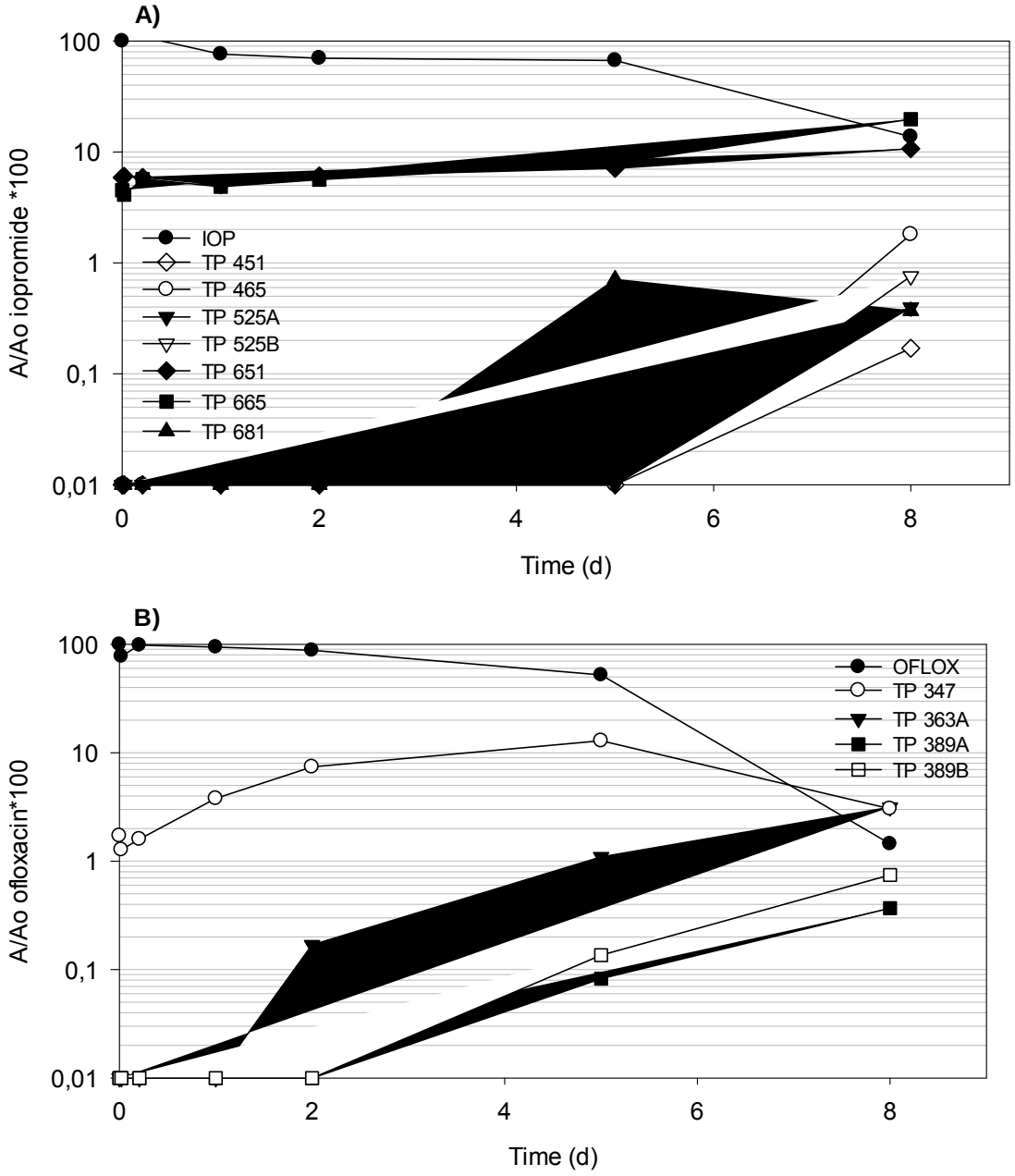


Figure X.4: Semilogarithmic plots for time-course degradation of IOP (A), OFLOX (B) and transformation products in a batch bioreactor fed with sterile real hospital wastewater. The legend refers to transformation products identified in Figure X.2.

Figure X.4 depicts the degradation of IOP and OFLOX in bioreactor load with sterile HWW and the evolution of the TPs identified in Erlenmeyer experiments. Regarding IOP TPs, almost all degradation products identified in Erlenmeyer

experiments were found in bioreactor samples (specifically TPs 465, 665, 651, 451, 681, 525 (A and B). However, for OFLOX, only TPs 363A, TP389 and TP347 were detected. The fact that not all TPs were identified in bioreactor samples can be due to that they are formed at concentrations under the detection limits since lower concentrations of parent compounds (105 μg L^{-1} and 32 μg L^{-1} were found in sterile HWW for IOP and OFLOX, respectively).

At the end of the experiment, only 13% IOP was remaining in the medium (8 days of treatment) while OFLOX was almost completely removed (98.5%). These results together with those obtained in Erlenmeyer evidence the high degradability demonstrated by both IOP and OFLOX when exposed to WRF.

For IOP, TP651 and TP665 are the most significant ones detected, forming at the beginning of the experiment and reaching fairly constant levels until 8 days of treatment. Those TPs were also the most significant ones (highest intensities) in the Erlenmeyer flask experiments. TP681 is formed after the second day of treatment while the other TPs are mostly generated at the last days of treatment.

For OFLOX, TP363A and TP389 appeared at approximately the second day of treatment and kept increasing until the end of the experiment. In contrast, the concentration of TP347 slightly increased along the experiments.

In an attempt to obtain further insights in the process under even more realistic conditions, the same batch bioreactor treatment was performed using non-sterile hospital wastewater.

IOP and OFLOX were detected in the non-sterile hospital wastewater at 419.7 μg L^{-1} and 3.3 μg L^{-1}, and as indicated in Figure X.5, they were also highly removed, achieving elimination percentages of 65.4% and 99%, respectively, after 5 d of the treatment and remaining constant until the end of the experiment.

In the non-sterile treatment, only 3 IOP TPs were detected (Fig. 5A). TP665 was initially found in the wastewater and its concentration increased concomitantly with IOP degradation until the end of the treatment. Both TPs 651 and 681 were detected after 5 d and remained in the medium until the end of the experiment. For OFLOX all

TPs identified in Erlenmeyer experiments were also detected under non-sterile conditions. While TP347 seems to follow the same profile than in above experiments (increasing during the first day of the treatment) the rest of them were not detected until day 5, with the exception of TP375 which was detected only after 8 d of the treatment.

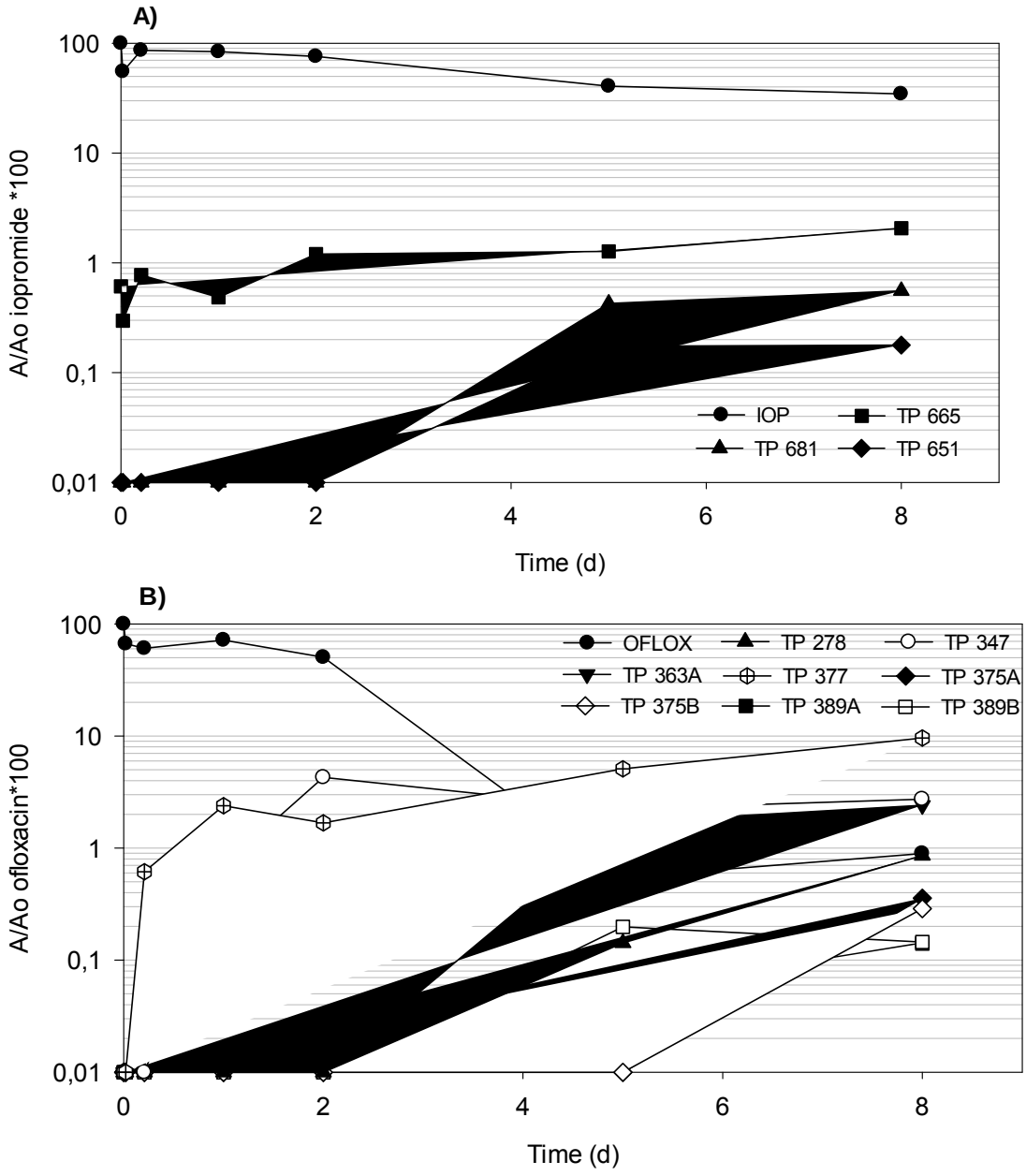


Figure X.5: Semilogarithmic plots for evolution of IOP (A) and OFLOX (B) with their respective transformation products in batch bioreactor fed with non-sterile real hospital wastewater.

Regarding the parameters of both sterile and non-sterile treatments (data not shown), laccase activity achieved its maximum around 90 U L^{-1} on day 2 and 300 U L^{-1} on day 5 respectively. However, very low activities were detected at the end of the experiment. Glucose was not accumulated during the experiment, which means that the fungus was active throughout the experiment, although consumption of glucose by other microorganisms in the non-sterile treatment cannot be ruled out.

X.3.4. Toxicity evaluation

Acute toxicity was determined with the bacterium *V. fischeri* (Microtox test) in order to evaluate whether transformation products were less toxic than the parent compounds, which is the most important goal of any treatment (see results in table X.4). In Erlenmeyer experiments, non-biotic IOP controls as well as the samples taken during the first hours of the IOP experiments, showed a 15 min EC_{50} below 2%, which indicates a high toxicity. After 1 day of treatment, the toxicity decreased to values of 43%, going down until reaching the lower toxicity level (110%) at the last day (day 12) of the experiment. These values indicate that the overall toxicity of the TPs generated from IOP were less toxic than the parent compound.

For OFLOX treatment, non-biotic control showed a 15 min EC_{50} of 40%, and during the treatment (first 4 d) the toxicity values decreased around levels of 80-90%. In the last samples of OFLOX treatment, the EC_{50} remained constant at 40%. Hence, it can be concluded again that, at the end of the experiment, the overall toxicity of OFLOX TPs is lower than the original parent compound.

Sterile hospital wastewater showed a 15 min EC_{50} of 4%. After 1 d the toxicity was reduced to values of 50% and on day 5 the lowest value of toxicity was observed (more than 100%). However, at the end of the treatment the toxicity rises to values of 12%. Nevertheless, toxicity is reduced by three times in comparison with the beginning of the treatment. In the case of the non-sterile hospital wastewater treatment, the toxicity followed the same profile as in sterile wastewater, but toxicity was already lower at the beginning of the treatment (30%). Toxicity remained constant until day 5,

where a considerable toxicity reduction was observed (EC_{50} of 25% at the end of the experiment).

Table X.4. Microtox test values in both Erlenmeyer and batch bioreactor treatments**.**

Erlenmeyer experiments (EC_{50}, %)		
Time	**Iopromide**	**Ofloxacin**
Non-biotic control	2	40
15 min	2	41
8 h	1	81
1 d	43	88
4 d	66	93
7 d	87	46
12 d	110	42
Batch bioreactor treatment (EC_{50}, %)		
Time	**Sterile**	**Non-sterile**
Initial	4	30
30 min	4	20
5 h	7	16
1 d	50	29
5 d	149	122
Final (8 d)	12	25

Overall, the toxicity values observed provide evidence that the degradation of IOP and OFLOX by *T. versicolor* leads to the formation of transformation products that are less or equally toxic than the parent compound themselves, and that a reduction of the toxicity can be obtained after the treatment of hospital wastewater.

X.4. Conclusions

Results derived from this study pointed out that *T. versicolor* is capable of efficiently degrading IOP and OFLOX in a defined medium in an air pulsed fluidized bed bioreactor load with real hospital wastewater under sterile and non-sterile conditions. Degradation of both compounds in all treatments was almost complete. Indeed, elimination was more efficient in the hospital wastewater treatment than in a defined medium with the contaminant spiked at high concentration. A significant number of TPs were identified in experiments at Erlenmeyer scale. In batch bioreactor experiments, only the most prominent OFLOX TPs were identified while almost all the IOP TPs were detected.

Acute toxicity tests revealed that the resulting treated broth in both Erlenmeyer and batch bioreactor experiments were less or equally toxic than the initial samples. Obtained results can help to take decisions in the application of possible continuous treatments. Applied treatment might be a good strategy for the degradation of relevant pharmaceuticals in highly polluted wastewaters, such as hospital wastewaters, and that it could be successfully applied as a pre-treatment of this potentially hazardous wastes.

X.5. References

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Chapter XI:

Hospital wastewater treatment by fungal bioreactor: removal efficiency for Pharmaceuticals and Endocrine Disruptor Chemicals.

Manuscript draft: Cruz-Morató, C., Lucas, D., Llorca, M., Gorga, M., Rodríguez-Mozaz, S., Barceló, D., Marco-Urrea, E., Sarrà, M., Vicent, T., Hospital wastewater treatment by fungal bioreactor: removal efficiency for Pharmaceuticals and Endocrine Disruptor Chemicals

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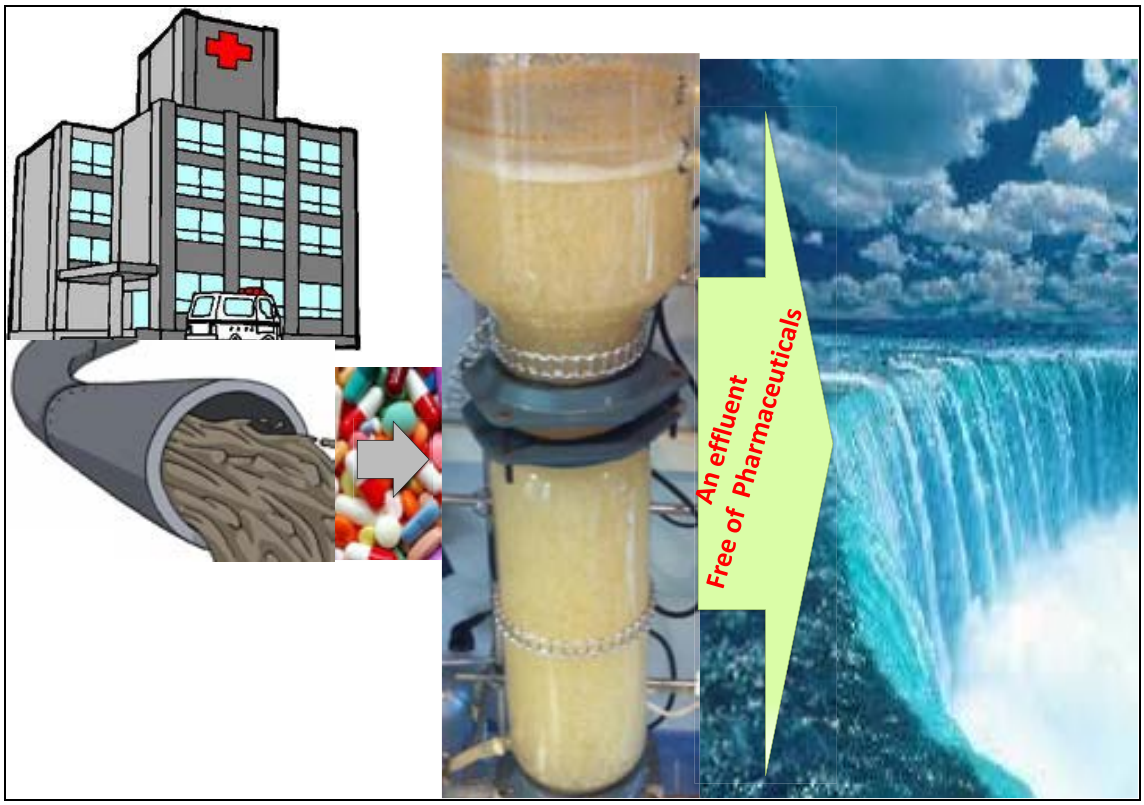

Abstract

Hospital effluents present a high load of pharmaceutical active compounds (PhACs) and endocrine disruptor chemicals (EDCs) are considered as the main source of the pollution of those micro-pollutants to the environment. Nowadays, hospital wastewaters are co-treated with urban wastewater; however, the dilution factor and the inefficiency of wastewater treatment plants in the removal of PhACs makes inappropriate co-treat both effluents. In this paper, a new alternative to pre-treat hospital wastewater concerning the removal of PhACs is presented. The treatment was carried out in a batch fluidized bed bioreactor under non-sterile conditions with *Trametes versicolor* pellets. Glucose and ammonium tartrate were continuously supplied as nutrients, and pH was maintained at 4.5. Results pointed out that 51 out of the 57 detected PhACs were partially (35 %) to complete removed. In addition, Microtox test showed reduction of wastewater toxicity after the treatment. Hence, the good efficiency of the fungal treatment regarding removal of the wide diversity of PhACs detected in hospital effluents is demonstrated.

Keywords: Pharmaceuticals, hospital wastewater, *Trametes versicolor*, degradation, bioreactor.

XI.1. Introduction

Pharmaceutical active compounds (PhACs) comprise one of the most common groups of organic micro-contaminants present in different environmental water compartments, detecting them on the range of ng L^{-1} to µg L^{-1} (Heberer, 2002; Tixier et al., 2003; Kasprzyk-Hordern et al., 2008; Sim et al., 2010). The primary source of PhACs pollution in the environment has been shown to be through wastewater treatment plant (WWTP) effluents (Suárez et al., 2009; Jelic et al., 2011). WWTPs are not designed to remove these contaminants and, therefore, many of them pass unchanged reaching surface water (Verlicchi et al., 2012a).

The main entry of PhACs into the water cycle occurs after their administration, in households or hospitals, and their subsequent excretion by the urine and faeces. Another portion of the contamination that some authors have reported is the contamination attributed to the direct disposal in drains (Emmanuel et al., 2005; Verlicchi et al., 2010), although the best practices adopted currently in the waste management in hospitals and research centers rule out this possibility. Since PhACs could be found at higher concentrations (up to mg L^{-1}) in hospital wastewaters, some authors consider this effluent as the most important source for this type of pollutants in the WWTPs influents (Verlicchi et al., 2012b). Nevertheless, other authors are in controversy because they say that the amount of PhACs contributed by the hospital effluent is insignificant compared to the large flow and the large amounts of PhACs presents in urban wastewater. Hospital effluents are considered, in general, to have the same pollutant load as urban wastewaters (in terms of DBO and nitrogen) and are discharged into public sewer networks being co-treated with urban wastewater (Corre et al., 2012). The scientific community recommends a pre-treatment of hospital effluents before being discharged into public sewage (Altin et al., 2003; Pauwels and Verstraete, 2006), which is a sustainable practice and follow the guidelines proposed by the European Union regarding treating the pollution at source. Hospital wastewater can be over 150 times more concentrated in micropollutants than urban wastewater (Verlicchi et al., 2010). The mixture of both effluents only would dilute the contaminants without solving the specific problem of contamination (Verlicchi et al.,

2010). The treatment of the hospital wastewater separately allows the specific degradation of these pollutants since they could be more accessible for biological treatments avoiding (Kovalova et al., 2012).

The detection of PhACs in the environment has been complemented with the evaluation of the impact of these pollutants on aquatic ecosystems. Ecotoxicity studies have shown the potential risk of the hospital wastewater (Jean et al., 2012; Orias and Perrodin, 2013). For example, Santos et al. (2013) identified several PhACs in hospital wastewater as potentially hazardous to aquatic organisms, showing that especial attention must be paid to antibiotics. Although chronic ecotoxicity data is scarce compared to acute studies, accumulative effects have been shown to damage of some ecosystems (Stuart et al., 2012).

Hence, the high load of PhACs in hospital wastewater and the possibility to reach the environment, causing bioaccumulation and ecotoxic problems (Verlicchi et al., 2012b), denotes the importance to find efficient treatments for removing them from the specific hospital effluent.

First studies regarding treatment of hospital wastewater by physicochemical processes, as advanced oxidation processes, nanofiltration and reverse osmosis, have been appeared during last 3 years (Beier et al., 2010; Koehler et al., 2012; Nielsen et al., 2013). Even though the majority of these techniques are very efficient in the PhACs removal, the main limitation is the formation of undesirable, and sometimes toxic, transformation products (Oller et al., 2011). Moreover, these processes require high energy consumption and, then, an effort must be done to find a suitable biological treatment. The only biological process that has been developed to treat hospital wastewater is membrane bioreactors (MBR). Beier et al. (2012) reported the high efficiency removing trace pollutants for hospital wastewater. Nevertheless, PhACs were not analyzed in their studies. However, it is noteworthy that studies with urban wastewater with MBR demonstrated their inefficiency to degrade some recalcitrant PhACs as for example psychiatric drugs (carbamazepine) (Radjenović et al., 2009).

On the other hand, White-Rot fungi (WRF) have demonstrated their capability to transform and/or remove PhACs (Cruz-Morató et al., 2012). In particular, *Trametes*

versicolor have shown to be an attractive fungus for designing effective bioremediation strategies for PhAC due to their unspecific oxidative enzymatic system, which includes ligninolytic extracellular enzymes as laccase and potential peroxidases, as well as intracellular enzymes as cytochrome P450 system (Asgher et al., 2008).

First studies of PhACs removal with *T. versicolor* were carried out under sterile conditions with defined medium, in Erlenmeyer scale and with single spiked pollutants. PhACs that have been removed by *T. versicolor* under above conditions include β-blockers (Marco-Urrea et al., 2010a), antiinflamatories (Marco-Urrea et al., 2009, 2010b, c and d), antibiotics (Rodríguez-Rodríguez et al., 2012; Prieto et al., 2011), psychiatric drugs (Jelić et al., 2012), iodinated contrast agents (Rode and Müller, 1998) and endocrine disruptors chemicals (Blánquez et al., 2008b; Cajthalm et al., 2009). In addition, continuous bioreactors for the elimination of the single lipid regulator clofibric acid under sterile conditions showed the good efficiency of the process to remove that pollutant (Cruz-Morató et al., 2013a). Ecotoxicological assessment of treated effluents has also to be performed since transformation products formed from the target contaminants during treatment may exhibit higher toxicity than parent compounds.

In order to approach real scale processes, the experiments must be performed in bioreactors with non-sterile conditions and using real wastewaters, where many contaminants (at different concentrations from ng L^{-1} to mg L^{-1}) and microorganisms are presents. However, to date few studies have been published regarding bioreactor process of real wastewater with fungi and none of them using hospital wastewater. As far as we know, there are few works attempting the elimination of PhACs in these conditions. Zhang and Geiβen (2012) observe 80% removal of carbamazepine (spiked at 5 mg L^{-1}) in non-sterile urban wastewater by the WRF *Phanerochaete chrysosporium,* immobilized in polyether foam and achieving stable continuous operation during 100 days in a bioreactor. Yang et al. (2013) performed a continuous fungal membrane bioreactor in a non-sterile environment, with *T. versicolor* and a hydraulic retention time of two days, obtaining stable removal of spiked bisphenol A (80-90%) and diclofenac (55%), loading at 475 and 112 µg L^{-1}, respectively. Cruz-Morató et al. (2013b) treated urban wastewater in non-sterile conditions in a batch

fluidized bed biorreactor containing *T. versicolor* pellets with PhACs presents at their environmental concentration. The authors observed the completely removal of 7 out of the 10 initially detected PhACs, and 2 out of that 10 were partially removed. A reduction in the toxicity analyzed by Microtox test was also observed, demonstrating the feasibility of the treatment. The batch fluidized bed bioreactor used in the urban wastewater treatment have not only showed the efficiency in the removal of PhACs, but also for specific industrial effluents as dyes demonstrating its potential in bioremediation (Blánquez et al., 2008b).

The aim of this study is the treatment of a hospital wastewater by the WRF *T. versicolor* to degrade PhACs presents in hospital wastewater at their environmental concentrations and at both sterile and non-sterile conditions.

XI.2. Materials and Methods

XI.2.1. Fungus and chemicals

T. versicolor (ATCC#42530) was from the American Type Culture Collection and was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at 25ºC. Subcultures were routinely made every 30 days.

Pellet production was done as previously described by Blánquez et al. (2004). Pellets obtained by this process were washed with sterile deionized water.

Pure standard of the target estrogens estradiol (E2) and its main metabolites estriol (E3) and estrone (E1), 17α-ethynylestradiol (EE2), diethylstilbestrol (DES), estriol 3-sulfate (E3-3S), estradiol 17-glucuronide (E2-17G), estrone 3-glucuronide (E1-3G), estriol 16-glucuronide (E3-16G), triclosan (TCS), methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), benzylparaben (BeP), labelled bisphenol A (BPA-d_{16}), 4-tert-octylphenol (OP), labelled octylphenol (OP-d_2), labelled 4-tert-octylphenol-3,5 d_2-diethoxylate (OP_2EO-d_2), labelled triphenyl-d_{15}-phosfate, caffeine and labelled caffeine C_{13} were purchased from Sigma-Aldrich (St Louis, MO, USA). Triclorocaraban (TCC), benzylparaben (BeP), bisphenol A (BPA) and 5-methyl-1H-benzotriaxole (tolytriazole; TT), tris(2-butoxyethyl) phosphate (TBEP) and tris(2-chlorethyl) phosphate (TCEP) were supplied by Aldrich (Milwaukee, WI, USA). Nonylphenol (NP), labelled nonylphenol (NP-d8), octyl- and nonlylphenol monocarboxylate (OP_1EC and NP_1EC, respectively),

octyl- and nonylphenol monoethoxylate (OP_1EO and NP_1EO, respectively), octyl- and nonylphenol diethoxylate (OP_2EO and NP_2EO, respectively) and labelled nonylphenol monoethoxylate (NP1EO d_2) were purchased from Dr. Ehrenstorfer (Germany). Labelled estradiol (E2-d_5), labelled estrone (E1-d_4), labelled 17α-ethynylestradiol (EE2-d_4) and labelled estriol 3-sulfate (E1-3S-d_4) were also obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Benzotriazole (BT), tris(clorosiopropyl) phosphate (TCPP), labelled ethynylhydroxibenzoate C_{13} and labelled benzotriazole (BT-d_4) were purchased from Fluka (Buchs, Switzerland).

Glucose, ammonium tartrate dibasic and malt extract were purchased from Sigma-Aldrich (Barcelona, Spain).

XI.2.2. Hospital wastewater samples

Hospital wastewater samples were collected from the main sewer of Girona Universitary Hospital Dr. Josep Trueta (Girona, Spain). Table XI.1 shows the characteristics of the wastewater. Sample 1 was sterilized at 121 ºC during 30 min. Sample 2 was directly used at non-sterile conditions. Those samples were taken at different days.

Table XI.1: Characteristics of the hospital wastewater samples used in the sterile and non-sterile treatment.

Hospital wastewater samples	COD (mg L^{-1})	TOC (mg L^{-1})	$N\text{-}NH_4^+$ (mg L^{-1})	TSS (mg L^{-1})	Conductivity (µg cm^{-1})	pH
Sample 1 (sterile treatment)	490	261	68	11.1	1455	8.3
Sample 2 (non-sterile treatment)	357	153	49	6.1	1194	8.1

XI.2.3. Batch bioreactor treatment

A glass air fluidized bed bioreactor with a useful volume of 10 L (Blánquez et al., 2008a) was used to carry out the treatment of hospital wastewater under sterile and non-sterile conditions. Approximately, 1.4 g d.w. biomass L^{-1} was inoculated into the bioreactor. Fungal biomass was maintained fluidized by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 s open, 5 s close) and the air flow was 12 L h^{-1}. The bioreactor was equipped with a pH controller in order to maintain pH at 4.5 by the addition of NaOH and the temperature was maintained at 25 ºC. As a nutrients source, glucose and ammonium tartrate were fed continuously from their stock solution (300 g L^{-1} and 675 mg L^{-1}, respectively) at a flow rate of 0.96 mL h^{-1} to ensure an uptake rate of 0.439 g glucose g^{-1} dry weight (d.w) pellets d^{-1} and 1.98 mg ammonium tartrate g^{-1} d.w. pellets d^{-1} (Casas et al., 2013). For the sterile treatment, the bioreactor and the wastewater were autoclaved at 121 ºC for 30 min. In both non-sterile and sterile treatments, 200 mL samples were taken from the middle of the bioreactor at times 0 min, 15 min, 8 h, 1 d, 2 d, 5 d and 8 d.

XI.2.4. Analytical procedures

XI.2.4.1. Analysis of pharmaceuticals in hospital wastewater

The method applied for the PhACs analysis was previously developed by Gros et al. (2012). Firstly the water samples were filtered through 0.45µm nylon membrane filters (Whatman, U.K.) in order to retain suspended solids. A certain volume of the chelating agent EDTA was added to all of the samples to a final concentration of 3% (ml solute ml^{-1} solution), as it is well known that enhances the extraction of some PhACs. Pre-concentration of samples was performed by SPE (Solid Phase Extraction) using a Baker (J.T.Baker®) and Oasis HLB 3cc, 60 mg, extraction cartridges (Waters Corp. Mildford, MA, USA). According to the method previously mentioned cartridges were conditioned using 5 mL of methanol followed by 5 mL of water HPLC grade at 1 mL min^{-1}; then 50 mL of each sample were loaded at 1 mL min^{-1}. Elution of the samples was done passing 6 mL of pure methanol at a flow rate of 2 mL min^{-1} through the cartridges. The extracts were evaporated under nitrogen stream using a Reacti-Therm 18824 system (Thermo Scientific) and reconstituted with 1 mL of methanol-water

(10:90 v/v). Lastly, 10 µL of standard of internal standard mix at 10 ng μL^{-1} were added in the extracts for internal standard calibration and to compensate, if it was necessary, a possible matrix effect.

Chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3 column (50 mm x 2.1 mm i.d. 1.7 µm particle size; Waters Corp. Mildford, MA, USA) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) for the ones analyzed under negative electrospray ionization (NI), both purchased from Waters Corporation. The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All transitions were recorded by using the Scheduled MRMTM algorithm and the data were acquired and processed using Analyst 2.1 software. Analytical parameters as limits of detection and quantification are showed in previous published article (Gros et al., 2012).

XI.2.4.2. Analysis of endocrine disruptors and related compounds in hospital wastewater

The EDCs and related compounds were analyzed by on-line turbulent flow chromatography coupled to a LC-(ESI)-MS/MS system through the methodology previously developed by Gorga et al. (2013). The compounds were analyzed according to their more efficient ionization mode in the ESI source (positive or negative mode). Very brief, 5 ml of samples were spiked with a mixture of internal standards (in methanol) for final concentration of 2 ng ml^{-1} (in vial). Then, all the volume was filtered through 0.45 µm Nylon syringe filters (ACEFESA) and introduced into on-line LC-vials. Thermo Scientific EQuan MAX Plus chromatographic system (Thermo Fisher Scientific) was used for purification and separation purposes. This system comprises an Accela Open AS auto sampler and two mixing quaternary pumps (eluting pump and loading pump). The entire system was controlled via Xcalibur 2.1 software. The system was adapted with different turbulent flow chromatographic columns (TFC) for purification

purposes (Cyclone P, 50 × 0.5 mm, 60 µm particle size, 60 Å pore size in negative mode and Cyclone MCX 50 × 0.5 mm, 60 µm particle size, 60Å pore size in positive mode (Thermo Fisher Scientific, Franklin, MA)); the separation of target analytes was achieved using a Hypersil GOLD (50 x 2.1; 1.9 µm) analytical column (Thermo Fisher Scientific). The procedure was adapted from a previous method developed using the EQuan on-line sample enrichment system (Gorga et al. 2013). The method consists in a first loading step of the sample into the TFC column and retention of the analytes followed by a transfer step in which the analytes of interest were desorbed from the TFC column onto the analytical column through the same gradient used for analytes separation in the LC system. More detailed information about the chromatographic method can be seen in Table XI.2 from the Supporting Information. The injected volume was 250 µl with a total run time for each injection of 13 min.

The chromatograph was coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a Turbo Ion Spray source. The ionization of the compounds was performed under negative or positive mode, depending on the analyte (Gorga et al. 2013). Acquisition was performed in selected reaction monitoring mode (SRM) to obtain enough identification points (IP) for confirmation of each analyte according to Commission Decision 2002/657/EC (European_Commission, 2002).

Table XI.2: Chromatographic conditions for the analysis of EDCs and related compounds.

On-line TFC							Connection	LC				
Time (min)	**Flow (ml/min)**	**A%**	**B%**	**D%**	**E%**	**Step**		**Time (min)**	**Flow (ml/min)**	**F%**	**G%**	**Step**
Positive ionization mode												
0.00	1.0	5	95			Loading	Out	0.00	0.3	50	50	Initial conditions
1.45	1.0	5	95			-	In	1.45	0.3	50	50	TFC Elution and LC separation
1.75	0.0	50	50			-	In	1.75	0.3	50	50	TFC Elution and LC separation
2.50	0.0	100	0			-	In	2.50	0.3	70	30	TFC Elution and LC separation
5.50	0.0	100	0			-	In	5.50	0.3	100	0	TFC Elution and LC separation
8.50	0.0	100	0			-	In	8.50	0.3	100	0	TFC Elution and LC separation
10.0	0.0	5	95			Initial conditions	Out	10.0	0.3	50	50	Initial conditions
11.0	0.0	5	95			Cleaning	Out	11.0	0.3	50	50	Initial conditions
Negative ionization mode												
0.00	1.0	0	0	100	0	Loading	Out	0.00	0.3	50	50	Initial conditions
0.25	1.0	0	0	0	100	Loading	Out	0.25	0.3	50	50	Initial conditions
1.25	1.0	0	100	0	0	-	In	1.25	0.3	50	50	TFC Elution and LC separation
1.75	0.0	0	100	0	0	-	In	1.75	0.3	50	50	TFC Elution and LC separation
2.00	0.0	0	100	0	0	-	In	2.00	0.3	50	50	TFC Elution and LC separation
7.00	0.0	0	100	0	0	-	In	4.00	0.3	70	30	TFC Elution and LC separation
8.00	0.5	85	15	0	0	Cleaning	Out	9.00	0.3	100	0	LC separation
9.00	0.5	100	0	0	0	Cleaning	Out	9.20	0.3	100	0	LC separation
9.50	0.5	50	50	0	0	Cleaning	Out	9.50	0.3	50	50	Initial conditions
10.0	0.5	0	100	0	0	Inintial conditions	Out	10.0	0.3	50	50	Initial conditions
11.0	0.5	0	100	0	0	Inintial conditions	Out	11.0	0.3	50	50	Initial conditions

Positive conditions:
A and **F**: methanol (20 mM ammonium formiate + 0.1% of formic acid); **B** and **G**: water (20 mM ammonium formiate + 0.1% of formic acid); **C** and **D**: -
Negative conditions:
A and **F**: methanol; **B** and **G**: water; **C**: water (pH 4 with formic acid); **D**: water (pH 8 with ammonia)

XI.2.4.3. *Vibrio fishceri* luminescence test (Microtox® test)

A Microtox bioluminescence assay was used to perform toxicity test. This method is based on the percent decrease in the amount of light emitted by the bioluminescent bacterium *V. fischeri* upon contact with a filtered sample at pH 7. The 50% effective concentration, EC_{50}, was measured after 15 min of exposure. Effluent toxicity was expressed in toxicity units. Toxicity units are calculated as $TU=100/EC_{50}$. The experimental samples tested were collected from both sterile and non-sterile reactor treatments at their corresponding times.

XI.2.4.4. Other analyses

Laccase activity was assayed using a modified version of the method for the determination of manganese peroxidase (MnP) as described elsewhere (Kaal et al., 1993). The reaction mixture used consisted in 200 µL of 250 mM sodium malonate at pH 4.5, 50 µL of 20 mM of 2,6-dimethoxiphenol (DMP) and 600 µL of sample. DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV-vis spectrophotometer at 30ºC. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

Biomass pellets dry weight was determined after vacuum-filtering the cultures through pre-weighed glass-fiber filters Whatman GF/A (Barcelona, Spain). The filters containing the biomass pellets were placed on glass plates and dried at 100ºC to constant weight.

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instrument and Co. (Yellow Springs, OH, USA).

Total organic carbon (TOC), total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to APHA (1995). The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analyzed by using commercial kits (LCH302 and LCK114 respectively, Hach Lange, Düsseldorf, Germany).

XI.3. Results and discussion

Analysis of PhACs in raw hospital wastewater showed that 54 (sample 1) and 57 (sample 2) out of the 112 analyzed PhACs were detected. Throughout the manuscript first values correspond with sample 1 (sterile treatment) and the second value correspond with sample 2 (non-sterile treatment). The more common families of PhACs detected were analgesics, antibiotics, psychiatric drugs, compounds that present endocrine disruptor effect and X-ray contrast media, which correspond with the main classes of drugs used in hospitals (Verlicchi et al., 2010). Results from the hospital wastewater treatment by *T. versicolor* showed a partial or total removal of 47 and 51 PhACs out of the 54 and 57 initially detected in both sterile and non-sterile treatment, respectively. The total amount of PhACs initially detected in both samples was 8,234.07 µg and 667,752.68 µg and after the treatment was obtained 1,436.92 µg and 3,984.33 µg corresponding with 82.5 % and 99.4 % removed, respectively.

Those general results indicate that PhACs present in hospital wastewater effluent can be removed in the sterile batch bioreactor treatment by *T. versicolor*, but also in a non-sterile treatment. Those results are in agreement with previous reported investigation of our group concerning the treatment of urban wastewater by *T. versicolor*, where complete removal of 7 out of the 10 initially detected PhACs was achieved (Cruz-Morató et al., 2013b).

Regarding bioreactor operation (figure XI.1), in sterile and non-sterile batch bioreactor treatments the glucose was totally consumed during all the experiment and laccase production was observed after 5 d of the treatment. This fact suggests that the fungus is active throughout all the experiment. $N\text{-}NH_4^+$ was totally consumed after 2 d and biomass concentration was increased to 2 g d.w. L^{-1} during last days, explained by the growing due to the fact that hospital wastewater contains nutrients as nitrogen.

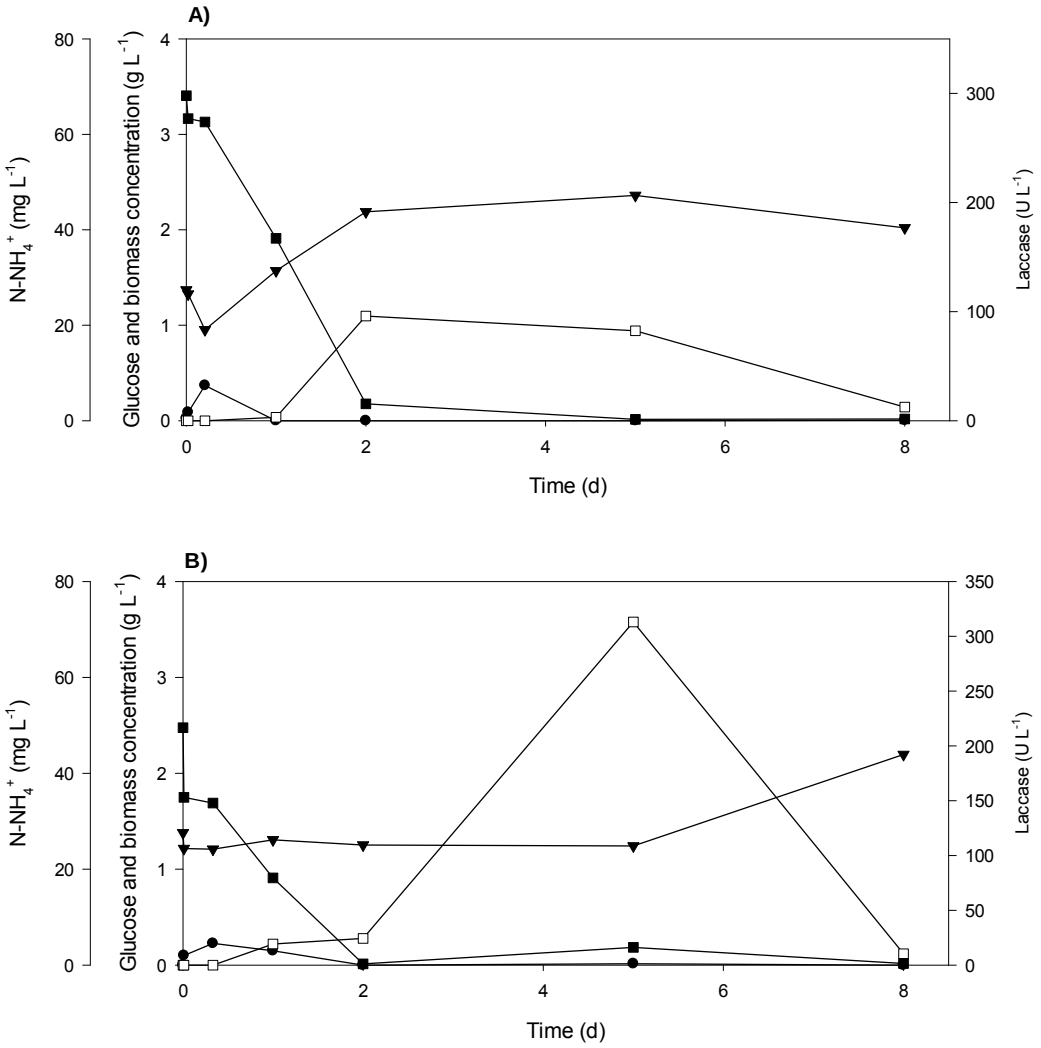

Figure XI.1: Bioreactor parameters during the sterile (A) and non-sterile (B) hospital wastewater treatment. Symbols: Glucose (●), biomass concentration (▼), laccase (□) and N-NH_4^+(■).

On the other hand, the treated wastewater became murky when the experiment at non-sterile conditions was carried out. A possible hypothesis is due to the growing of bacteria, but also it was observed the breakdown of the pellets morphology into free mycelium, leading to an increase in viscosity. This effect was not observed in the sterile treatment. Hence, further research is needed in order to find a solution to develop a longer treatment, for example with biomass renovation or removing bacteria in a pre-treatment.

In following sections are presented the detailed discussion of the PhACs removal during the treatment by families of PhACs.

XI.3.1. Analgesics/anti-inflammatories

In general, the initial amount of analgesics was 1,478.57 µg (18 % from the total PhACs amount) and 660,757.60 µg (98 % from the total amount), respectively in both sterile and non-sterile treatment, leading a reduction up to 9.12 µg and 17.66 µg corresponding to eliminations of 99.4 % and 99.1 %, respectively. Among analgesics/anti-inflammatories, the highest concentrations correspond to acetaminophen (114.4 µg L^{-1} - 27.9 mg L^{-1}, respectively in samples 1 and 2) and ibuprofen (10.3 µg L^{-1} - 38.1 mg L^{-1}). Due to their high human consumption the detection of these compounds around 10-100 µg L^{-1} is not surprising. However, it is quite staggering to find values ranging mg L^{-1}. This high concentration could be explained by the disposal of drugs leftovers to sewage, which would suggest doubts concerning the good praxis in the way to dump PhACs in hospitals. In descending order of concentration, naproxen, ketoprofen, diclofenac, phenazone, codeine and salicylic acid were found, with values ranging from 0.05 to 13.7 µg L^{-1}.

All detected anti-inflammatory drugs were, in general, completely removed (Figure XI.2) in both treatments. In detail, ibuprofen, acetaminophen, naproxen, diclofenac and phenazone were removed over 80% after 1 d and reaching total elimination on the following days. Removal rate of ketoprofen and codeine was slower than the formers, since they were totally removed after 5 d. On the contrary, no removal was observed for salicylic acid and dexamethasone. Piroxicam was not detected in the raw wastewater; however, its concentration starts to increase after 5 h of the non-sterile treatment and rise to the value of 0.15 µg L^{-1} at day 5 remaining constant until the end of the treatment. Despite of last analgesics are not removed, they are in low concentration and the completely elimination of all others analgesics even the highest concentrations ranging mg L^{-1}, demonstrates the feasibility of the treatment to remove this kind of PhACs.

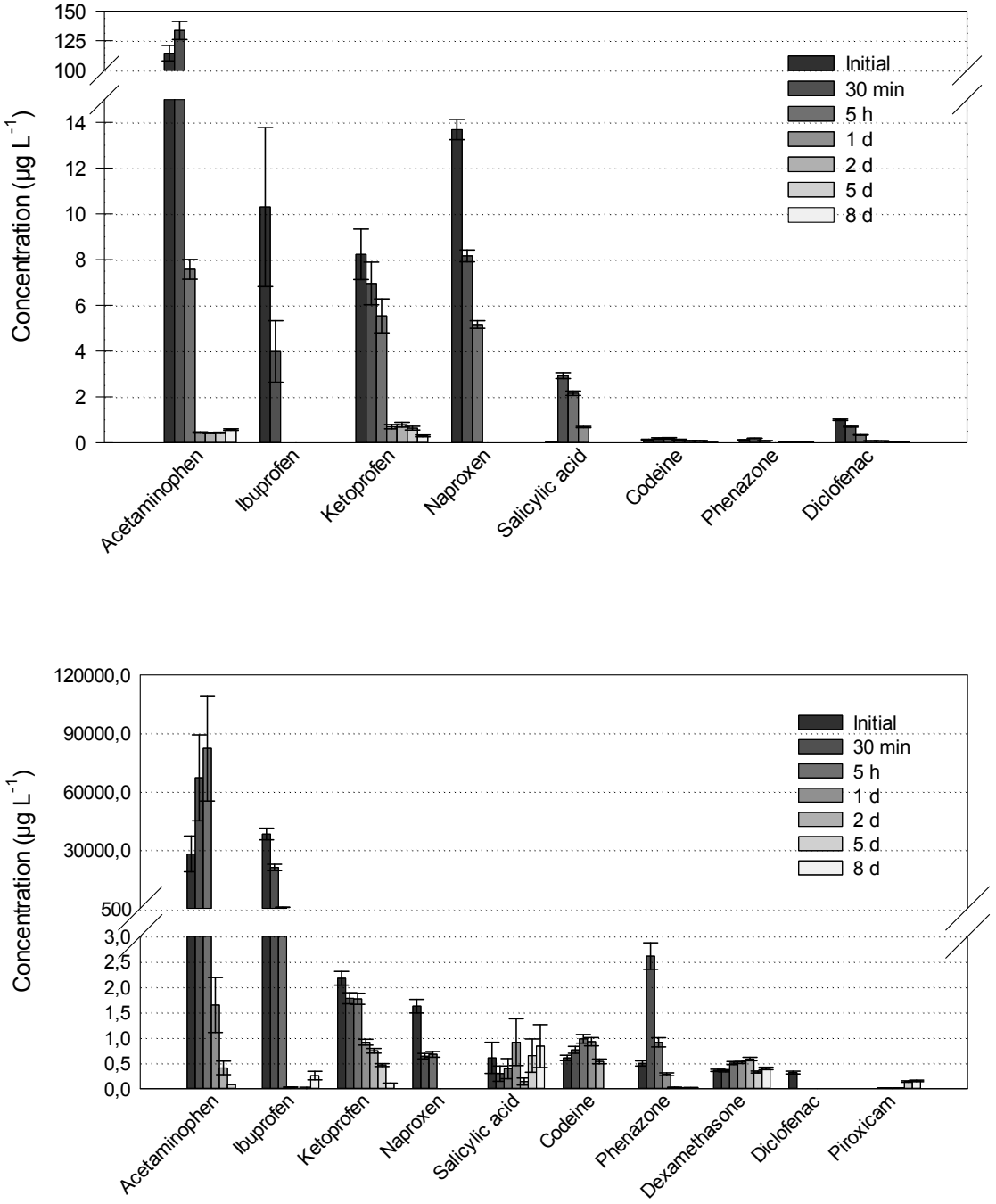

Figure XI.2: Anti-inflamatories profile during the hospital batch bioreactor treatment. Sterile treatment (A) and non-sterile treatment (B). Black to white correspond the treatment times in which was taken the sample.

In conventional biological treatment, the anti-inflammatory drugs acetaminophen, ibuprofen and ketoprofen also are successfully removed, but partial elimination (around 50 %) was observed for the others (Verlicchi et al., 2012a). On the other hand, it has been published that the removal efficiency of these compounds is

strongly related to the initial influent concentration of each target compound when extended sludge age biological process is used (Yu et al., 2009). It is maybe due to the fact that induction of enzyme synthesis in bacteria is dependent of the pollutant concentration (Harms et al., 2011). In contrast, the induction of the enzymatic system in fungus is not dependent on the concentration and on the chemical, therefore, allows degrading contaminants to near non-detectable levels even different initial concentrations (Barr and Aust, 1994).

XI.3.2. Antibiotics

The initial amount of antibiotics was 568.2 μg (7 % from the total PhACs amount) and 239.4 μg (0.04 % from the total amount) with a reduction up to 79.3 μg and 22.3 μg corresponding to eliminations of 86.0 % and 91.1 %, respectively. Similar as analgesics, almost all antibiotics were from partial (30%) to complete removal (figure XI.3). However, the rate of elimination was lower, since more days (after 5 days) were needed to achieve values of total elimination. Initial concentration in the non-sterile treatment (ranging from 0.008 μg L^{-1} to 1 μg L^{-1}) of some antibiotics as sulfamethoxazole, trimethoprim, metronidazole and its hidroxilated metabolite, dimetridazole and erithromycin was relatively significant lower than in the sterile treatment (ranging from 2 to 5 μg L^{-1}).

It must be highlighted the highest initial analgesic concentration observed for ofloxacin (31.9 and 3.34 μg L^{-1} respectively for sample 1 and 2) and ciprofloxacine (12.05 and 13.04 μg L^{-1}). They were completely removed in both treatments; with an exception of ciprofloxacin in sterile treatment that was removed 69% after 8 d. Prieto et al. (2011) reported values over 90% of ciprofloxacin degradation after 6 d in a defined medium by *T. versicolor* spiking the contaminant at 2 mg L^{-1}, which despite of the higher spiked concentration is in agreement with our results. Clarithromycin (2.2 μg L^{-1}) and azithromycin (1.37 μg L^{-1}) detected only in sample 2 of the hospital wastewater was removed over 80 % after 5 d in the non-strile treatment.

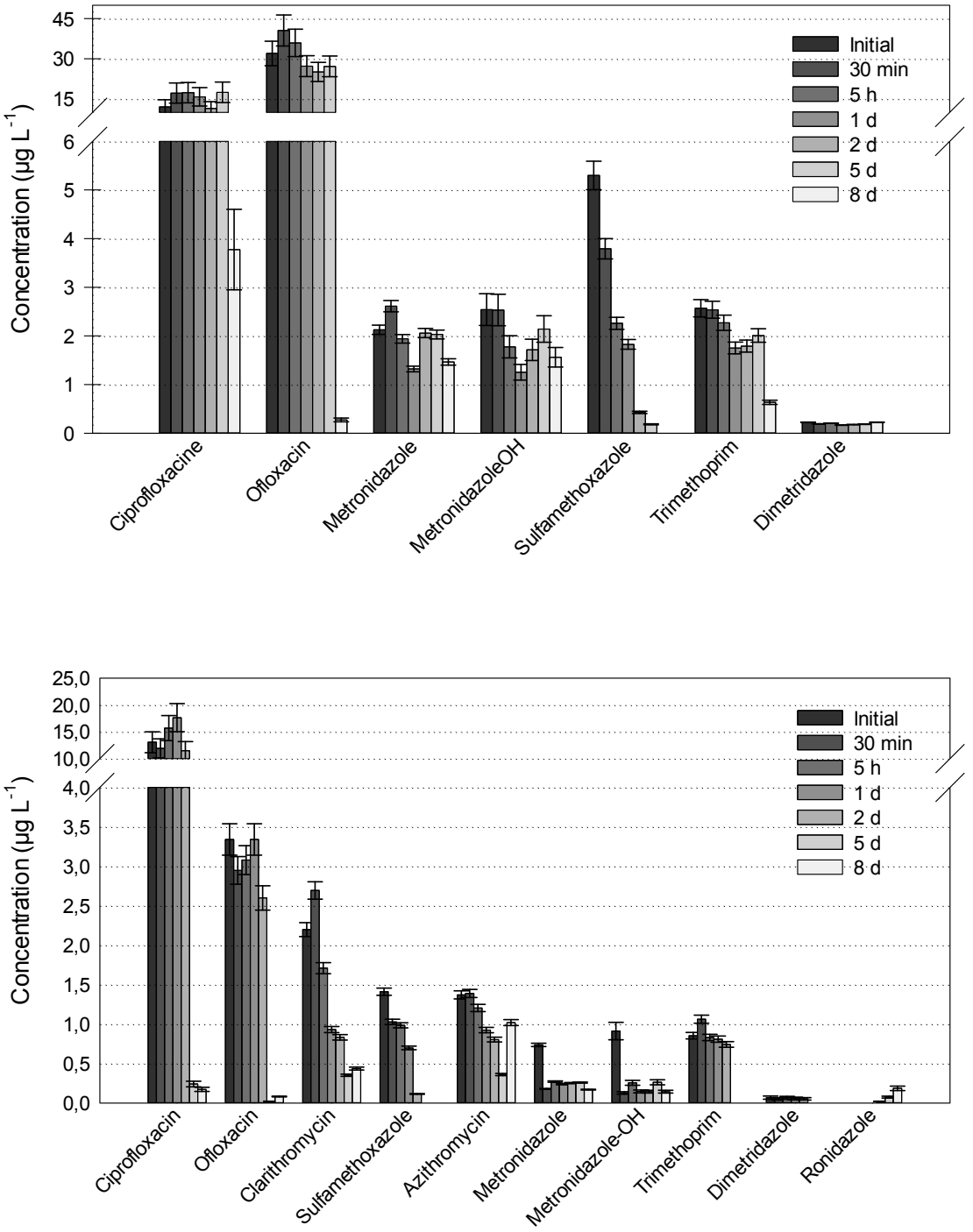

Figure XI.3: Antibiotics profile during the hospital batch bioreactor treatment. Sterile treatment (A) and non-sterile treatment (B). Black to white correspond the treatment times in which was taken the sample.

In conventional wastewater treatments plants the ranges of removal efficiency variability of antibiotics are generally wide (Verlicchi et al., 2012a). The corresponding average values of removal for the antibiotics detected in the present study vary from 15% to 98% in conventional treatments. Ciprofloxacin and ofloxacin, as average, are removed in conventional treatment around 70%, mostly attributed to the adsorption in the sludge (Jia et al., 2012), sulfamethoxazole around80 %, trimethoprim and metronidazole around 40%. Comparing this data with our results it can be concluded that a treatment with fungi could remove the part of antibiotics than cannot be removed by conventional treatments, but also could prevent the accumulation of some of this PhACs (as ciprofloxacin) into the sludge, used after as compost and may discharging this compounds to farmlands.

XI.3.3. Psychiatric drugs

In general, the initial amount of psychiatric drugs was 3,654.9 μg (44 % from the total PhACs amount) and 107.67 μg (0.02 % from the total amount) with a reduction up to 232.2 μg and 5.29 μg corresponding to eliminations of 93.6 % and 95.2 %, respectively. In figure XI.4 are depicted the profiles of psychiatric drugs during both sterile and non-sterile treatment by *T. versicolor*. Carbamazepine has been one of the most studied compounds concerning its persistence in conventional wastewater treatments and its ubiquitous occurrence in the environment (Verlicchi et al., 2012a, Jelić et al., 2011). In the present study, carbamazepine has been detected at 0.44 μg L^{-1} and 0.06 μg L^{-1} in samples 1 (sterile) and 2 (non-sterile) respectively from the hospital wastewater. Results showed that this compound it is not removed in both sterile and non-sterile treatments, even its concentration seems to increase. This behavior is similar in previous reported experiments regarding the treatment of urban wastewater (Cruz-Morató et al., 2013b), but also in conventional wastewater treatments (Verlicchi et al., 2012a). It is attributed to the fact that deconjugation with glucuronides or the transformation of its metabolites present in the raw wastewater back to the parent compound. On the other hand, experiments carried out in defined medium and with the individual compound present, showed that *T. versicolor* is able to degrade

carbamazepine (Jelić et al., 2012), which is in controversy when the treatment with the fungus is applied to a real matrix observed in the present study.

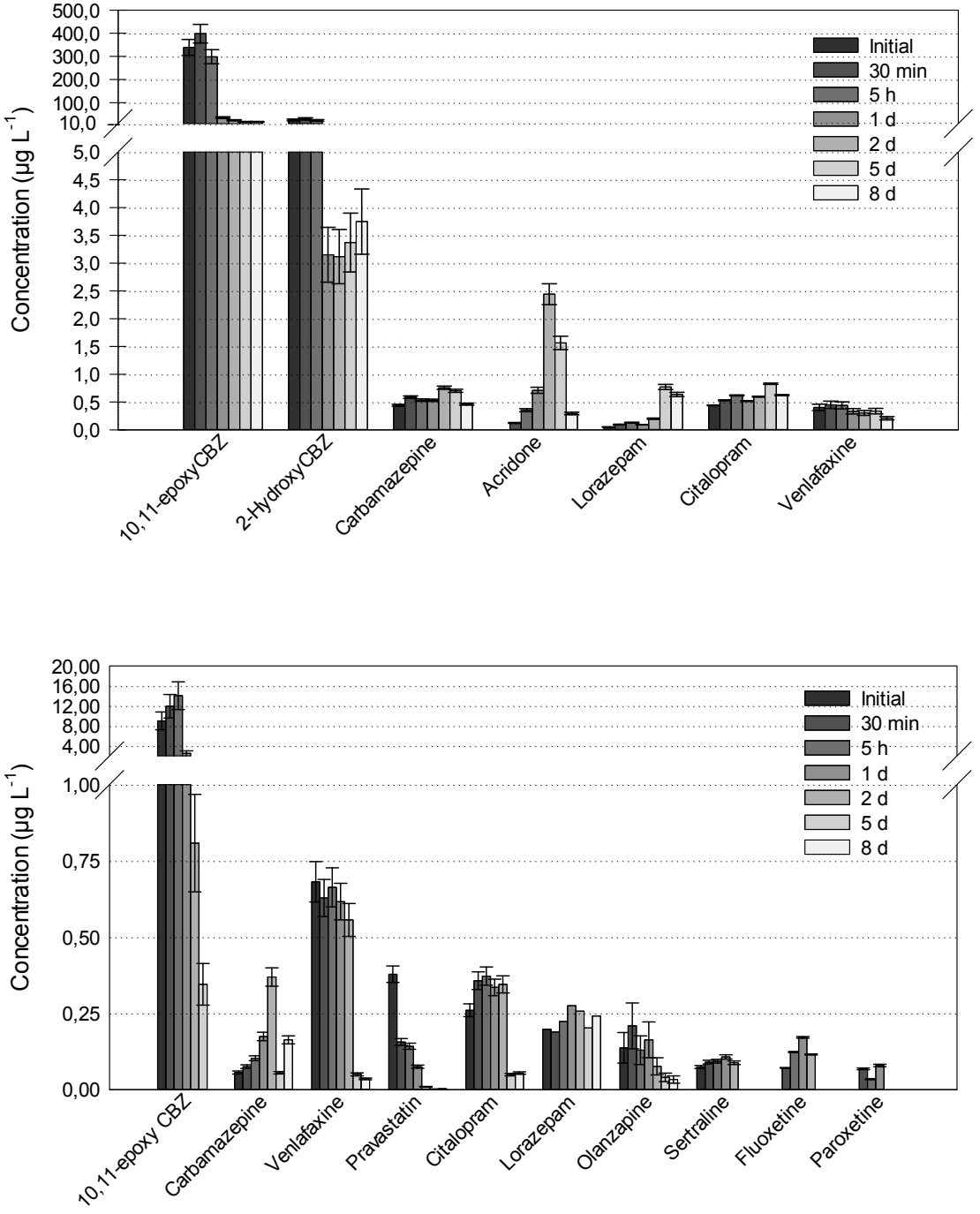

Figure XI.4: Psychiatric drugs profile during the hospital batch bioreactor treatment. Sterile treatment (A) and non-sterile treatment (B). Black to white correspond the treatment times in which was taken the sample.

The highest psychiatric drug concentration observed in both samples of the hospital wastewater correspond with 10,11-epoxycarbamazepine (338.9 and 8.98 μg L^{-1} samples 1 and 2 respectively). It is the main metabolite of carbamazepine in humans, in fact, the metabolism of mammalians transform carbamazepine into this compound in a first step and subsequently it is transformed to hydroxylated and other metabolites (Lertratanangkoon and Hornin, 1982). In addition, the percentage of excretion after the carbamazepine intakes has been established in 3 %, therefore the 97 % is excreted in form of metabolites (Verlicchi et al., 2012a). Due to the high consumption of carbamazepine in hospitals and the above explained, it is not surprising that 10,11-epoxycarbamazepine could be one of the principal compounds detected in this kind of effluent. Total elimination of this compound even 2-hydroxycarbamazepine (the other human metabolite of carbamazepine detected at 25.2 μg L^{-1} only in sample 1 and hence treated only at sterile conditions) was observed after both treatments presented in this study. It is interesting to comment the production of acridone, other metabolite of carbamazepine (Jelić et al., 2012), detected during the performance of the sterile bioreactor treatment, which reached values of 2.5 μg L^{-1} after 1 d and consecutively removed to be remained at 0.29 μg L^{-1} at the end.

Other psychiatric drugs as lorazepam (0.05 μg L^{-1} and 0.2 μg L^{-1}) and citalopram (0.44 μg L^{-1} and 0.26 μg L^{-1}) were not removed in the batch bioreactor treatment by *T. versicolor*. The exception was in the non-sterile treatment where the citalopram concentration was reduced around 80 % after 5 d. Lorazepam concentration increase to 0.64 μg L^{-1} after 8 d of sterile treatment. The same behavior was observed in conventional treatments (Gracia-Lor, et al., 2012).

Venlafaxine was detected at 0.4 μg L^{-1} and 0.68 μg L^{-1} in samples 1 and 2 respectively. This compound is one of the most psychiatric drugs detected its occurrence in wastewaters around the world at a significant range of ng L^{-1} to mg L^{-1} on the last years (Rúa-Gómez and Püttmann, 2012; Yuan et al., 2013). Therefore, special attention should be paid in this compound due to its absolutely non-removal in conventional wastewater treatment plants (Gracia-Lor et al., 2012) and therefore, reaching surface water and being able to cause problems in the environment. In the

sterile hospital wastewater treatment venlafaxine was 50 % removed, demonstrating almost its partial removal by *T. versicolor*. In the non-sterile treatment more than 90 % was removed after 5 d. As far as author's knowledge, it is the first time that it is reported the degradation of velafaxine by white-rot fungus. Only as hypothesis the synergy of other microorganisms could help to remove venlafaxine, such as more removal is observed in the non-sterile treatment, but further research is needed about this topic.

Paravastatin (0.38 μg L^{-1}), olanzapine (0.14 μg L^{-1}) and sertraline (0.07 μg L^{-1}) were only found in sample 2 of the raw hospital wastewater and they were totally removed after 8 d of the treatment. In addition, fluoxetine and paroxetine were not found initially, but were detected during the treatment at concentration lower than 0.125 μg L^{-1}. However, at the end of the treatment were totally removed.

XI.3.4. Endocrine disruptor chemicals and related compounds

The initial amount of EDCs was 928.3 μg (11 % from the total PhACs amount) and 2,273.2 μg (0.34 % from the total amount) with a reduction up to 783.4 μg and 1,146.3 μg corresponding to eliminations of 16.3 % and 50.1 %, respectively. Among all chemicals that present endocrine disruptor effect, the highest concentration detected in the hospital wastewater corresponds to benzotriazole (5.57 μg L^{-1} and 56.0 μg L^{-1} for samples 1 and 2 respectively). However, the highest values were detected for caffeine. This compound does not have any disrupting effect but is used as a biomarker of other EDCs (75.7 μg L^{-1} and 149.2 μg L^{-1}). In descending order of concentration, OP_2EO, ethynylestradiol, OP, BPA, estriol, estriol-3-sulfat and estriol-16-glucuronide were also detected in the raw hospital wastewater (ranging values from 0.11 to 7.5 μg L^{-1}). The profile of these compounds during sterile and non-sterile batch bioreactor treatments are depicted in Figure XI.5. Regarding elimination rates, the biomarker caffeine was the only compound that was not eliminated (8%) by *T. versicolor* under sterile conditions. However, partial removal (38%) was observed in the non-sterile treatment. This is an indicator that maybe many other microorganisms could help to remove

caffeine. Removal efficiencies over 98% have been observed in conventional wastewater treatment plants remarking that bacteria can remove this analyte (Ratola et al., 2012). Hence, although no elimination has been observed during a possible pre-treatment by *T. versicolor*, this can be removed in conventional sludge systems. Most of the EDCs were partial (over 70%) or completely removed after the treatment. However, the concentration of ethynylestradiol, estrona, dietilbestrol and OP_2EO increased thorough the treatment. A hypothesis based on the generation of these compounds through the biological degradation of other related analytes is formulated. As an example, the transformation of estradiol into ethynylestradiol through the estrone compound (Kleemann et al., 2009).

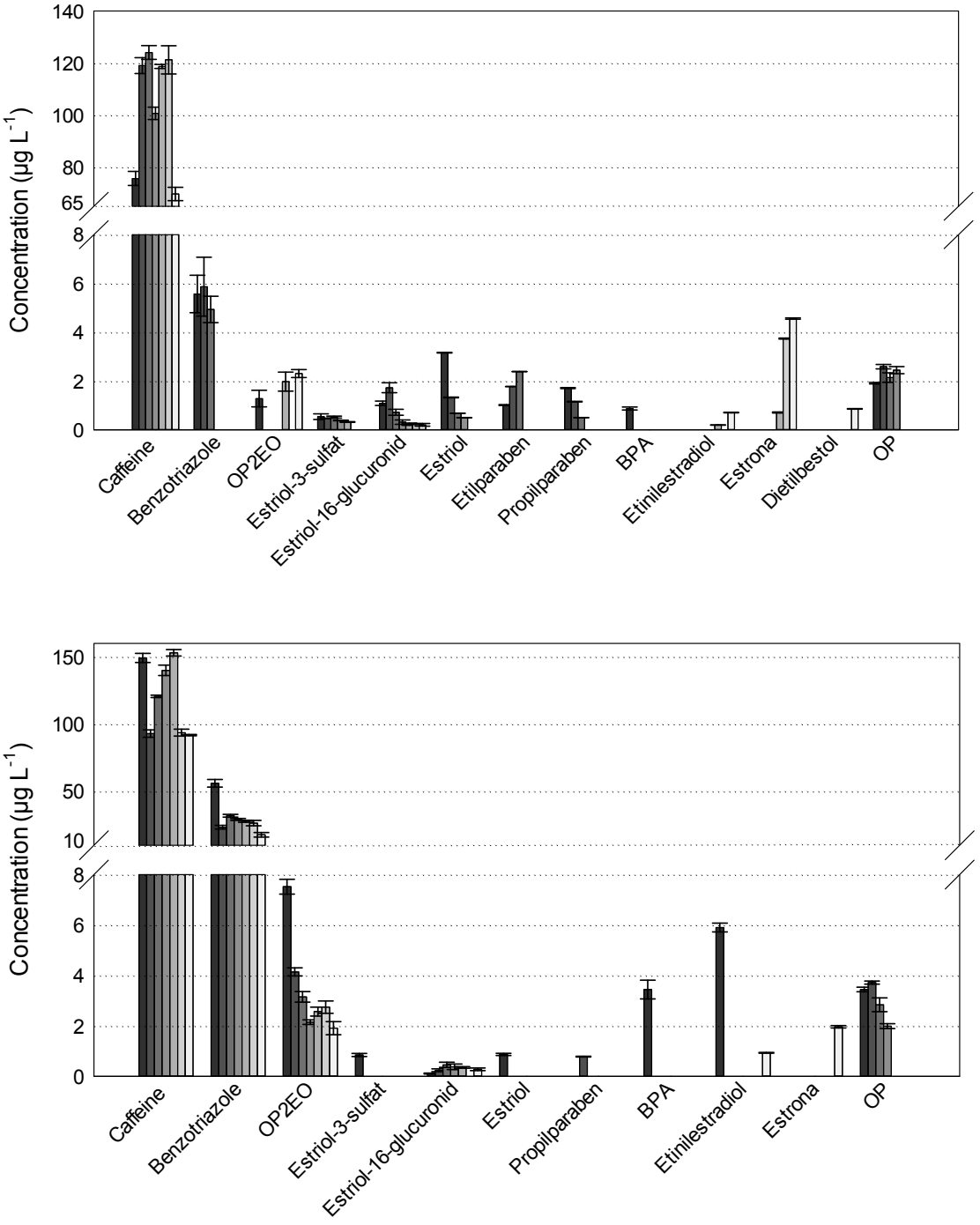

Figure XI.5: Endocrine disruptors and compounds that present endocrine disruptor activity profiles during the hospital batch bioreactor treatment. Sterile treatment (A) and non-sterile treatment (B). Black to white correspond the treatment times in which was taken the sample.

XI.3.5. Other pharmaceuticals

The X-ray contrast agent iopromide is also one with the highest concentration detected in the raw hospital wastewater (104.6 and 419.7 μg L^{-1}). The total initial amount was 1,046.4 μg (13 % from the total PhACs amount) and 4197.3 μg (0.63 % from the total amount) with a reduction up to 255.9 μg and 2,768.7 μg corresponding to eliminations of 75.5 % and 34.2 %, respectively.

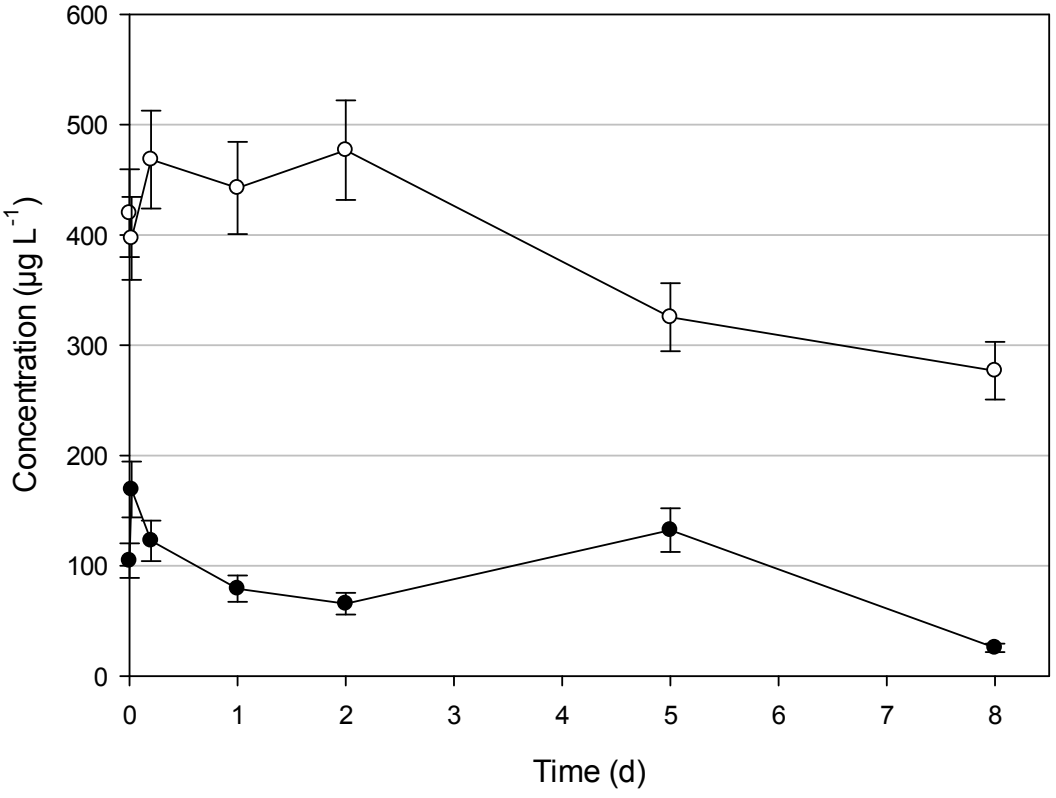

Figure XI.6: Iopromide profiles during the hospital batch bioreactor treatment. Symbols: Sterile treatment (●) and non-sterile treatment (○).

Iopromide was scarcely removed by conventional biological process (Miège et al., 2009; Verlicchi et al., 2012a). Its persistence is due to the fact that, as diagnostic agent, it has been designed to be highly stable. However, in the present treatment it was achieved removal rates of 76% when only the fungus was present and 34% in the non-sterile treatment (Figure XI.6). Engels-Matena (1996) showed that *T. versicolor* is

able to remove iopromide in a defined medium demonstrating partial elimination (60%) after 7 d with a subsequently total elimination after 15 d. Hence, the percentage removal achieved in the present study could be increased by a longer treatment.

The total initial amount of the rest of PhACs was 558.1 μg (7 % from the total PhACs amount in sample 1) and 177.5 μg (0.03 % from the total amount in sample 2) with a reduction up to 77.3 μg and 24.12 μg corresponding to eliminations of 86.1 % and 86.4 %, respectively. Figures XI.7 and XI.8 show the removal profile of anithipertensives, loop diuretics, β-blockers, β_2-adrenergic receptor agonist, lipid regulators, histamine H1 and H2 receptors antagonist, calcium chanel blockers, anthelmintic, antiplatelete agents and antidiabetic families of PhACs during the batch bioreactor treatment by *T. versicolor*. Among all PhACS of this section, valsartan, furosemide, atenolol, gemfibrozil, and ranitidine were the more significant compounds detected in raw hospital wastewater which concentration range from 4.52 to 15.8 μg L^{-1}. All of them were completely removed (more than 90% eliminated) in both sterile and non-sterile treatments. An exception was observed for atenolol. This β-blocker drug was partially removed around 41% and 75% in sterile and non-sterile treatments respectively. The other compounds were detected below 1 μg L^{-1} and most of them were totally removed after the treatment with the exception of hydrochlorothiazide (50% removed at non-sterile treatment) and losartan (increased its concentration more than 4 times with none reasonable justification identified).

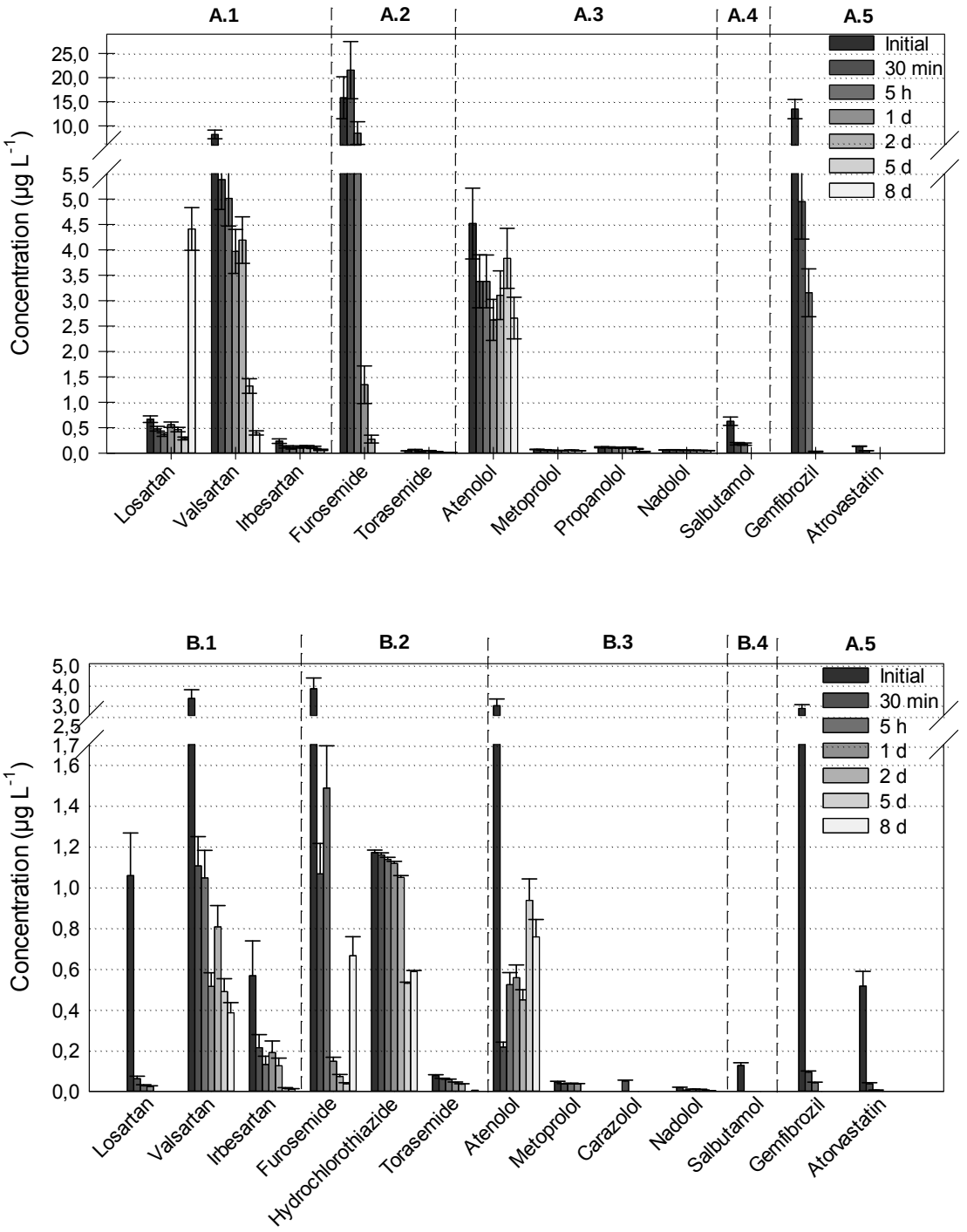

Figure XI.7: Pharmaceutical profiles during the hospital batch bioreactor treatment. Sterile treatment (A) and non-sterile treatment (B). PhACs families: Antihipertensives (A.1 and B.1), loop diuretics (A.2 and B.2), β-blockers (A.3 and B.3), β_2-adrenergic receptor agonist (A.4 and B.4) and lipid regulators (A.5 and B.5).Black to white correspond the treatment times in which was taken the sample.

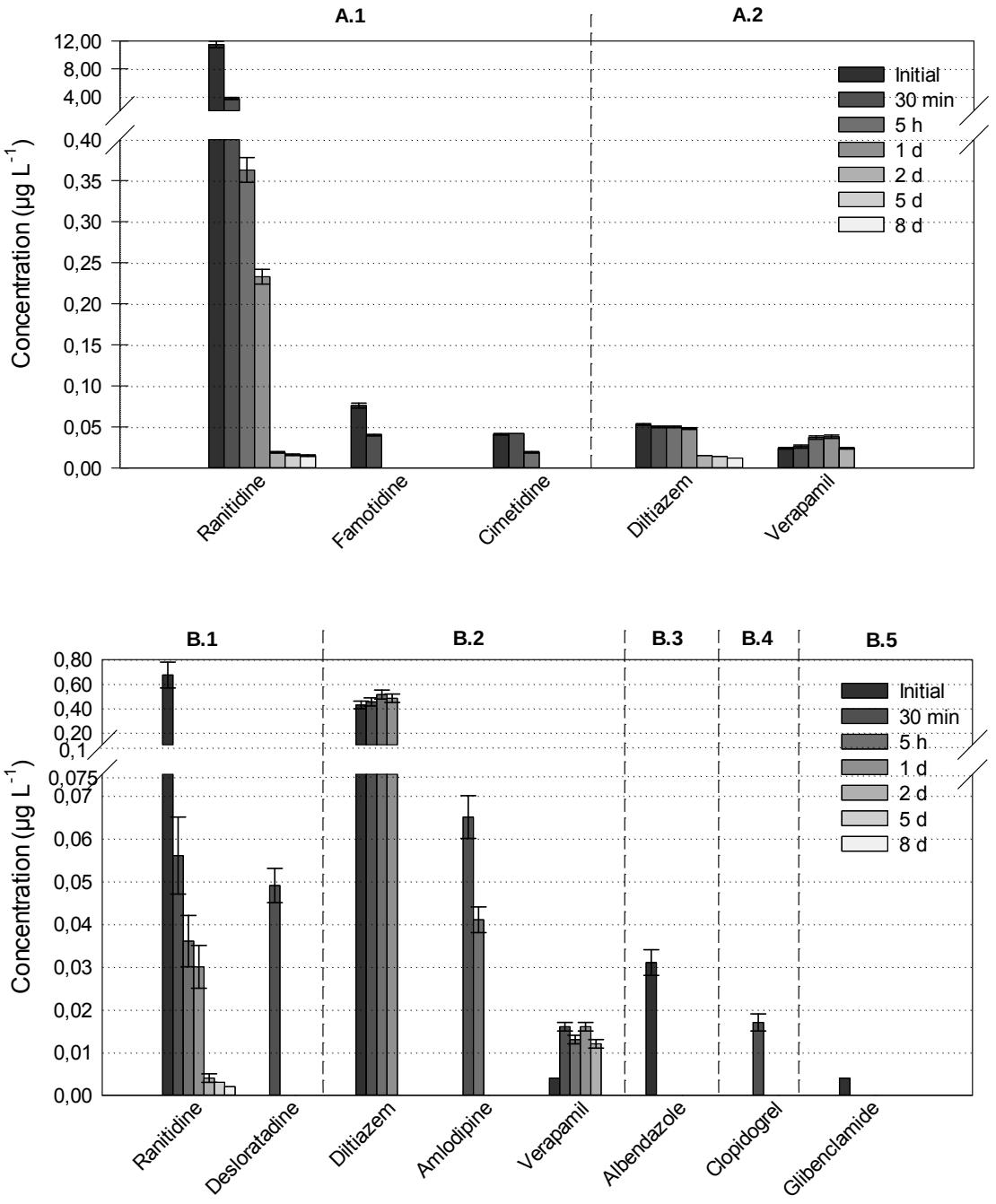

Figure XI.8: Pharmaceuticals profiles during the hospital batch bioreactor treatment. Sterile treatment (A) and non-sterile treatment (B). PhACs families: Histamine H1 and H2 receptors antagonist (A.1 and B.1), calcium chanel blockers (A.2 and B.2), anthelmintic (B.3), antiplatelete agent (B.4) and antidiabetic (B.5). Black to white correspond the treatment times in which was taken the sample.

The removal variability observed in conventional wastewater treatment plants of the last PhACs discussed in this section is very wild (from 10% to 98%). However, all of them showed, as an average, removal efficiencies of 40-60 % in Verlicchi et al. (2012a). These values are lower than the ones obtained with the application of *T. versicolor* in the bioreactor.

XI.3.6. Toxicity assessment (Microtox® test)

One of the main goals of any treatment dedicated to remove pollution from wastewater is to decrease the effluent toxicity. Therefore, bioluminescence assay using *V. fischeri* photobacteria (Microtox® test) was performed to determine the change in the toxicity during the PhACs treatment from hospital wastewater.

Raw hospital wastewater presented different values of toxicity. Sample 1 showed 25 toxicity units, which it is considered toxic by the Environmental Protection Agency (EPA, 2004). They consider a toxic effluent when it present values of toxicity over 10. However, sample 2 showed values of 3 toxicity units. Emmanuel et al. (2005) carried out experiments in order to assess the ecotoxicological risk of raw hospital wastewater by *V. fischeri*. They measured values of 15 min EC_{50} over 50%, corresponding with 2 toxicity units, which is in controversy with our results observed in sample 1. The difference in the toxicity values could be explained by the fact that some chemicals presenting more toxicity could be disposed to sewage the day in which was taken the sample 1, but was not present in sample 2. On the other hand, the toxicity in both treatments follows the same profile (Figure XI.9). The toxicity remained constant along the first hours in both treatments, but after 1 d the toxicity decreased to values of non toxicity (below 1 toxicity unit). However, at the end of the treatment the toxicity increased, returning to initial values in the case of the non-sterile treatment (4 toxicity units). Slightly low toxicity values (8 toxicity units) were observed after 8 d for the non sterile treatment, indicating a reduction of 66% compared to initial sample

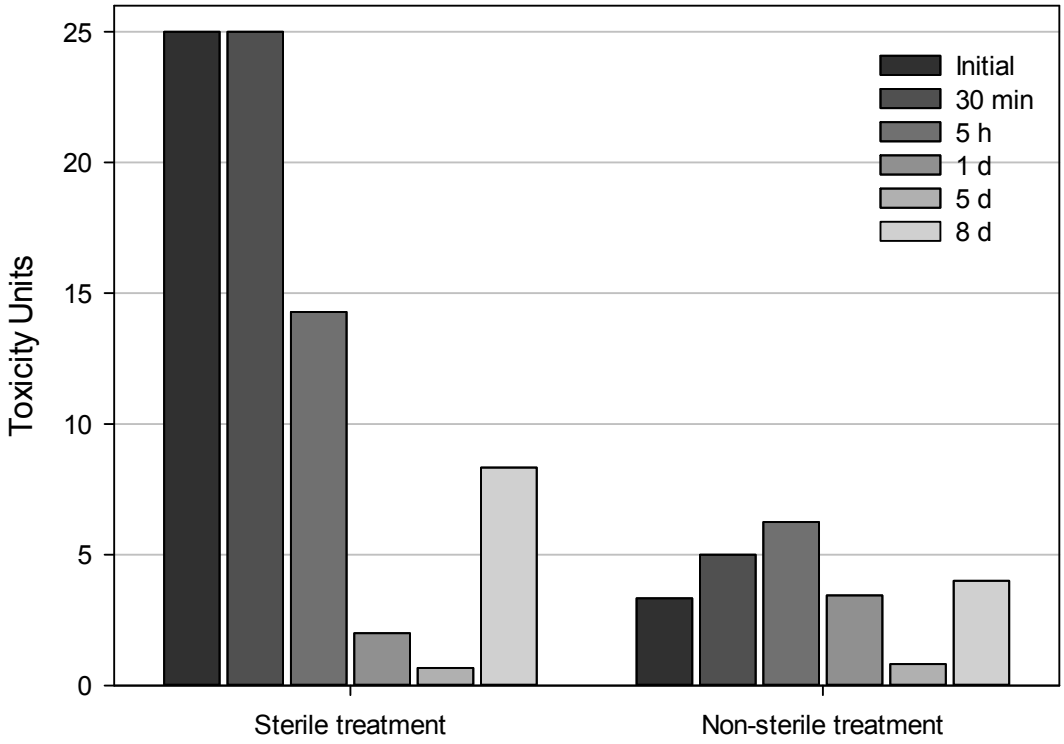

Figure XI.9: Microtox values (toxicity units) during the hospital wastewater treatment in a batch bioreactor. From black to pale gray correspond the treatment times in which was taken the sample.

XI.4. Conclusions

Non sterile hospital wastewater treatment in a fluidized bed bioreactor by *T. versicolor* demonstrates to be an efficient strategy for removing PhACs. 51 out of the 57 detected PhACs were partially (35 %) or completely removed. Analgesics are one of the most commonly detected drugs in hospital wastewater with the highest concentrations (from 0.6 µg L^{-1} to 28 mg L^{-1}) and were completely removed after the treatment. All the studied antibiotics, detected between 0.08 and 32 µg L^{-1}, were removed over the 77% with the exception of azithromycin (partially removed 26 %). Psychiatric drugs, including carbamazepine metabolites and the most recalcitrant drug venlafaxine in conventional treatments, were detected ranging from 0.006 to 8.9 µg L^{-1} and removed over 80%. The endocrine disruptor chemical biomarker caffeine was partially removed around 38% while the others disruptors drugs were from 75 to 100% eliminated. Iopromide showed one of the highest concentrations in this study (419 µg

L^{-1}) and was partially removed (34%). The other PhACs detected in the hospital wastewater were removed from 50% to 100%. On the other hand, increasing concentration was observed for some of PhACs, i.e carbamazepine, estrone, piroxicam. One hypothesis suggested is that this increase could be consequence of the transformation of any metabolite to the parent compound or desconjugation of other compounds that release the target compound. The promising results obtained show the possibility to act directly from the basis of the problem, as for example hospital or industrial effluents, avoiding the dilution of other effluents with least load of PhACs. Higher removal percentages obtained in the present treatment compared with conventional sludge systems have been observed. On the other hand, the decrease of the toxicity along the time supports the suitability of this treatment and the necessity to carry on with the study of the effluents treatment containing PhACs with *T. versicolor*, as for example continuous treatments in pilot plants, in order to reach real approaches.

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SECTION 3:

CONCLUSION & FUTURE PROSPECTS

Chapter XII:

Concluding remarks and future prospects

XII.1. Concluding remarks

The ability of the white-rot fungus *T. versicolor* to remove a wide range of PhACs in liquid phase has been demonstrated in the present thesis. Firstly, almost complete removal of single anti-inflamatories (ketoprofen and diclofenac), lipid regulator (clofibric acid), psychiatric drug (carbamazepine), antibiotic (ofloxacin) and X-ray contrast agent (Iopromide) by this fungus was achieved in Erlenmeyer flasks scale under sterile conditions. During time-course degradation experiments, transformation intermediates were identified and results showed either disappearance of parent compound and byproducts, suggesting possible mineralization (i.e. diclofenac and ketoprofen) or accumulation of byproducts in the culture medium (i.e. carbamazepine). One of the advantages of using whole mycelia cells over free/immobilized enzymes is the possible combined effect of oxidative enzymes able to transform a target compound, as evidenced in removal of diclofenac in *in vitro* experiments by laccase and *in vivo* experiments with inhibitors also suggesting a first oxidation step catalyzed by cytochrome P450.

A fluidized bed bioreactor, operated in both continuous and batch mode, was satisfactorily employed to scale up the degradation process of carbamazepine and clofibric acid by *T. versicolor*. These chemicals were chosen due to their low or even negligible removal in wastewater treatment plants. Comparison of the removal efficiency of both bioreactor scale and Erlenmeyer scale indicates that the former is more efficient, as completely eliminations were observed earlier.

Because of the great versatility of *T. versicolor* in degrading PhACs, it is important to study bioremediation applications on real effluent streams to simulate real conditions, i.e. PhACs present at environmentally low concentrations and growth limitations found in non-sterile habitats. The treatment of non-sterile urban wastewater using a 10 L fluizided bed bioreactor completely eliminated 7 out of the 10 initially detected PhACs. In addition, specific hospital wastewater was treated in the fluidized bed bioreactor at non-sterile conditions, where almost totally removal of iopromide and ofloxacin was achieved. The remarkable reduction in the final toxicity of both urban and hospital wastewater also supports the possibility of employing a treatment with WRF as an eco-friendly strategy to reduce the release of toxic contaminants into the environment.

XII.2. Future prospects

With the aim to obtain real approaches for the wastewater treatment by *T. versicolor*, further research should focus on:

- Identification of the transformation products of pollutants that were present in wastewaters but still not tested for white-rot fungus degradation and assessment of the toxicity of the treated medium.

- Study the relationship between white-rot fungi and microbiota present in real wastewaters, and their combined effect on the degrading capacity.

- Optimization of the process to extent and improve the treatment.

- Study different configurations as combination of the fungus and conventional bacteria treatment, in order to implement the treatment to conventional wastewater treatment plants.

- Scale up the process to pilot plants.