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Fungal treatment for biodegradation of recalcitrant compounds in wastewaters

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Abstract

Recalcitrant compounds are defined as pollutants that tend to resist conventional wastewater treatments and, therefore, require advanced processes to be removed from waters. Their presence is ubiquitous inside industrial and urban wastewater: typical examples of such compounds are dyes, tannins, phenols, synthetic polymers, solvents, pharmaceuticals and many others. Existing technologies for their removal comprise mainly chemical-physical processes such ad adsorption, membrane filtration and chemical oxidation. These technologies however present some disadvantages because they are expensive, very often are based on the transfer of the compounds into another phase (such as adsorption), which become an hazardous waste to dispose of or, as in the case of chemical oxidation, often no complete mineralization is achieved and some intermediates are formed, which can be even more toxic than the former compounds. Fungi can represent an alternative to chemical-physical processes thanks to their capability of expressing several extracellular lignin-modifying enzymes (such as laccases and peroxidases). These enzymes have a broad substrate specificity and have been shown to be able to oxidize different types of xenobiotics such as pesticides, polycyclic aromatic hydrocarbons, synthetic dyes, synthetic polymers and many other pollutants.

The present work focused on the use of white rot fungi for the removal of naphthalenesulfonic acid polymers (NSAP) and drugs from wastewater.

In the first case, the fungi *Bjerkandera adusta* and *Pleurotus ostreatus* were tested for the treatment of a petrochemical wastewater containing a mixture of NSAP as the largest fraction of organic recalcitrant pollutants. NSAP are commonly employed in several industrial sectors and are characterized by low biodegradability, which make them a remarkable group of recalcitrant compounds.

Experiments on petrochemical wastewater were conducted firstly in Erlenmeyer flasks and then scaled-up to bioreactors at lab scale. Early experiments in Erlenmeyer flasks showed that *Pleurotus ostreatus* was able to depolymerize NSAP in presence of an additional carbon source (glucose, corn starch, cellulose and lignin) up to 70%, while no degradation occurred with no external carbon source addition. This depolymerization led to an overall increase in wastewater biodegradability (the bCOD/COD ratio rose from 9% to 40-55%). These results suggested the possibility to use fungi as a pretreatment for an activated sludge process.

Further tests were conducted in bioreactors running under non-sterile conditions.

In the first phase several problems were encountered regarding bacterial overgrowth which, very often, led to poor or none enzymatic activity and, consequently, poor or none NSAP depolymerization/removal. These problems led to the conclusion that simple and easily biodegradable carbon sources, such as glucose, do not bring to acceptable degradation in non-sterile conditions, and that a possible strategy to enhance fungal biodegradation could be the use of attached biomasses.

Another generation of reactors were straw-bed bioreactors, which joined the use of attached biomasses and the use of straw as carbon source. These reactors, using complex lignocellulosic materials as carbon and energy source for fungi gave stable results in non-sterile environment in terms of enzymatic activity and degradation capacity. The conducted respirometric tests allowed to assess the potential of a two-phase process. In fact the combination of fungal treatment and an AS process was able to remove up to 40% of the initial COD. Nevertheless the use of straw as co-substrate caused the release of by-products, which contribute to non-biodegradable COD.

For this reason, the last set of reactors was set up using another biodegradable carrier, *Luffa cylindrica*, with lower content in lignin, which can cause the release of non-biodegradable compounds, and higher content in cellulose, which represent a good carbon source for fungi. Reactors treating wastewater showed stable enzymatic activity, polymer removal (20-35%), COD removal after combined treatment with AS (40-50%) and allowed to conclude that long HRT are not necessary for carry on the process, while, on the other hand, lower pH values seems to enhance the process. Nevertheless, in the final period of operation, a lack of performance of the reactors were observed, probably due to the depletion of *Luffa*. It is therefore reasonable to think that the initial amount of *Luffa* was not sufficient to allow *P. ostreatus* to degrade NSAP for all the period of observation.

For what concerns the removal of drugs, *Pleurotus ostreatus* was tested for the degradation of Diclofenac (DCF), Ketoprofen (KTP) and Atenolol (ATL).

Also in this case, experiments were performed firstly in flasks and then in sterile labbioreactors. Erlenmeyer tests pointed out that Diclofenac and Ketoprofen were degraded very fast by alive fungus, while Atenolol is more recalcitrant to biodegradation. Experiments in bioreactors showed that, during the batch stage, DCF was removed completely, KTP was removed up to 90%, while ATL concentration was scarcely reduced (< 20%). During the continuous treatment the DCF was fully removed, the KTP removal was about 70%, while again no ATL degradation was detected. When the HRT of the system was increased from 1.63 d to 3 d, DCF was still completely removed, KTP removal was increased up to 85% and also the ATL was removed up to 80%.

SECTION I INTRODUCTION

1. OBJECTIVES AND CONTENT OVERVIEW

The main objective of the present work is to contribute to the development of fungal processes for wastewater treatment. In particular, the work aims to evaluate fungal processes towards different types of wastewaters, from early stages (Erlenmeyer flasks tests) to non-sterile bioreactors at lab scale.

Specific objectives of the work can be summarized as follows:

- 1. To define the state of the art of using fungi for recalcitrant compound removal, with the simultaneous objective of understanding the factors governing the biodegradation processes through bibliographic researches;
- 2. To evaluate fungal biodegradation of different types of compounds, investigating whether or not they represent a suitable substrate for the growth of fungi and if they can be transformed in a co-metabolic process;
- 3. To scale up these processes into lab scale bio-reactors, studying the influence of different operational parameters;
- 4. To investigate the passage from sterile to non-sterile conditions;
- 5. To evaluate kinetic parameters of fungi, useful for the design of pilot-scale plants;
- 6. To evaluate the feasibility of a combined process with fungi and activated sludge.

This thesis is organized into three sections. In the first section, "Introduction", some general aspects and background information are given. In particular, chapter 1 presents objectives and contents of the thesis, chapter 2 contains a review on existing removal technologies for recalcitrant compounds in wastewaters, while chapter 3 and 4 give, respectively, information about fungi, their biology and their enzymatic systems and the state of the art of their use in the field of wastewater treatment.

Section II, "Experimental work" is organized into 3 chapters, reporting the conducted research work. Chapter 5 presents methods of fungal cultures and the study of some kinetic parameters of selected fungal strains, chapters 6 describes fungal treatments of a petrochemical wastewater and chapter 7 fungal treatments of a pharmaceuticals-containing wastewater. In these chapters are presented materials, methods and result of different experiments, from the early stages (fungal screening) to bioreactor applications.

Finally, Section III presents some concluding remarks (chapter 8) and future perspectives (chapter 9).

2. RECALCITRANT COMPOUNDS IN WASTEWATERS: REMOVAL TECHNOLOGIES

2.1. INTRODUCTION

Until the middle of the last century, wastewater treatment objectives were mainly concerned with the removal of suspended, colloidal and floatable materials, the degradation of biodegradable compounds and the elimination of pathogenic microorganisms [1]. Since the early 1970s, thanks to a great improvement in global analytical skills, a remarkable number of new compounds has been addressed in several different wastewaters. New evidences demonstrate the impact of many different pollutants originating from a broad range of industrial activities on human health and on the environment [2]. Among other constituents, recalcitrant organics deserve special attention. These are defined as compounds that tend to resist conventional wastewater treatments and, therefore, require advanced treatments to be removed from waters. Typical examples of such compounds are dyes and surfactants in textile wastewater, tannins in tannery wastewaters, phenols, synthetic polymers, solvents, and much more [2]. More recently, another class of compounds, the so-called "micro-pollutants", have been addressed inside waters and wastewaters. These compounds are found at very low concentrations (from µg/L up to pg/L) but still can present a threat to the aquatic environment as well as a range of possible adverse effects on human health [3]. Micropollutants include biologically active compounds (pesticides, herbicides, and pharmaceuticals), heat stabilizers, plasticizers, personal care products and more [4].

All these compounds can present a wide range of adverse effects inside the wastewater treatment plants (WWTPs): give rise to non-biodegradable chemical oxygen demand (nbCOD) and color in the effluent, generate toxic and/or inhibitory effects on the biomass in the sludge and interfere with oxygen transfer (e.g. in the case of surfactants) [5].

Conventional WWTPs using activated sludge (AS) process are partially able to remove the contained recalcitrant compounds, but very often do not achieve a satisfactory degradation. In particular, heavy metals are removed from the liquid essentially by biosorption on the biomass and then removed with the excess sludge [6]. A similar destiny is the one of dioxins and polycyclic aromatic hydrocarbons which, thanks to their low volatility and high affinity with organic phase, are efficiently adsorbed into sludge biomass [6]. On the contrary, volatile organic compounds such as benzene, toluene and styrene are removed by

volatilization during the process. It is important to underline that these removals are only apparent because these compounds are not degraded into less toxic forms [6].

Actual biodegradation of recalcitrant compounds (meaning transformation into less complex and/or less toxic compounds) in AS process is achieved only for some compounds (such as surfactants [7]) and in case of very long sludge retention time (SRT) [8], which allow the growth of organisms with very slow kinetics and/or degrading slow biodegradable compounds.

In this chapter, several treatments commonly used for removal of different classes of compounds from waters are presented.

2.2. REMOVAL TECHNOLOGIES

Activated sludge process constitute the most common, versatile and economical technology for treatment of wastewaters, but, as reported in the previous paragraph, it does not allow the removal of many compounds commonly founded in wastewaters. It is therefore necessary, for their complete removal, to couple existing processes with non-conventional biological treatments (such as fungal treatments, as reported in the following chapters) or, more commonly, with physical and/or chemical processes, such as adsorption (e.g. on activated carbon), coagulation-flocculation processes, chemical precipitation, membrane filtration and reverse osmosis, chemical oxidation and, more recently, advanced oxidation processes.

2.2.1. ANAEROBIC PROCESSES

Many toxic compounds present in industrial wastewaters such as electrophilic aromatic pollutants with multiple chloro, nitro, and azo groups have proven to be persistent to biodegradation by aerobic bacteria [9]. On the contrary, these compounds are readily reduced by anaerobic consortia [9]. For example, under anaerobic conditions many dye molecules can be reduced by azo bond cleavage (in azo dyes) or quinone-group reduction (for anthraquinone dyes) [10]. The result is a breakdown of the chromogenic group with a consequent decolourization. However, it is important to underline that these reduced compounds are less coloured but may be more toxic that the former dye [7]; for example, azo dyes are irreversibly reduced, leading to the formation of carcinogenic aromatic amines, which are usually not degrade further [10].

Among other recalcitrant compounds, Razo-Flores et al. [11] tested an Upflow Anaerobic Sludge Blanket (UASB) reactor for the treatment of a petrochemical wastewater containing benzoate, phenol, toluene, propanol, ethylbenzene, cumene, methylphenylcarbinol and methylphenylketone and they find out that the tested process was able to remove between 80% and 95% of the initial chemical oxygen demand (COD) due to the contained organic compounds.

2.2.2. CHEMICAL OXIDATION PROCESSES

In water treatment, chemical oxidation processes are used for the treatment of specific inorganic or organic species found in waters. For organic compounds, the purpose is to convert them into harmless forms, while inorganic metal species (e.g. iron or manganese) are oxidized to insoluble forms and removed by precipitation [5].

Because of the many different types of oxidation processes that have been developed, it is useful to note some important differences between conventional and advanced oxidation processes (AOP). Conventional chemical oxidation processes employing such oxidants as chlorine, chlorine dioxide, or potassium permanganate do not produce highly reactive species, such as the hydroxyl radical (HO•), produced in the AOP. These radicals are reactive electrophiles that react rapidly and non-selectively with most organic compounds by undergoing addition reactions with double bonds or extracting hydrogen atoms from organic compounds [5]. The reaction rates for conventional oxidants are much slower compared to the reactions and conventional oxidants are more substrate specific in terms of the types of organic molecules that may be oxidized. Nevertheless, conventional oxidation processes can be effective in oxidizing certain organic and inorganic compounds [5].

Given the uncertainty of the toxicity of the byproducts of chemical oxidations, any oxidation process used to remove recalcitrant pollutants should completely oxidize these compounds into carbon dioxide, water and mineral acids (e.g. HCl) [5]. On the other hand, it is important to mention that partially oxidation of recalcitrant compounds often lead to a formation of a simpler and more biodegradable molecule; in the same way, many toxic chemicals for biomass become less toxic after a chemical oxidation. For these reasons chemical oxidations can be also used as a pre-treatment for biological processes with the purpose of increasing the biodegradability of recalcitrant and/or toxic compounds: if complete oxidation is not achieved during the chemical oxidation stage, the partially oxidized compounds can then be biodegraded by the biological process [2,12].

Advantages of chemical oxidation over other chemical-physical techniques lie in the fact that very often other processes are aimed at removing contaminants from the water by transferring them into another phase (e.g. adsorption, coagulation-flocculation, etc), while chemical oxidation can actually transform them into harmless compounds.

General disadvantages of chemical oxidation processes are linked to the chemicals and energy consumption, which depends on the amount of recalcitrant pollutants to be oxidized, but also on the amount of other organic compounds which act as scavengers for the oxidant [12]. Moreover, plant cost for processes such as ozonation is between 5 and 20 times more than the one of a biological process [13]. A possible approach to achieve recalcitrant compounds removal and contain treatment costs is to couple the two processes, using partial chemical oxidation followed by an activated sludge process as suggested above [13].

Conventional oxidation processes

The principal oxidants used in water treatment are chlorine, chlorine dioxide, permanganate, ozone and hydrogen peroxide [5].

Among those, ozone is the one with the higher redox potential [7] and it is commonly employed in the textile industry for removal of color, COD, surfactants, phenols, biphenyls and organochlorides [14]. As an example, ozonation has been employed successfully in a full-scale plant for textile wastewater treatment as reported by Valeri et al. [15]. In that study the purpose of ozonation was to remove textile dyes from the water in order to reuse the plant effluent for industrial purposes.

Mezzanotte et al. [16] proposed an ozonation as a pre-treatment for activated sludge process for the removal of antibiotics from an industrial wastewater and their findings clearly show that chemical oxidation increased the biodegradability of the antibiotics which were removed in the second stage.

Advanced oxidation processes

AOPs are probably the most common practice to remove recalcitrant compounds from wastewaters [2]. AOPs are feasible for full-scale use to destroy organic compounds because they generate hydroxyl radicals at ambient temperature and atmospheric pressure. These radicals can be obtained by the combination of: 1) ozone and hydrogen peroxide, 2) UV light and ozone, 3) UV light and hydrogen peroxide and 4) UV light and titanium dioxide. Pharmaceutical compounds are effectively removed with AOPs: for the treatment of an hospital wastewater Verlicchi et al. [17] tested a combination of a biological membrane

reactor and an AOP with O_3 and UV on membrane permeate and with such combination they managed to remove between 50% and 90% of the contained analgesics and antibiotics. Also recalcitrant dyes are successfully mineralized by AOPs, and these processes are often chose when a complete mineralization is desirable but not achievable by standard chemical oxidation (e.g. with ozone alone) [7].

Another example of AOP efficiency is shown by Cortez et al. [18] who used a combination of hydrogen peroxide and ozone for the treatment of mature landfill leachate, obtaining 72% of COD removal and an increase in the ratio between biochemical and chemical oxygen demand (BOD₅/COD ratio) from 0.01 to 0.24.

2.2.3. COAGULATION-FLOCCULATION PROCESSES

The objective of a coagulation-flocculation process is the removal of suspended, colloidal and some dissolved organic constituents. Coagulation by the addition of hydrolyzing chemicals such as alum and iron salts and/or organic polymers can destabilize small suspended and colloidal particulate matter and allow them to form flocculant particles that can then be removed by sedimentation or filtration. As a result, this process is very useful for removing some classes of recalcitrant compounds from waters.

One of the main disadvantages of this process lies in the fact that pollutant removal is not associated with a mineralization or degradation, but the contaminants are accumulated in the sludge, which become an hazardous waste to dispose of.

Pollutants that can be removed with this process are of different nature: regarding pharmaceuticals compounds, coagulation-flocculation processes are effective only on lipophilic compounds [17], while good results have been obtained for removal of polycyclic aromatic hydrocarbons (PAH) from marine waters by Genovese et al. [19]. In that study, almost 90% of initial hydrocarbons were removed by using ferric chloride and this removal was associated with a reduction of the COD value.

Riera-Torres et al. [20] finds out that coagulation-flocculation process of a textile wastewater could remove between 85% and 95% of the color, with a simultaneous decrease of the COD.

2.2.4. MEMBRANE FILTRATION

Membrane processes are physicochemical separation techniques that use differences in permeability of water constituents as a separation mechanism [5]: during treatment, when water is pumped against the surface of the membrane, permeable components pass through, while impermeable components are retained on the feed side. As a result the product stream is relatively free of impermeable constituents and the waste stream is concentrated in impermeable constituents. The four types of pressure-driven membrane commonly used in wastewater treatment are microfiltration, ultrafiltration, nanofiltration and reverse-osmosis membrane [5]. Depending on the type of membrane, it is theoretically possible to remove from water any type of constituent. The main disadvantage of this technology lies in the energy cost necessary for pumping the water through the membrane, which is strongly dependent on the type of membrane and the amount of non-permeable components to be removed.

Nanofiltration has found a place in the removal of recalcitrant organic compounds and heavy metals from landfill leachate where removal percentages can be over 99% for metals and 65% for organic compounds [21].

Very often membrane filtration is used in combination with other processes, such as biological processes (e.g. in Membrane BioReactors, MBRs) or coagulation-flocculation processes. As an example, Ali Alturki et al. [22] tested a combination of MBR and nanofiltration unit for the removal of trace organic compounds (forty compounds among pharmaceutically active compounds, steroid hormones and pesticides). In that study authors find out that hydrophobic organic compounds were removed by the MBR due to adsorption on the biomass, while the membrane unit effectively removed hydrophilic compounds and removal percentages were over 95% for all the compounds.

Riera-Torres et al. [20] used a combination of coagulation-flocculation and a nanofiltration for dye removal of textile wastewater and, while membrane alone removed between 40% and 80% of dyes, combined treatment allowed to increase these percentages between 95% and 98%.

2.2.5. ADSORPTION

Adsorption techniques are mass transfer operations in which substances present in a liquid phase are removed from it by adsorption on a solid phase. The primary adsorbent material used in these processes is granular activated carbon (GAC), but also powder activated carbon, zeolites and synthetic polymeric adsorbents can be used [5]. One of the main disadvantage of this technology is the fact that after treatment, exhausted adsorbent material become an hazardous waste to dispose of. Nevertheless, GAC is among the technologies with bigger potential for trace organic compounds removal.

Nguyen et al. [23] reported that hydrophilic and persistent compounds carbamazepine, diclofenac, and fenoprop could be removed by GAC up to 95%. Yang et al. [24] tested a combined treatment made by MBR, GAC and ozonation and found out that some compounds recalcitrant to the biological treatments (erythromycin and carbamazepine) could be removed with GAC by up to 88%, while some other compounds, such as primidone, diethyltoluamide (DEET) and caffeine were not amenable to adsorption by GAC.

Adsorption techniques can also be used in textile wastewater treatment, where they can be useful for the removal of organochlorinated compounds, metals and phenols [14] or, after ozonation, for retain possible harmful partially oxidized molecules [7].

2.3. THE CASE OF NAPHTHALENE SULFONIC ACID AND ITS DERIVATIVES

Naphthalene sulphonic acid and its derivatives have been used by the chemical industry since the 19th century, mainly in the synthesis of azoic colorants [25]. They are now commonly employed in many industrial sectors covering a wide range of activities from textile mills and leather tanning operations to production of pharmaceuticals, pesticides, cosmetics, polymers, optical brighteners, dispersants, stabilizers, wetting agents and construction materials [25–27]. These compounds are known to have an highly hydrophilic nature [25–27] and a very low biodegradability [25,28], resulting in their transport and accumulation in both surface and groundwaters [25–28]. Most of naphthalene sulfonic acid derivatives are labeled as toxic chemicals [26,28,29], exert genotoxicity activity [25,26] and have the ability to remobilize toxic hydrophobic compounds [26,28].

Conventional biological treatments do not appear effective in removing these compounds [25,29]; in fact, a study conducted by Song & Burns [28] demonstrated that four activated sludge and two single bacterial strain (*Arthrobacter* sp. and *Comamonas* sp.) degraded only

the monomers of a mixture of naphthalene sulfonic acid polymers. The same study demonstrated, on the other hand, that the fungus *Cunninghamella polymorpha* was able to degrade also oligomers from n=2 to n=11.

Among chemical-physical treatment ozonation has been proposed by Shiyun et al. [29] who removed more than 40% of COD of 11 naphthalene sulfonic acids with an ozone dosage of 5.5 mg/min for 2 h, obtaining an increase of the BOD₅/COD ratio for all compounds. Also Germirli-Babuna et al. [26] used ozonation (89 mgO₃/min for 30 min) on a mixture of naphthalene sulphonic acid derivates, reducing the original COD up to 70%, but toxicity tests on the effluent towards the marine algae *Phaeodactylum tricornutum* indicated that the toxicity of the effluent increased considerably. Rivera-Utrilla et al. [25] tested instead a combined use of ozone and activated carbon in a single process for naphthalene sulfonic acids treatment and obtained almost complete depletion of the compounds, with a 80% removal of the total organic carbon (TOC) and a reduction in the genotoxicity. Another option of treatment is the one tested by Avetta et al. [27] which used a photodegradation on four naphthalene sulfonates, obtaining a depletion of 70-80% of the investigated compounds.

3. FUNGI

3.1. INTRODUCTION

The kingdom of fungi comprises molds, mushrooms, rusts, smuts and yeasts with remarkably diverse life histories that make essential contributions to the biosphere, human industry, medicine and research [30].

In terms of biodiversity, there are estimated to be at least 1.5 million different species of fungi [31], but only about 100'000 species (less than 7% of the total) have been described yet. Fungi in the environment can exist as terrestrial and aquatic saprobes, parasites, symbiotic partners of lichens, and mycorrhizal symbionts associated with plant roots [32]. All fungi have a range of features that separate them from other organisms [33]:

- All fungi are eukaryotic, which means that they have membrane-bonded nucleus in the cell.
- The main group of fungi (filamentous fungi) typically grow as filaments (hyphae), and exhibit apical growth (they extend only at their extreme tips), in contrast to many other filamentous organisms (e.g. filamentous green algae). Fungal hyphae branch repeatedly, giving rise to a network termed "mycelium". However, some fungi grow as single-celled yeasts, and some can switch between a yeast phase and a hyphal phase.
- Fungi are heterotrophs, which means that they need organic compounds as energy and carbon source for cellular synthesis. Fungi absorb simple, soluble nutrients through the wall and cell membrane. In many cases this is achieved by secreting enzymes at the hyphal tips to degrade complex polymers and then absorbing the nutrients released by the depolymerase (polymer-degrading) enzymes.
- Fungi have a distinctive range of wall components, including chitin and glucans.
- Fungi have a characteristic range of soluble carbohydrates and storage compounds, including mannitol and other sugar alcohols, trehalose (a disaccharide of glucose) and glycogen.
- Fungi typically have haploid nuclei. However, fungal hyphae often have several nuclei within each hyphal compartment, and many budding yeasts are diploid.
- Fungi reproduce by both sexual and asexual means, and typically produce spores.

Depending on their way of growth, fungi have a great range of activities, which have made them gain a lot of attention during history. The main fields in which fungi are involved are:

- Crop disease: fungi are one of the most important causes of crop diseases, causing severe economical damage every year. Few examples of fungal pathogens for crops are *Fusarium* spp., and *Rhizoctonia* spp. [33].
- Biological control: fungi can be used as commercial biological control agents for fighting insect pests, nematodes, and plant-pathogenic fungi. Species commonly employed as biological control agents are *Trichoderma harzianum*, *Metarhizium anisopliae* and *Beauveria bassianas* [34].
- Decomposition of organic matter: fungi are the main agent of decomposition and recyclers of organic matter, playing a key role in the breakdown and recycling of cellulose, hemicellulose and lignin.
- Human disease: fungi can cause a wide spectrum of diseases in humans, ranging from harmless skin diseases to life-threatening infections.
- Food industry: fFungi are widely used for mushrooms cultivation, bread-making, in several fermented foods, for beer and other alcoholic drinks production.
- Biotechnology: fungi are also used for production of antibiotics (e.g. penicillins), steroids (for contraceptives), ciclosporins (used as immunosuppressants in transplant surgery), and enzymes. A specific branch of biotechnology consists in the use of fungi for bioremediation. Chapter 4 is dedicated to this specific topic.

As one of the primary decomposers of organic matter in nature, fungal species catabolize a wide diversity of substrates, including cellulose and lignin, through several unique and unusual biochemical pathways [35]. The products of these pathways include important pharmaceuticals such as penicillin, potent poisons such as aflatoxins and industrially interesting enzymes such as laccases. All these compounds, very diverse in structure and perform function, that are not always know, are called "metabolites" [36]. Fungal metabolites are generally classified into primary, which are metabolites essential for growth and reproduction, and secondary, which include ecologically important metabolites not essential to cellular life [37]. However, this distinction is often arbitrary, because generally the pathways that generate primary and secondary metabolites are not mutually exclusive [35]. Another distinction can be made between those pathways shared by most organisms, which can be considered as belonging to general metabolism, and those specialized pathways that have evolved only in certain species. It is interesting to consider that most

secondary metabolites are produced after that the fungus has completed its initial growth phase and it is beginning a stage of development represented by the formation of spores [36].

A specific kind of metabolites, useful for bioremediation of contaminated matrix, are a class of oxidative enzymes secreted by a particular group of fungi (white-rot fungi), which will be discussed in the following paragraph 3.2.

3.2. ENZYMATIC SYSTEM OF WHITE-ROT FUNGI

3.2.1. INTRODUCTION

A specific group of fungal metabolites, as mentioned, are the enzymes. In particular, a specific class of enzymes is of interest for biodegradation of toxic chemicals and/or for treatment of contaminated effluents. These enzymes, called Lignin-Modifying-Enzymes (LMEs), are commonly secreted by a specific group of fungi, the White-Rot Fungi (WRF). WRF are a physiological rather than a taxonomic group of fungi, which include Basidiomycota and Ascomycota [33]. WRF are those fungi that are capable of extensively degrading lignin within lignocellulosic substrates (e.g. wood). The name "white-rot" derives from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance to the substrate [38].

However, it is the presence of lignin that make wood an exceptionally difficult substrate to degrade, because lignin is a complex aromatic polymer, highly resistant to breakdown by conventional enzyme systems, non-hydrolysable, and water-insoluble [33]. The recalcitrance of lignin in comparison to other natural molecules resides in its low porosity that do not allow large enzymatic molecules to penetrate. Moreover, the free radical coupling mechanism responsible for its biosynthesis results in a polymer interconnected through diverse carbon–carbon and ether bonds that are not hydrolysable under biological conditions [39]. In addition to its lignin content, wood also has a very low nitrogen and phosphorus content and it contains potentially toxic compounds for fungi, such as tannins, well known for their ability to cross-link proteins, and phenolic compounds such as terpenes, stilbenes, flavonoids and tropolones [33]. Despite this list of obstacles, fungi largely degrade woody tissues, and this ability is due to their LMEs.

The lignin degrading-system of white rot fungi is extracellular, non-stereoselective and non-specific, able to cleave the carbon-carbon and carbon-oxygen bonds, regardless of the conformation of the chiral carbon of lignin [40]. This characteristic is partly due to a highly

reactive free radical mechanisms of degradation, which would be ideal for the biodegradation of organic pollutants in the environment.

It is however interesting to consider that oxidation of lignin does not provide net energy gain to the fungus; this means that lignin is not a substrate in primary metabolism, but it is degraded during secondary metabolism in order to access wood polysaccharides locked in lignin-carbohydrate complexes (hemicelluloses and cellulose), providing in this way an energy source to which other organisms do not have access [38].

This factor explain one of the main differences between fungi and bacteria in degradation of xenobiotics: while bacteria generally utilize organo-pollutants as a nutritional carbon and/or nitrogen source, very often an additional carbon and nitrogen source is required for primary metabolism of WRF [38]. This feature, very important in the process design of fungal treatment of contaminated effluents, is called "cometabolism".

3.2.2. LIGNIN MODIFYING ENZYMES

From a chemical point of view, enzymes are defined as substances that alter a reaction rate and/or a reaction activation energy without being present in the reaction products [41]. Every enzyme has active sites where it can bond to a substrate and catalyze a reaction: after the temporary bond is formed, this substrate-enzyme complex loosens the bonds that hold the substrate together, allowing the bonds to break. (see Figure 1). Once this occurs, the substrate pieces are released, and the enzyme is ready to catalyze another reaction [41].



Figure 1. Enzymatic reaction process [41].

For what concerns enzymes produced by WRF, the most interesting from an environmental point of view are extracellular oxidoreductases (which catalyze redox reactions), with relatively nonspecific activities. WRF produce one or more than three LMEs, but the most studied LMEs are two glycosylated heme-containing peroxidases, Lignin peroxidase (LiP, E.C. 1.11.1.14) and Manganese peroxidase (MnP, E.C. 1.11.1.13), and a copper-containing phenoloxidase, Laccase (Lac, E.C. 1.10.3.2), although other enzymes are involved in the mineralization process of lignin [38].

The physiology of LME production by WRF has been studied extensively during the last decades. Despite the wide variety of fungal species and experimental conditions reported in literature, many authors agree with the statement that synthesis and secretion of LMEs is often induced by limited nutrient levels (carbohydrate, sulfur and, particularly, nitrogen) [38,40,42]. This characteristic is probably an evolutionary adaptation of the white rot fungi to the low nutrient levels in the wood (the carbon/nitrogen ratio of most woody tissues is high, in the order of 350-500:1) [40]. During evolution, white rot fungi became extremely efficient in their use of nitrogen, developing efficient mechanisms of assimilating, utilizing and conserving the low supply of nitrogen.

Lignin Peroxidases

Lignin peroxidases (LiPs) were first discovered in the extracellular medium of *Phanerochaete chrysosporium* grown under nitrogen limitation [39] They are monomeric hemoproteins with molecular masses between 38 and 47 kDa [42], contain heme in the active site and show a classical peroxidase mechanism (see Figure 2).



Figure 2. Catalytic cycle of peroxidases (modified from Wesenberg et al. [42]).

The catalytic cycle of LiPs is similar to that of other peroxidases and consists of the three reactions listed below [39,43]:

- LiPs are oxidized by H₂O₂ to give a two electron-oxidized intermediate (Compound I) in which the iron is as Fe(IV) and a free radical is present. Hydrogen peroxide is the natural electron acceptor of peroxidases, acting as a two-electron oxidizing substrate for the ferric enzyme, which results in formation of compound I.
- 2. Compound I then oxidizes a donor substrate by one electron, yielding a substratefree radical and Compound II, in which the iron is still present as Fe(IV), but no radical is present.
- 3. Compound II then oxidizes a second molecule of donor substrate, giving another substrate-free radical and the resting state of the peroxidase.

LiP is relatively unspecific for its reducing substrates, a characteristic shared with other peroxidases, but differ from them because its preferred substrates are non-phenolic aromatic compounds [43]. Another important difference between LiP and other peroxidase is that LiP can oxidize aromatic rings that are only moderately activated by electron-donating substituents [39].

It is interesting to consider that, even if LiP is involved in the mineralization of lignin, it is not essential: several highly active WRF and litter-decaying fungi (e.g., *Ceriopsis subvermispora*, *Dichotomitus squalens*, *Panus tigrinus*, *Rigidosporus lignosus*) do not secrete this enzyme [42].

Manganese Peroxidases

The most common ligninolytic peroxidases produced by almost all white-rot basidiomycetes are manganese peroxidases (MnP). These glycoproteins, with a molecular weight between 32 and 62.5 kDa [42] are also strongly oxidizing and undergo the same classical peroxidase cycle as LiP (Figure 2), but with few important differences. The peculiarity of MnPs cycle is that these enzymes do not oxidize non-phenolic lignin-related structures directly, but they preferentially oxidize Mn^{2+} from Compound I [39]. The product, Mn^{3+} , is released from the active site. The purpose of this reaction is evidently to transfer the oxidizing power of MnP to a small agent (Mn^{3+}), which can diffuse into the lignified cell wall and attack it from within. Mn^{3+} is a strong oxidant, but quite unstable in aqueous solutions; on the other hand it has been demonstrated that WRF are able to secrete some organic acids such as oxalic acid, which act as Mn^{3+} chelators. Chelated Mn^{3+} acts as

a highly reactive (up to 1510 mV in H₂O), low molecular weight, diffusible redox-mediator [42].

Regarding MnP production, it has been demonstrated for *Phanerochaete chrysosporium* that it is stimulated by nutrient limitation and presence of Mn^{2+} [39].

Some limitation in the use of this enzyme are represented by the fact that, by contrast with LiP, MnP is quite specific for reducing substrates and only Mn^{2+} efficiently supports enzyme turnover [43]. Moreover, the enzyme requires high concentrations of Mn(III) [44].

Laccases

Laccases are part of a larger group of enzymes termed the multicopper enzymes [45] and are produced by almost all wood- and litter-transforming basidiomycetes and several ascomycetes [42]. The presence of laccase has been documented in insects, bacteria, plants and virtually every fungus examined for it [46]. In particular, in many fungal species the presence of both constitutive and inducible laccases have been reported [46].

Fungal laccases often occur as isoenzymes that oligomerize to form multimeric complexes and the molecular mass of the monomer ranges from about 50 to 100 kDa [47].

These enzymes contain four copper atoms in the active site (as Cu^{2+} in the resting enzyme), that are distributed among different binding sites [42]. Although the structure of this active site seems to be similar in all the fungal laccases, there is great diversity in the rest of the protein structure [46].

The range of substrates which various laccases can attack is very wide; basically any substrate with characteristics similar to a *p*-diphenol will be oxidized by laccases [46], with the concomitant reduction of oxygen to water [45].

Moreover, laccases can also oxidize monophenols such as cresol [46], can decarboxylate phenolic and methoxyphenolic acids, and attack their methoxy groups (demethylation) [42]. It is important to underline that sometimes the oxidation of substrates by laccases creates reactive radicals that can undergo non-enzymatic reactions, such as ring cleavage of aromatics and depolymerization of natural and synthetic polymers [47]. This latter reaction takes place because generated reactive radicals cleavage the covalent bonds and lead to the release of monomers. However, because of steric hindrance, the enzymes might not come directly into contact with some polymers. Instead, small organic compounds or metals that can also be oxidized and activated by laccases, e.g. veratrylalcohol, 3-hydroxy-anthranilic acid and manganese, mediate the radical-catalyzed depolymerization [47]. These compounds, known as mediators, are better described in the following paragraph 3.2.3.

In conclusion we can state that the dependence of laccases by oxygen rather than hydrogen peroxide, its intrinsic stability, and the capability to be active on a great number of substrates thanks to mediators, makes it extremely interesting for biotechnology applications.

3.2.3. MEDIATORS

Given the random polymer nature of lignin and the molecular size of LMEs, direct and specific interactions between lignin and LME are highly improbable [42]. Instead, low-molecular weight, diffusible redox mediators of internal or external origin can migrate from the enzymes and oxide lignin from inside the lignocellulose complex [48]. These compounds, once oxidized by the enzyme, are stable radicals that can oxidize even compounds that in principle were not substrate of the enzyme (such as non-phenolic compounds for laccase), extending the range of possible substrate of the enzymes [49,50].

It is important to mention that mediators are especially important during the initial steps of lignin degradation, since the compact molecular architecture of the intact plant cell-wall prevents the penetration of enzymes to be in direct contact with lignin [43].

Important characteristics of a good mediator are [51]: a) it has to be a good enzyme substrate; b) its oxidized and reduced forms must be stable, but must be not inhibit the enzymatic reaction; c) its redox conversion must be cyclic (be oxidized by the enzyme to a stable high-potential intermediate, oxidize another compounds and be reduced to the initial form, closing the cycle).

It is interesting to underline that mediators are often produced as a result of fungal metabolism, such as veratryl alcohol, oxalate, malate, fumarate and 3-hydroxyanthranilic acid [48].

Lignin peroxidase mediators

The most studied LiP mediator by far is veratryl alcohol (VA, 3,4-dimethoxy benzyl alcohol), a secondary metabolite of several WRF [42]. The oxidation of VA by LiP results in the production of a cation radical (VA \cdot ⁺), which can then react with several substrates. However, because its half life is extremely short, its function as an independent diffusible mediator is highly dubious [48]. Mediating properties of VA could be enhanced if the radical is somehow complexed to the LiP [48]. Nevertheless, LiP is stimulated by VA probably by protecting the enzyme against the damaging effect of H₂O₂ [42].

Another natural metabolite of WRF, reported to act as a redox mediator in the LiPcatalyzed oxidations is 2-Chloro-1,4-dimethoxybenzene [42].

Manganese peroxidase mediators

The activity of MnP is dependent on Mn^{2+} because it has been demonstrated that MnP oxidizes Mn^{2+} to Mn^{3+} , which in turn oxidizes lignin. It is known that MnP is able to oxidize only phenolic compounds, but, in presence of suitable mediators it could also oxidize non-phenolic compounds such as veratryl, anisyl, and benzyl alcohols [52].

Examples of these mediators are unsaturated fatty acids, thiols (gluthanione, cysteine), organic acids (oxalate, malate, malonate, fumarate), and also a laccase mediator, 3-hydroxy-1,2,3-benzotriazin-4(3H)-one [32,42,48,52].

The action of organic acids, secreted by several fungal organisms, is to chelate and stabilize Mn^{3+} . MnP was found to simultaneously decompose these acids and oxidize Mn^{2+} to Mn^{3+} even in the absence of H_2O_2 [42]. Thus, organic acids are postulated to be the origin of different types of radicals, that could be a source of peroxides, which can be used by MnP as substrates instead of H_2O_2 . Consequently, even fungi lacking H_2O_2 -generating oxidases could be efficient lignin-degraders and, by extension, useful in the degradation of xenobiotics [42].

Laccase mediators

In the past years several works on laccase-mediator systems has been published and more than 100 possible mediator compounds have already been described, but the most commonly used are still 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) [53].

These compounds are oxidized by the enzyme to the radical HBT•, the cation radical $ABTS^{+}\bullet$, and the ABTS dication $ABTS^{2+}$ [53]. These species can then enabling the oxidation of non-phenolic compounds which cannot be oxidized by laccases on their own, thereby expanding the range of applications of these enzymes [46,54]. It is interesting to consider that often these redox mediators own a redox potential higher than laccase itself [55].

Most laccase mediators are compounds containing the structural groups N-OH or N-O, which are oxidized to reactive radicals. They include the already mentioned HBT, TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxyl), N-hydroxyphthalimide, violuric acid, N-hydroxyacetanilide, 3-hydroxy-1,2,3-benzotriazin-4(3H)-one and N-hydroxyphthalimide [48–52].

Other classes of organic compounds are also used as laccase enhancers such as nitroso compounds, phenols and aromatic amines [49,51]. Examples are: 3-hydroxyanthranilic

acid, 3-hydroxyanthranilic acid, 2-nitroso-1-naphthol-4-sulfonic acid, 1-nitroso-2-naphthol-3,6-disulfonic acid and 4-hydroxy-3-nitroso-1-naphtalenesulfonic acid [48,49,52].

In spite of the proven efficiency of laccase-mediator systems to degrade recalcitrant aromatic compounds of biotechnological and environmental interest, the application of these enzymatic systems is in part hindered by the economic cost of the artificial mediators and the generation of possible toxic species [56]. For these reasons, many studies have focused on searching alternative mediators which could present environmental and economic advantages. Indeed, a number of compounds, either produced by fungal metabolism or present during the degradation of lignocellulosic substrates (e.g. oxidized lignin units), are effective natural laccase mediators [48,49,51]. Examples of these compounds are phenol, aniline, veratryl alcohol, benzaldehyde, 4-hydroxybenzoic acid, pvanillin, acetovanillone, methyl vanillate, syringaldehyde coumaric acid, and acetosyringone [49,51,53].

It is however important to remember that, due to the different chemical properties of every compound, the use of a mediator instead of another is strictly dependent on the nature of the substrate to be oxidized.

3.2.4. OTHER ENZYMES

Ligninolytic enzymes (laccase, manganese peroxidase and lignin peroxidase) are by far the most studied enzymes produced by WRF due to their ability to degrade a broad spectrum of recalcitrant compounds. Nevertheless several other intracellular and extracellular enzymes are produced, which may play a role in biodegradation processes. Without claiming comprehensiveness, in the present paragraph a brief description of some of these systems is given.

Versatile Peroxidases

Besides LiP and MnP, another type of ligninolytic peroxidase called versatile peroxidase (VP) has been first observed in *Pleurotus* and *Bjerkandera* species [57] and, more recently, in some *Panus* and *Trametes* species [58]. VP is also a ligninolytic peroxidase but with MnP–LiP hybrid properties and thus capable of oxidizing both the typical substrates of MnP (Mn²⁺) and LiP (VA). Its hybrid molecular structure that provides multiple binding sites is the basis for the oxidation of different substrate types, including some aromatic compounds (both phenolic and non-phenolic) and dyes that are not efficiently oxidized by LiP and MnP [57,58].

Dye-Peroxidases

Dye-decolorizing peroxidases (DyP) represent a new superfamily of heme peroxidases identified in fungi and in bacteria [59]. A characteristic feature of all DyPs studied so far is their ability to oxidize synthetic high-redox potential dyes of the anthraquinone type, which can only hardly be oxidized by other peroxidases [59]. This catalytic feature makes DyP-type peroxidases interesting for applications in the wastewater treatment field. Although the catalytic cycle of DyP and the cleavage of dyes are not fully understood yet, there are indications for a unique mechanism that includes, besides typical peroxidative reactions, the hydrolytic fission of the anthraquinone back bone [60]. The high stability of DyPs is another remarkable property of these enzymes and makes them attractive for applications under rather harsh conditions [60].

Aromatic Peroxygenases

Aromatic peroygenases (APOs) is a group of heme-thiolate proteins which efficiently transfer oxygen from peroxides to various organic substrates including aromatic, heterocyclic, and aliphatic compounds. In addition to oxygen transfers, APOs catalyze also one-electron oxidation of phenolic substrates (peroxidative activity) [60]. A catalytic cycle has been proposed, which combines elements of that of heme peroxidases with the pathway of P450 monooxygenases [60]. In all cases, the APO enzyme needs merely hydrogen peroxide as co-substrate for function.

Among pollutants which have been successfully transformed by APOs it is important to mention toluene, naphthalene, fluorene, anthracene, phenanthrene, pyrene, dibenzofuran, propranolol, acetanilide, and diclofenac [60].

Cytochrome P450

Cytochrome P450 (cytP450) is a family of intracellular monooxygenases found in all three domains of life, involved in the metabolism of a diverse array of endogenous and xenobiotic compounds [61]. Catalytic activity of P450 family consists in the incorporation of a single atom from O₂ into a substrate molecule, with concomitant reduction of the other atom to H₂O. In this way cytP450 can perform epoxidation and hydroxylation of aromatic or aliphatic structures of many pollutants, including polycyclic aromatic hydrocarbons, polychlorinated dibenzodioxins, alkanes and alkyl-substituted aromatics [32].

The P450 family was classified in at least ten classes according to proteins participating in the electron transfer. Regarding fungi, three classes have been identified, involved in both

primary and secondary metabolism, as well as in xenobiotic degradation [62]. It has been demonstrated that cytP450 can be involved in fungal metabolism of anti-inflammatory drugs, lipid regulators, anti-epileptic and anti-analgesic pharmaceuticals, and diphenyl ether herbicides [32].

In order to assess the involvement of cytP450 family in degradation experiments very often it is used as inhibitor 1-Aminobenzotriazole (ABT), which is thought to inactivate P450 enzymes nonselectively by covalent modification of the heme prosthetic group following bioactivation [63].

Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular flavo-enzyme produced by several ligninolytic fungi which has been proposed to play a role in the early events of lignocelluloses degradation and wood colonization [64].

Typical basidiomycete CDHs show a strong preference for cellobiose and cellooligosaccharides, which are oxidized at the anomeric carbon atom, while glucose and other monosaccharides are very poor substrates [65]

Due to its ability to facilitate the formation of free hydroxyl radicals, CDH has been reported to display *in vitro* a synergism with laccases in the decolorization of many dyes [66].

Glucose oxidase

Glucose oxidase (GOD) is a flavoprotein which catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide by utilizing molecular oxygen as an electron acceptor [67]. The main function of GOD is to act as antibacterial and anti-fungal agent through the production of hydrogen peroxide [68].

GOD it has been successfully used *in vitro* in combination with lignin peroxidase for removal of dyes from water [69] because produced H_2O_2 can be used by peroxidases in their catalytic cycle.

4. FUNGAL WASTEWATER TREATMENT: STATE OF THE ART

4.1. TARGETED COMPOUNDS

Researches on the use of fungi and their enzymes for treatment of wastewaters containing recalcitrant compounds started about thirty years ago and several comprehensive reviews on the feasibility of their use has been published [32,38,70]. During this decades, several compounds and wastewaters has been treated with fungi and the interest of scientific community in their possible application has grown. Firsts compound successfully degraded by WRF were polycyclic aromatic hydrocarbons (PAH), organochlorides (such as polychlorinated biphenyls and dioxins), pesticides and munitions wastes (such as nitrotoluenes). During late 1990s and the beginning of 2000s several articles were published addressing the ability of WRF to mineralize phenols (commonly found in olive mill wastewaters) and to decolorized many different dyes (such as azo dyes, nitro and nitroso dyes, anthraquinone dyes). More recently, a massive work has been made in order to prove WRF ability to degrade the so-called "emerging contaminants" such as endocrine disrupting chemicals (EDC), pharmaceutical compounds, hormones and much more.

Other streams successfully treated by WRF are heavy metals containing wastewaters, landfill leachate, coking wastewaters, sugar refinery wastewaters and paper-mill wastewaters.

In the following paragraphs a brief description of fungal treatment of the above-mentioned compounds is given.

4.1.1. POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAH) are hydrocarbons composed of multiple aromatic rings, which present serious problems of toxicity and persistence in the environment.

A considerable number of individual PAH are found in wastewaters, arising from domestic and industrial wastes, rainfall and runoff waters and also street sweeping waters that collect atmospheric dry deposits, dusts from asphalt erosion, residues from petroleum products and various trash [71].

Several studies have shown that WRF are capable of PAHs degradation. The most studied fungi belonged to the genera *Phanerochaete*, *Pleurotus* and *Trametes*, but during years other WRF were added as candidates for PAH remediation, such as *Irpex* spp., *Polyporus* spp., *Stereum* spp., *Lentinus* spp., *Bjerkandera* spp. and *Phlebia* spp. [72].

Experiments with cell-free enzyme extracts have confirmed that LME are involved in PAH attack [38]. In particular, experiments with LiP from *P. chrysosporium* were among the first to be performed and demonstrated that benzo(*a*)pyrene, benzo(*u*)anthracene, anthracene, pyrene, and perylene are directly oxidized by LiPs to quinone-type products and that the mechanism for PAH oxidation by LiP is fundamentally the same as that for lignin-substructure compounds [70]. Further experiments with MnP and Lac in presence of HBT and ABTS showed that also these two enzymes are able to efficiently mineralize different PAHs [38]. More recently, it has been proposed that some intracellular monooxygenase such as cytP450 may play a role in fungal PAH metabolism [73].

4.1.2. ORGANOCHLORIDES

Chlorinated alkanes and alkenes

A first class of organochlorides is chlorinated alkanes and alkenes. Typical examples are the aliphatic halocarbons trichloroethylene (TCE) and perchloroethylene (PCE), widely used as degreasing solvents, which represent a serious environmental pollution problem, being on the Environmental Protection Agency list of priority pollutants [74]. *P. chrysosporium* was the first organism tested for TCE removal and it has been proposed that TCE is subject to reductive dehalogenation catalyzed by LiP, leading to the production of the corresponding reduced chlorinated radicals [75]. More recently, other authors indicates a possible involvement of cytP450 system in TCE and PCE degradation by *T. versicolor* [74].

Polychlorinated Biphenyls

Another important class of organochlorinated pollutants is polychlorinated biphenyls (PCBs), aromatic compounds containing multiple chlorines per biphenyl molecule. These compounds are no longer in production due to their environmental toxicity and persistence, but in the past they were widely used in heat transfer fluids, dielectric fluids, coolant fluids, carbonless copy paper, solvent extenders, flame retardants and plasticizers [38]. Some PCB congeners have been shown to be transformed by bacteria; however, higher chlorinated congeners are generally recalcitrant to biological degradation [38].

Also in this case *P. chrysosporium* was the first fungus that was shown to degrade *in vivo* a wide range of PCB congeners, but several other fungi were later found to have similar abilities, such as *Pleurotus* spp., *Trametes* spp., *Bjerkandera* spp., *Phlebia* spp. and *Coriolopsis* spp. [38,72,74]. Most fungi produced chloride ions during degradation, indicating dehalogenation of the molecule [72]. In general it is possible to state that the
extent of degradation/mineralization of PCBs decreases with increase in the number of chlorines on the biphenyl nucleus [74].

It was also studied the capacity of WRF to degrade the hydroxylated form of PCBs and it has been shown that, unlike PCB, hydroxylated PCBs are transformed by laccase, but also in this case, the degradation rate decrease with increase in the degree of chlorination, leading to the need of laccase mediators to achieve the transformation of highly chlorinated hydroxylated PCBs [74].

Chlorinated dioxins

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), commonly known as dioxins, are formed from various sources and are toxic environmental pollutants and confirmed humans carcinogens. These compounds have been shown to be degraded by several species of white-rot fungi such as *Phanerochaete* spp., *Phlebia* spp., and *Bjerkandera* spp.

It has been proposed that the pathway for *in vivo* degradation of certain PCDDs is an hydroxylation and/or a methoxylation [72]. *In vitro* mineralization of 2,7-PCDD was achieved with LiP and MnP carrying on a multi-step pathway involving sequential oxidation, reduction and methylation reactions to remove the two Cl atoms and carry out ring cleavage [38]. Moreover, some inhibition studies suggested a possible involvement of cytP450 enzyme in dioxin degradation [74].

4.1.3. PESTICIDES

Chlorinated Herbicides

One of the most important groups of chlorinated herbicides is represented by triazine herbicides. These compounds are chloro-derivates of triazine and are among the most widely used agricultural chemicals worldwide. Atrazine is the most common triazine herbicide found in groundwaters, possible cause of several types of cancer and a proven endocrine disruptor [76].

Biological degradation of atrazine has been achieved by white rot fungi such as *Phanerochaete* spp. and *Pleurotus* spp., yielding hydroxylated and *N*-dealkylated metabolites. Atrazine oxidation by these two species appears to involve cytP450 system, while *in vitro* degradation with purified LiP and MnP seems not to occur [74]. *Trametes versicolor* is another fungus which seems to be able to degrade atrazine in non-sterile soil

and to effect *N*-dealkylation of triazine herbicides simazine, propazine and terbuthylazine [74].

Another group of organochlorinated herbicides are phenoxyalkanoic herbicides, which includes 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). These compounds have also been widely used, with the concomitant generation of dioxins [38]. They were components of the "Agent Orange", used extensively as a defoliant during the Vietnam War and they are suspected to cause a wide range of disease such as chloracne, Hodgkin disease and cancer [77]. Biodegradation of 2,4-D and 2,4,5-T by *P. chrysosporium* has been demonstrated both in liquid media as well as in soil [38], although ligninolytic enzymes seems to be not involved in the process [74].

Other chlorinated herbicides degraded by WRF are alachlor, cylanilides and phenylurea, degraded by *P. chrysosporium* [70], propanil, sulfentrazone and chlornitrofen, degraded by various ectomycorrhizal and white rot fungi [72].

Chlorinated Insecticides

One of the most toxic chlorinated insecticides is by far DDT (1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane), the first of the chlorinated organic insecticides, used quite heavily after the World War II. This compound has been extensively studied and also its biodegradation by white rot fungi has been widely reported. Several fungi from the genera *Phanerochaete*, *Pleurotus* and *Trametes* has proven to be able to mineralize this compound [74,78], but the role of LMEs remains unclear.

Another important insecticide, widely used in the past before being banned is aldrin, which is extremely persistent in soil and waters [79]. Aldrin is converted by common soil microorganisms to dieldrin, which is the active form of the insecticide. Some WRF (such as *Phlebia* spp. and *Pleurotus* spp.) seems to be able to carry out the epoxidation of aldrin to dieldrin and to achieve partial dieldrin mineralization [78,79]. It is of interest that also other fungi (not white-rot) show a high ability to degrade DDT: *Gloeophyllum* genus, *Daedalea* genus, and *Fomitopsis* genus [72].

Another group of chlorinated insecticides, widely used in the past and very persistent in the environment, is represented by derivatives of hexachlorocyclopentadiene: lindane, chlordane, heptachlor and endosulfan. Different fungi are reported to mineralize lindane, such as *P. chrysosporium*, *T. hirsutus*, *P. sordida*, *B. adusta* and various *Pleurotus* spp. [72,74,78]. Lindane degradation was not observed *in vitro* using purified LiP and MnP from *P. chrysosporium* [74], while lindane degradation from *P. ostreatus* is proposed to be

achieved thanks to ligninolytic enzymes [78]. However, in the presence of the cytochrome P450 inhibitor 1-aminobenzotriazole, lindane degradation was reduced drastically suggesting the involvement of cytP450 in lindane degradation by *P. chrysosporium* [74]. Chlordane is reported to be partially mineralized by *P. chrysosporium* [70], while heptachlor is reported to be degraded by various *Pleurotus* spp. [78]. Regarding endosulfan, a model for its degradation by *P. chrysosporium* was proposed in the past, in which distinct hydrolytic and oxidative (including cytP450-mediated) pathways are involved [38]. Other fungi reported to be able to efficiently degrade endosulfan are *Chaetosartorya stromatoides*, *Aspergillus* spp. and *Pleurotus* spp. [72,78].

Among other chlorinated insecticides degraded by fungi, it is important to mention β cyfluthrin, a synthetic pyrethroid insecticide, biotransformed by *Aspergillus nidulans* and *Sepedonium maheswarium*, imidacloprid, a class of neuro-active insecticide, degraded by *Calocybe indica* and toxaphene, degraded by *Bjerkandera* spp. [72]

Other pesticides

Other classes of pesticides, including organophosphorous, methylcarbamate, and synthetic pyrethroid insecticides, that were developed to replace organochlorines, also present problems of toxicity and persistence. Among these, carbofuran, one of the most toxic carbamate pesticides, is efficiently degraded by several fungi, including *Fusarium* spp. and *Aspergillus* spp. [80].

Organophosphate insecticides are generally quickly degraded in the environment, but some of them are moderately persistent. Chlorpyrifos, fonofos and terbufos are degraded by *P. chrysosporium*, but no confirmation of LME involvement was done [38], while *Fusarium* sp. is able to degrade glyphosate [72]. Other non white rot fungi able to degrade organophosphate compounds are *Penicillium* spp., *Trichoderma* spp., *Aspergillus* spp. and *Alternaria* spp. [81].

4.1.4. PHENOLS

Other groups of important aromatic contaminants are phenols and derivatives. The major sources of phenol contamination are the chemical and petrochemical industries, agriculture (e.g. olive oil production), wood processing as part of papermaking technologies and textile industry [72]. The ubiquitous nature of phenols, their toxicity even in trace amounts and the stricter environmental regulations make it necessary to develop processes for their removal from wastewaters.

White-rot fungi have been shown to exhibit unique biodegradation capabilities for phenols. A broad screening of fungi [82] showed that *Pycnoporus coccineus*, *Pleurotus sajor caju*, *Coriolopsis polyzona* and *Lentinus tigrinus* were very active in decolourisation and COD removal of olive mill wastewater (OMW). Another *Pleurotus (P. ostreatus)* has been widely used for phenols removal and OMW detoxification [83–85]. It is interesting to consider that *Pleurotus* spp. seems to be able to grow in OMW containing polyphenols without any additions and that phenol content could induce laccase expression [86].

Also the fungus *Phanerochaete chrysosporium* was investigated for OMW treatment and it was demonstrated that this fungus is capable of reducing colour, chemical oxygen demand and phenol content [87].

Another fungus, *Panus tigrinus*, was investigated for its ability to reduce the polluting load of OMW, with a significant presence of phenolic components [72].

4.1.5. DYES

Synthetic dyes are used commonly in different industries ranging from food, textile production, paper printing, color photography, cosmetics, leather industries and pharmaceuticals [88]. All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents, and microbial attack [42]. For these reasons the majority of dyes are recalcitrant and some of them have been shown to be carcinogenic [89]. As a consequence, treatment of dyes-containing effluents (e.g. textile wastewaters) become very complex and often requires advanced processes such as ozonation [90]. Among biological processes proposed in literature white-rot fungi are by far the most efficient organisms in degrading synthetic dyes. The abilities of white rot fungi and their ligninolytic enzymes to degrade synthetic dyes have been widely proved during the past years and some of these many evidences are summarized in Table 1.

Fungus	Enzyme	Dye	Reference
Aspergillus spp.	n.d.	Direct Red, Malachinte Green, Reactive Red	[91]
Bjerkandera spp.	LiP, MnP	Orange II, Poly R-478, Reactive Blue, Reactive Orange, Reactive Violet, Reactive Black, Remazol Brilliant Blue	[92–96]
Ceriporia spp.	n.d.	Poly R-478, Remazol Brilliant Blue	[95]
Daedalea spp.	n.d.	Brilliant Green, Congo Red, Cresol Red	[97]
Daedaleopsis spp.	n.d.	Poly R-478, Remazol Brilliant Blue	[95]
Dichomitus spp.	Lac, MnP	Brilliant GreenTPM, Congo Red, Cresol Red, Crystal Violet, Orange II	[97]
Funalia spp.	n.d.	Acid Black, Acid Red, Acid Violet, Lanasol Black, Reactive Black, Reactive Blue, Reactive Orange, Reactive Red	[66,98]
Fusarium spp.	n.d.	Crystal violet, Malachite green	[99]
Geotrichum spp.	n.d.	Reactive Blue	[100]
Irpex spp.	Lac, LiP, MnP	Bromophenol Blue, Chicago Sky Blue, Congo Red, Cu- phthalocyanine, Methyl Red, Naphthol Blue Black, Poly R- 478, Reactive Black, Reactive Orange, Remazol Brilliant Blue	[95,101,10 2]
Lentinus sp	Lac, MnP	Bromophenol blue, Indigo carmine, Methyl Red, Orange II, Poly R-478, Reactive Blue, Remazol Brilliant blue	[96,103]
Myocacia spp.	n.d.	Poly R-478, Remazol Brilliant Blue	[95]
Penicillium spp.	n.d.	Cotton blue	[104]
Phanerochaete spp.	Lac, LiP, MnP	Acid Green, Acid Red, Amaranth, Azure Blue, Brilliant Green, Brilliant Yellow, Bromophenol blue, Chlorazol Yellow, Chrysophenine, Congo Red, Cu-phtalocyanine, Cresol Red, Crystal Violet, Everdirect Supra Yellow, Everzol Red, Everzol Turquoise Blue, Everzol Yellow, Indigo, Indigo Carmine, Mordant Yellow, Navilene Black, Navione Blue, Neutral Red, New Coccine, Orange G, Orange II, Orange K, Poly R-478, Reactive Black, Reactive Blue, Reactive Orange, Reactive Red, Reactive Violet, Reactive Yellow, Reactofix Blue, Reactofix Golden Yellow, Reactofix Orange, Remazol Brilliant Blue, Remazol Turquoise Blue, Sulphur Red, Sulphur Green, Tartrazine, Vat Brown	[92,95– 97,105– 112]

Table 1. White rot fungi and their enzymes for dyes degradation.

(continued on the next page)

Fungus	Enzyme	Dye	Reference
Phellinus spp.	n.d.	Indigo, Poly R-478, Remazol Brilliant Blue	[95,105]
Phlebia spp.	Lac, LiP, MnP	Brilliant Green, Cibacron Orange, Cibacron Red, Congo Red, Cresol Red, Crystal Violet, Orange II, Poly R-478, Reactive Blue, Remazol Black, Remazol Blue, Remazol Golden Yellow, Remazol Navy Blue, Remazol Red, Remazol Turquoise Blue	[96,97,113]
Piptoporus spp.	n.d.	Acid Green, Acid Red, Brilliant Green, Brilliant Yellow, Chlorazol Yellow, Chrysophenine, Cibacron Brilliant Red, Cibacron Brilliant Yellow, Crystal Violet, Cu-phtalocyanine, Indigo Carmine, Orange II	[112]
Pleurotus spp.	Lac, LiP, MnP	Acid Green, Amaranth, Brilliant Green, Brilliant Yellow, Chrysophenine, Cibacron Brilliant Red, Cibacron Brilliant Yellow, Crystal Violet, Cu-phtalocyanine, Indigo, Indigo Carmine, Mordant Yellow, Neutral Red, New Coccine, Orange G, Orange II, Poly R-478, Reactive Black, Reactive Blue, Reactive Violet, Remazol Brilliant Blue, Tartrazine	[93– 95,105,108 ,112]
Polyporus spp.	Lac, MnP	Brilliant Green, Congo Red, Crystal Violet, Orange II, Poly R- 478, Reactive Blue	[96,97]
Pycnoporus spp.	n.d.	Amaranth, Bromophenol Blue, Indigo, Malachite Green, Orange G, Trypan blue	[105,114,1 15]
Stereum spp.	Lac, MnP	Orange II, Poly R-478, Reactive Blue, Remazol Brilliant Blue	[95,96]
Thelephora spp.	n.d.	Amido black, Congo Red, Orange G	[116]
Trametes sp	Lac, LiP, MnP	 Acid Green, Acid orange II, Amaranth, Amido black, Brilliant Green, Brilliant Yellow, Bromophenol Blue, Chlorazol Yellow, Chrysophenine, Cibacron Brilliant Red, Cibacron Brilliant Yellow, Congo Red, Crystal Violet, Cu- phtalocyanine, Everdirect Supra Yellow, Everzol Red, Everzol Turquoise Blue, Everzol Yellow, Fast blue, Indigo Carmine, Mordant Yellow, Neutral Red, Acid Red, Orange G, Orange II, Orange K, Poly R-478, Poly S119, Reactive Black, Reactive Blue, Reactive Orange, Reactive Violet, Remazol Black, Remazol Brilliant Blue, Remazol Brilliant Red, Remazol Orange, Tropaeolin 	[92,95,111, 112,117– 121]

Table 1 (continued)

Most of the dye molecules have a polyaromatic structure with a high molecular weight, but they exhibit considerable structural diversity. The chemical classes of dyes employed more frequently on industrial scale are the azo, anthraquinone, sulfur, indigoid, triphenylmethyl (trityl), and phthalocyanine derivatives [122]. Thanks to their nonspecific LMEs fungi are able to degrade a wide range of classes; in the case of azo dyes, laccases seem to be the most promising enzymes [89], while for anthraquinone dyes the involvement of MnP has been proposed [102].

Anyway, it is clear that LME play significant roles in dye metabolism by WRF thanks to several *in vitro* experiments with LME systems from WRF culture supernatants [42]. This is not surprising, given the structural similarity of most commercially important dyes to lignin (sub)structures.

4.1.6. EMERGING CONTAMINANTS

Micropollutants (MPs) such as pharmaceuticals (e.g. antidepressants, anti-inflammatory, analgesics, antibiotics), personal care products, hormones, solvents and detergents, are biologically active substances which can present a threat to the aquatic environment with effects such as acute and chronic toxicity to aquatic organisms, accumulation in the ecosystem and loss of habitats and biodiversity, as well as a range of possible adverse effects on human health [3]. Some of them are classified as endocrine disrupting chemicals (EDCs) because at certain doses, they can interfere with the endocrine (or hormone) system in mammals. These disruptions can cause cancerous tumors, birth defects, and other developmental disorders [123]. Although they occur in the environment at concentrations ranging from a few nanograms to several micrograms per litre, MPs may still cause harm to aquatic species [4].

Among the developed bioprocesses, fungi and their enzymes (mostly laccases and peroxidases) have been successfully used for degrading several MPs. Without a claim of comprehensiveness, Table 2 summarize the work made by the scientific community in this field.

Fungus	Compound	Wastewater	Removal Percentages	Reactor Type	Ref.
Trametes versicolor	Pharmaceuticals	Urban wastewater	80 - 100%	Fluidized bed bioreactor	[124]
Trametes versicolor	Clorifibric Acid	Synthetic wastewater	80%	Fluidized bed bioreactor	[125]
Trametes versicolor	Carbamazepine	Synthetic wastewater	54%	Fluidized bed bioreactor	[126]
Transtas	Pharmaceuticals		30 - 100%		
versicolor	Flame retardants	Sewage sludge	0-53%	Bioslurry reactor	[127]
versicolor	UV Filters		0-100%		
	Pharmaceuticals		20 - 100%		
T	Steroid hormones	Construction	75 - 100%	Maarlaan	
I rametes	Pesticides	Synthetic	13 – 95%	Nembrane	[128]
versicolor	UV filters	wastewater	85 - 95%	Bioreactor	
	Phytoestrogens		95 - 98%		
Trametes	Diclofenac	Synthetic	95%	Membrane	
versicolor	Bisphenol A	wastewater	99%	Bioreactor	[129]
Aspergillus versicolor	4- <i>n</i> -nonylphenol	Synthetic wastewater	87%	Batch reactor	[130]
Phanerochaete chrysosporium	Carbamazepine	Synthetic wastewater	90%	Fixed bed Bioreactor	[131]
	Anti-inflammatory drugs		30 - 100%	Endbatch CTD	
Phanerochaete	Carbamazepine	Synthetic	0-64%	Fed batch STR	[100]
chrysosporium	Anti-inflammatory drugs	wastewater	35 - 95%	COTTO	[132]
	Carbamazepine		58-93%	CSTR	
	Anti-inflammatory drugs		65 - 93%	Stirred tank	
Phanerochaete	Psychiatric drugs	Synthetic	24 - 63%	Reactor	
chrysosporium	Anti-inflammatory drugs	wastewater	> 90%		[133]
	Carbamazepine		60 - 90%	Fixed bed reactor	
Laccase from	Phenolic Endocrine-	Model polluted	80 - 90%	Rotating reactor	[134]
Trametes spp.	Disrupting Chemicals	soil	00 7070	1.0000000	[10.]
Laccase from	Endocrine-Disrupting	Synthetic	80 - 100%	Packed bed	[135]
C. polyzona	Chemicals	wastewater		reactor	[]
Laccase from T. versicolor	17-β-estradiol	Synthetic wastewater	60 - 70%	Packed bed reactor	[136]
Laccase from M. thermophila	Endocrine-Disrupting Chemicals	Synthetic wastewater	60 - 80%	Packed bed bioreactor	[137]
Laccase from Trametes sp	Nonylphenol	Model polluted soil	95%	Rotating reactor	[138]
Laccase from C. polyzona	Endocrine-Disrupting Chemicals	Synthetic wastewater	85 - 95%	Fluidized bed reactor	[139]

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I able 2	. Emerging	contanunants	Temoval Dv	winte rot	Tungi anu	then enz	vmes.

The removal of MPs by WRF varies widely from compound to compound. The physicochemical properties of the target molecules appear to be a key reason for such variation: some compounds are easily biosorbed due to their high hydrophobicity; some have molecular features that render them readily biodegradable by WRF, while others are resistant to the WRF enzymatic system due to certain features of their molecular structure [3]. For example, the presence of electron withdrawing functional groups, such as amide, carboxylic, halogen, and nitro group generates an electron deficiency and thus renders the compounds less susceptible to oxidative catabolism, while electron donating functional groups such as amine, hydroxyl, alkoxy, alkyl and acyl group on the other hand, render the molecules more prone to electrophilic attack by oxygenases [3].

4.1.7. OTHER COMPOUNDS

Among the pollutants that have been successfully treated with fungi, heavy metals are one of the most toxic. It has been demonstrated that the common filamentous fungi can absorb heavy metals (Zn, Cd, Pb, Fe, Ni, Ag, Th, Ra and U) from aqueous solutions to varying extents. Fungal biosorption largely depends on parameters such as pH, physical or chemical pretreatment of biomass, metal ion and biomass concentrations [140]. Biomass can then be regenerated by using various eluents [140].

Species with good metal binding potential are *Allescheriella* spp., *Stachybotrys* spp., *Phlebia* spp., *Pleurotus pulmonarium*, *Botryosphaeria rhodina*, *Phanerochaete chrysosporium*, *Aspergillus* spp. and *Lentinus edodes* [72].

Another important compound, widely studied in the past, is the explosive TNT (2,4,6 - Trinitrotoluene), which is founded in soils and waters at military facilities. Some authors had shown that WRF are capable of oxidative TNT attack, offering potential for mineralization of explosives. In particular, it has been demonstrated that *Phanerochaete chrysosporium* is capable of TNT mineralization to CO₂ and that this degradation is carried out by its ligninolytic enzyme MnP [38].

Different fungi, such as *Ganoderma australe* [141], *Porostereum spadiceum* [142], *Dichomitus squalens* [143] and *Trametes versicolor* [144] has been efficiently used also for the treatment of landfill leachate, being capable of decolorization, COD removal and reduction of toxicity.

4.2. PROCESS CONDITIONS

Despite the massive work already done by the scientific community, summarized in the previous paragraph 4.1, the use of fungi in the field of wastewater treatment still find poor application. Most works are still at the lab scale and/or done under strict limitations for full scale application (e.g. in sterile conditions). It is therefore necessary, in order to successfully implement fungal technologies into full-scale plants, to make an effort into scaling-up the process and take knowledge in such fields as physiology, biochemistry, enzymology and engineering in order to successfully design an engineered ecosystem composed mainly by fungi which can allow long-term operations under real conditions. In the present paragraph some information about existing literature for fungal processes will be given, with particular attention on the type of process (using enzymes only or using

whole fungal cells), reactor types, process condition and the existing interactions between fungi and other microorganisms in a non-sterile environment.

4.2.1. PROCESS TYPE

A first classification of fungi-based processes for wastewater treatment can be made between processes which use enzymes only or processes which use whole fungal cells.

The first approach consists of a two-stage treatment: growth of fungi and enzymes production in a side-stream reactor and then addition of the extracellular liquid containing the enzymes in the mainstream reactor, treating the wastewater. The second approach consists of a direct treatment of the wastewater inside a reactor containing fungal biomass, free or immobilized. Both these approaches can be integrated with the conventional activated sludge process as a pre- or post- treatment, depending on the type of recalcitrant compounds to be removed, if they are removed in a co-metabolism or not, type of wastewater, amount of biodegradable COD, etc.

Wastewater treatment using enzymes

The use of extracellular enzymes for industrial processes began in the 1960s and now it has become a staple in such industries as textiles, food production and detergent [41], while in the field of wastewater treatment this technology is still not mature for real applications and some research is still needed.

Comparing the use of microorganisms for wastewater treatment and the use of enzymes, these latter present some advantages: while the microbial population needs specific growth conditions to degrade certain compounds (such as temperature, oxygen, pollutant concentrations, pH, etc), enzymes are able to act in a large range of environmental conditions [41]. Other very important benefits of enzymatic treatment is that the enzymes themselves are biodegradable proteins, meaning that the enzymes that are not recovered will be degraded in the environment and that, unlike other processes, there is no buildup of biomass or chemicals that must be removed. Moreover, enzymatic treatment of wastewaters presents different types of positive side effects, depending on the type of enzymes used: it can help disinfection (some enzymes, such as protease can kill pathogens through the degradation of their cell walls [145,146]), can increase the biodegradability of the sludge by break apart large sludge particles [147] and can make greases and fats soluble (e.g. using lipases [148]).

On the other hand, there are some disadvantages in the use of enzymes only. Microorganisms can reproduce and increase their population in order to consume a large amount of substrate, but extracellular enzymes cannot, meaning that any increase in enzyme concentration must come from the outside. Moreover, it has been shown that enzymes may actually lose some reactivity after they interact with pollutants and could eventually become completely inactive [149,150]. For these reasons, in a full-scale system it is important that enzyme concentration is monitored and controlled.

One of the main disadvantage of using extracellular enzymes for bioremediation is the high cost of the enzymes themselves [50], which comes mainly from their purification [41], but, if a crude enzyme solution is used, the production process is much less expensive [151]. Moreover, crude culture filtrates may include natural mediators secreted by the fungus and other factors which may stabilize the enzymes [151].

Particular attention must be paid if there are several different contaminants present. In this case enzymes with a low specificity (e.g. laccases) can be used, and enzymatic degradation of several compounds at once is feasible, but it must also be noted that the degradation rate

of the enzyme for each compound is reduced and the treatment will probably take longer and require a higher enzyme dosage [41]. Also in the case the use of crude enzyme solution can be helpful because it may contain more than one ligninolytic enzyme which may attack different chemical structures [151].

In an enzymatic wastewater treatment enzymes can either remain mobile or be immobilized onto some type of carrier. Mobile enzymes are much less robust than immobilized ones, making them more susceptible to temperature, substrate concentration and pH swings and more likely to deactivate relatively quickly [152]. Another disadvantage is that mobile enzymes cannot be recovered and reused. Enzymes can be immobilized onto different types of carriers, including natural materials like alginate, chitin, chitosan, collagen, gelatin, cellulose, starch, pectin, and inorganic materials like zeolites, ceramics, silica, glass, activated carbon and charcoal [153].

Before enzymatic technology can be used for wastewater treatment on a large scale, further studies must be done. A vast majority of the studies that have been done focus on using one specific enzyme to degrade one specific compound at time, while in real wastewaters very often a great number of different compounds, ranging from the easily biodegradable compounds to extremely recalcitrant ones are present, and one single type of enzyme may not be effective for the whole system. Another important difference between lab studies and real conditions is that very often the former have been performed in sterile-environments, while in a real plant the enzymatic reactions take place in presence of different types of microorganisms, which may help or hinder the process. Furthermore, many studies were done at optimum temperature and pH for the enzyme, which can be very different from the ones commonly found in wastewater treatment plants.

Wastewater treatment using fungal biomass

A different approach for using fungal processes for degradation of recalcitrant compounds consists of direct treatment of the wastewater in a main-stream reactor containing whole fungal cells.

During the design of this type of process there are several aspects to consider and evaluate.

A first important point is the evaluation of the removal mechanism of the targeted compounds, whether it happens in a co-metabolism or not (see paragraph 3.2.1.). If the recalcitrant compounds to be removed do not represent a growth substrate for fungi, an additional carbon and maybe nitrogen source is required for primary metabolism of fungi [154]. This source can be a simple and soluble substrate like glucose. Glucose is probably

the most used growth substrate in fungal treatment studies and often it is used in very high concentrations, in the order of grams per liter [155–159]. Such concentrations does not fit economical industrial constraints because of the high cost of glucose. Moreover, glucose is a simple and easily biodegradable molecule that could induce competitions phenomena with other microorganisms [160]. A possible solution could be the use of minor concentrations of glucose, as already reported by other authors [161,162], maybe feeding fungi with glucose with a rate as close as possible to their consumption rate, as hypothesized by Blánquez et al. [156]. On the other hand, this strategy must be used carefully because, while in some basidiomycetes glucose starvation can induce laccase production, other times this is stimulated by high levels of glucose [163].

Another strategy could be the use of complex carbon sources such as ligno-cellulosic materials. These substrates are already been proved to successfully sustain fungal biodegradation; examples are pinewood chips [164], hay, rye, spelt grains, peanut shells [165], grape seeds [166] and wheat straw [167,168]. The use of these materials could bring some advantages: they could be selective carbon source for fungi and help them to outcompete bacteria [165], represent a physical support in case of attached growth bioreactors (see paragraph 4.2.2.), enhance the expression of LMEs [165] and be source of natural mediators [49]. While choosing the external carbon source to be used in the fungal process, it is important to take into account the total C/N ratio of the growth substrate and of the wastewater [169] (discussed in paragraph 4.2.3.). It is important as well to evaluate the presence of biodegradable compounds in the wastewater that could act as growth substrate; in this way there would be no longer need of external additions. This is particularly important in the case of fungal treatment on a raw wastewater, before the activated sludge process.

A different aspect to consider is the will to use single species or a consortium of fungi. Most times in designing a fungal process the first step is an accurate selection of a fungal strain with good degradative abilities toward a specific compound or class of compounds [170]. Single strain cultures are easy to obtain and manage in labs, but in the long term and in a real environment a microorganisms consortium will establish. The problem is to design an engineered ecosystem that allows the growth of every specie of the consortium. This aspect will be discussed in the paragraph 4.2.4. It is also possible to decide from the beginning to create a fungal consortium that creates a useful synergy for the degradation of the targeted compounds. For examples, Chen et al. [171] used a consortium of white rot

fungi to degrade a mixture of PAH, Pant & Adholeya [168] used a consortium of six fungi (*Penicillium pinophilum, Alternaria gaisen, Aspergillus flavus, Fusarium verticillioides, Aspergillus niger* and *Pleurotus florida*) for the treatment of molasses distillery wastewater and Ehler & Rose [164] used three white rot fungi (*P. chrysosporium, T. versicolor* and *L. edodes*) for phenol degradation.

The last aspect to consider is the enzymatic one. When designing a fungal process, it is necessary to understand which is/are the enzyme/enzymes involved. Moreover, it is necessary to understand if a direct correlation between the amount of enzyme and the amount/velocity of degradation exists [101,172]. If a correlation is found it is important to create inside the reactor the conditions to maximize the expression of the enzyme/enzymes involved or its/their functionality (e.g. varying the pH, temperature, source of C or N, aeration, presence of inducers and/or mediators, etc.) [173].

4.2.2. REACTOR TYPES

Various types of bioreactors have been tested in the treatment of wastewaters with fungi during the years, summarized in Table 3 and Table 4. A first classification can be made between suspended growth (Table 3) and attached growth reactors (Table 4).

Fungus	Reactor type	Targeted	Removal	Co-substrate	Ref.
		compound	efficiency		
P. chrysosporium	CSTR (2L)	Pharmaceuticals	93%	Glucose 3-6 g/L	[132]
T. versicolor	Air-lift (1.5L)	Tannery dye	86 - 89%	Glucose	[174]
T. versicolor	Air-lift (1.5L)	Clofibric acid	80%	Glucose	[125]
T. versicolor	Air-lift (10L)	Dyes	78%	Glucose	[156]
T. versicolor	Air-lift (10L)	Pharmaceuticals	53 - 83%	Glucose	[175]
P. ostreatus	Air-lift (5L)	Polyphenols	95%	Potato dextrose 2.4 g/L	[83]
T. versicolor	MBR (5.5L)	Pharmaceuticals	55-90%	Glucose 6g/L	[129]
T. versicolor	MBR (11.8L)	Dyes	93%	Starch 2g/L	[176]
P. chrysosporium	MBR (900 mL)	Dyes	62%	-	[177]

Table 3. Reactor used in the field of wastewater treatment using suspended fungal biomass.

Fungus	Reactor type	Targeted compound	Removal efficiency	Carrier	Co-substrate	Ref.
I. latux	Trickling filter (250 mL)	Dyes	35 - 91%	PUF and straw	Straw Glucose 10 g/L	[155]
Fungal consortium	Trickling filter (6.3L)	Phenols	> 98%	Pinewood chips	Pinewood	[164]
Fungal consortium	Trickling filter (6.3L)	Phenols	> 98%	Foam glass	Glucose 2g/L	[164]
P. chrysosporium	RBC (3L)	Dyes	85 - 100%	PUF	Glucose 10 g/L	[178]
P. chrysosporium	Plate bioreactor (2L)	Carbamazepine	20 - 68%	PUF	Malt extract 10g/L Glucose 10 g/L	[131]
T. hirsuta	Fed-batch (1L)	Dyes	82%	Stainless steel sponges	Glucose 3g/L	[179]
Fungal consortium	Novel reactor (14L)	Molasses distillery wastewater	65% (COD removal)	Straw	Straw	[168]
Fungal consortium	FBBR (4.5L)	Dyes	65-94%	Polyethylene fibers	Glucose 0.5- 5g/L	[180]
P. chrysosporium	FBBR (400 mL)	Pharmaceuticals	60-100%	PUF	Glucose 3-6 g/L	[133]
A. niger	FBBR	Dyes	58-94%	Synthetic sponge	Glucose 860 mg/L	[181]
B. adusta	FBBR	Dyes	45-84%	Stainless steel sponges	-	[182]
T. pubescens	MBBR	Dyes	30%	PUF	Glucose 100 mg/L	[161]
P. chrysosporium	MBBR (15L)	Dyes	54%	PU-DSCM	-	[183]
T. versicolor	MBBR (2L)	Dyes	89%	PVAL- hydrogel beads*	Glucose 10 g/L	[159]
P. variotii	Biofilter (2L)	VOC Mixture	20-90%	Perlite	-	[184]
N. crassa	MBfR	Phenols	92-100%	Membrane	Sucrose 5g/L	[185]
T. hirsuta	Air-Lift (2L)	Dyes	65-95%	Alginate beads*	Glucose 10g/L	[186]

Table 4. Reactor used in the field of wastewater treatment using attached fungal biomass.

*Fungus encapsulated in the beads.

Among suspended growth reactors, air-lift reactors are widely used for wastewater treatment with fungi. These reactors are simple to design, reliable, provide a low shear environment for enzyme production by free mycelial pellets and do not require a mechanical stirrer, lessening the energy demand [187]. On the other hand, they can present some operational problems due to clogging [188] and bacterial overgrowth due to the necessity to add a soluble carbon source such as glucose [156]. A different type of suspended growth reactor is the MBR which allows a suspended solids and macro-colloidal material free permeate, retains high biomass concentration and reduces excess sludge production [189]. On the other hand, these systems present a relatively high energy demand. A similar approach is the one used by Luke & Burton [185] which used a capillary membrane-immobilized fungal biofilm to degrade phenolic pollutants.

Immobilized mycelia present several advantages compared to free cells: they are more resistant to mechanical stresses, pH changes or toxic pollutant concentration [161,188,190], present higher degradation capacity [161,188,190], are easy to separate from liquid stream [161,188] and cause minimal clogging in continuous flow systems [161]. Moreover, cell immobilization reduces protease activity [191] and make fungal biomass more effective in suppressing bacterial growth [192]. However, the immobilization not only increase the cost of inoculum preparation but also affect the mass transfer kinetics of organics uptake [188]. Moreover, the use of a support for the degradation of certain compounds has to take into consideration the potential adsorption of these compounds onto the support surface, which may render for limited biodegradation and the need of an extraction stage to desorb them [133].

Among attached growth reactors the most used are the Moving Bed Biofilm Reactor (MBBR) and the Fixed Bed Biofilm Reactor (FBBR). The former have the advantage to be highly resistant to shocks, due to the complete mixing [183], but present lower volumetric loading rates. Also the trickling filters are used for fungal treatment [155,164], which can enhance biodegradation with submerged, partially submerged and non-submerged carriers (semi and solid state fermentation), increasing the contact between compounds and mycelia [164], but present high incidence of clogging and low volumetric loading rates [1].

Other types of reactor tested were rotating biological contactors (RBC) [178] and specifically designed reactors [131,168].

Regardless of reactor configuration, there are many different types of available carriers. Many works used biodegradable carriers, such as ligno-cellulosic materials, pursuing the double goal of being a physical support and providing a carbon source [164,168], together with many other advantages such as enhancing LMEs expression and providing natural mediators, as discussed in the previous paragraph. On the other hand these types of carriers present some operational problems (due to the fact that their structural behavior change during time [168]) and can release some soluble by-products in the liquid which can be non biodegradable, toxic and give rise to a colored solution.

Among the inert carriers, many are available, which differ in size, structure and material. It is important to underline that the use of a specific carrier depends strictly by the microorganism tested [193]. Polyurethane foam (PUF) is probably the most used because it allows homogenous and persistent fungal colonization and it is easy to keep in movement inside the reactor [193].

A different type of immobilization is the encapsulation of the fungus inside a carrier such as alginate, gelatin, agarose, carrageenan and chitosan [170]. This technique can increase the stability of fungal biomass [186] and protect the enzyme and the fungus from bacterial attack, as shown in a study by Leidig et al. [159]. On the other hand, it is important that the material chosen for encapsulation is stable in a wide range of environmental conditions; for example alginate beads are unstable in contact with phosphate and citrate and at pH higher than 8 [193].

Regarding the feeding strategy (continuous or discontinuous mode) Ehler & Rose [164] showed that sequencing batch operations allowed to achieve homogenous biomass distribution throughout the reactor and greater tolerance to fluctuating influent concentrations, increasing the operational flexibility. Moreover, Hai et al. [176] demonstrated that sequencing batch operation led to a better granulation of *T. versicolor* and higher enzymatic activity. However, Libra et al. [165] showed that sequencing operations can retain bacterial biomass inside the reactor and foster its growth.

4.2.3. PROCESS PARAMETERS

Besides the choice of the organism and reactor configuration, the performance of a wastewater treatment depends on the parameters which govern the process.

Probably the most important parameter in fungal processes is the pH, which play a key role for the functioning of ligninolytic enzymes [172]. Most works were made in acidic environment, with a pH ranging from 3.5 to 5.5 [129,175], which is the optimum pH range for fungal growth [183,192] and for ligninolytic enzymes activity [182,183]. Very few works were made using basic pH. For example, Pant et al. [168] successfully treated a molasses distillery wastewater with a fungal consortium at pH 8.2 while Park et al. [183] tested two pH, 7 and 12, for dyeing wastewater treatment, finding that enzymatic activity and consequent dye degradation was almost null at pH 12.

Another important parameter is temperature, which is maintained in most works between 25°C and 30°C [132,177], with few exceptions. For example, Zhang et al. [131] used a temperature of 34°C while Anastasi et al. and Leidig et al. used a temperature of 20°C [182,159]. Since most wastewater treatment plant generally work in colder environment, more studies at lower temperature are needed.

Also the amount of active biomass is important in a biodegradation process. Fungal concentration is generally kept between 1 and 5 g/L (dry weight) [175,184], regardless of being in attached or suspended growth. Few authors worked with lower biomass concentration such as Hai et al. [176] who used 200 mg/L of *Trametes versicolor* in a MBR and Olivieri et al. [83] who also used, for olive mill wastewater treatment, only 200 mg/L of *Pleurotus ostreatus*, which however grew sensitively inside the reactor.

For what concerns oxygen, many works do not measure its concentration inside the liquid, but the ones who do it generally present high dissolved oxygen concentration, between 3 and 8 mg/L [129,131].

Regarding cellular retention time, very rarely this parameter is taken into account. For example, Blánquez et al. [156] estimated in their air-lift reactor a cellular retention time of 21d.

On the contrary, hydraulic retention time (HRT) is controlled more often in the range of 1÷4 d [125,178,183], even if some authors tested successfully shorter HRT [180]. Hai et al. [176] tested in a MBR two different HRTs, 1d and 7d and find out that the higher retention time exhibited better granulation, higher efficiency and lower enzyme washout.

Also carbon and nitrogen content as well as their ratio (C/N) may play a key role in enzyme production [163,172] and, consequently, in reactor performance [169]. How these components and their ratio influence enzyme production and biodegradation abilities is a controversial issue, since examples of activity increases have been described under both high and low C/N ratios. For example, it is reported that ligninolytic enzyme activity is stimulated by low C/N ratio for *Pleurotus ostreatus* and *Trametes trogii* [151,169], while, on the contrary, low C/N ratio inhibit enzymatic activity and decolorization abilities of *Phanerochaete chrysosporium* [151] and *Bjerkandera adusta* [172].

Moreover, the type of nitrogen source influence the amount and type of ligninolytic enzymes produced by several white-rot fungi [151]. For example, it is reported that inorganic nitrogen sources lead to low levels of laccase with sufficient biomass production, while organic nitrogen sources give high laccase yields with good fungal growth [163].

4.2.4. INTERACTIONS WITH OTHER ORGANISMS

A very important issue when dealing with fungal reactors in real conditions is the presence of interactions with other organisms (mainly, but not exclusively, bacteria) and how to manage them. Many scientific works in this field are made under sterile conditions, which are useful to study the potential abilities of a single strain or a consortium and to better understand the process, but for full scale application it is mandatory to take into account the interactions between fungi and other organisms. The main issue is how to engineer an ecosystem composed by fungi and bacteria in the context of wastewater treatment, since the typical environmental conditions are favorable to bacteria and fungi are easily outcompeted.

It has been largely demonstrated that the presence of bacteria very often compromise fungal activities [156,160,176]. In particular, it has been shown that bacteria 1) affect fungus morphology [3,194], 2) lead to cessation of fungal growth [129], 3) inhibit enzyme secretion capacity [129], 4) denaturate the secreted fungal enzymes [129] and 5) outperform fungi in the competition for the substrate [3].

However, if a good synergy is achieved, it is possible to obtain better results with combination of bacteria and fungi rather than with one culture only [161,194,195].

Many authors studied some strategies to foster fungal growth and suppress bacterial ones, by changing process parameters or culture conditions. In particular, pH seems to play a key role in maintaining fungal biomass active [165,192] and one strategy could be to set the pH in the interval $3.5 \div 5.5$, which is the optimum for growth and enzyme secretion of many

fungal species [183,192]. Other strategies could be the use of nitrogen-limiting conditions, which may, in some cases, foster fungal growth and inhibit bacterial one [165,192], the use of selective carbon sources such as ligno-cellulosic materials [165] and the use of immobilized or encapsulated mycelium, more resistant to bacterial attacks [159,192]. Moreover, it could be helpful the use of low substrate concentration, as highlighted by Yang et al. [180], who found out that in a non-sterile bioreactor treating textile wastewater, the ratio between fungal and bacterial biomasses increased after a decrease in glucose feeding.

SECTION II

EXPERIMENTAL WORK

5. FUNGAL CULTURES AND KINETIC STUDIES

5.1. PREPARATION OF BIOMASSES

In the present paragraph, used techniques for preparation of fungal biomass are described. Every phase of the preparation of inoculums and crude extracts, from growth in solid culture to the use of mycelium into the experiments, were made under sterile conditions.

5.1.1. SOLID CULTURE

All the fungi used in the present thesis were stored at 4° in MEA plates (Malt Extract Agar, ATCC medium 325):

- 20 g/L Malt extract
- 20 g/L Glucose
- 2 g/L Tryptone
- 20 g/L Agar

Periodically, sub-cultures were made, kept at 28° for 10 days and then stored at 4° or used as inoculums for liquid cultures.

5.1.2. LIQUID CULTURE

Liquid cultures were prepared transferring 10 plugs, equivalent to an eighth of plate, into 250 mL Erlenmeyer flask containing 100 mL of Basidiomycete Rich Medium (BRM) [196]:

- 0.6 g/L Asparagine
- 1 g/L KH₂PO₄
- 0.5 g/L KCl
- 0.5 g/L Yeast extract
- 10 g/L Glucose
- 10 g/L Tryptone
- 3 mg/L MnSO_4
- 10 mg/L FeSO_4
- 3 mg/L CuSO₄
- 2 mg/L ZnSO4
- 1 g/L MgSO₄·7H₂O
- 50 mg/L Ca(NO₃)₂

Flasks were then incubated in the dark at 26°C under continuous stirring at 110 rpm for seven days.

5.1.3. INOCULUM PREPARATION

Free cell biomass

After the growth phase in BRM (see 5.1.2.) if the mycelium is going to be used as free cell biomass, it is separated from the liquid media with the help of a strainer, rinsed with deionized water and used in the experiment.

For the evaluation of dry weight of biomass additional flasks were prepared and sacrificed: the content of these flasks were filtered through 0.45 μ m filters, kept at 60° until dry and then weighted.

Immobilized mycelium on PUF

Some experiments were made using fungi immobilized on open cell Polyurethane Foam (PUF) cubes (see Figure 3). For this type of inoculum, the mycelium, after liquid culture (see 5.1.2) is separated from BRM with the help of a strainer, rinsed with deionized water and transferred in another 250 mL flask together with 100 mL of modified Czapek Dox:

- 6g/L NaNO₃
- 0.52 g/L KCl
- 0.52 MgSO₄·7H₂O
- 1.52 g/L KH₂PO₄
- 10 mg/L FeSO₄
- 3 mg/L CuSO₄
- 2 mg/L ZnSO_4
- 10 g/L Glucose*

*This latter was omitted in several experiments, see description case by case.

The mycelium is then homogenized with a blender and incubated in flask together with eight PUF cubes, in the dark under the same agitated conditions (110 rpm, 26°C) for 7 days. After checking that the glucose concentration is zero, the colonized carriers are rinsed with deionized water and used in the experiment. Also in this case, for the evaluation of dry weight of biomass additional flasks were prepared and sacrificed.



Figure 3. Picture of a PUF cube before (left) and after (right) colonization by fungi.

Immobilized mycelia on straw

In order to prepare immobilized mycelium in straw, after fungal growth in liquid culture (as described in 5.1.2.) fungi are separated from liquid media with a strainer, rinsed with deionized water and transferred in 250 mL flasks together with 100 mL of modified Czapek Dox prepared without glucose addition. The mycelium is then homogenized with a blender and incubated in flask together with 7 g of washed, chopped and autoclaved straw and then incubated in agitated conditions (110 rpm) at 26° for 3 days. Also in this case, for the evaluation of dry weight of biomass additional flasks were prepared and sacrificed.

Immobilized mycelia on Luffa cylindrica

The procedure for preparation of immobilized mycelium on *Luffa cylindrica* is the same used for immobilization on straw, with the difference that the content of one flask, homogenized in 100 mL of mineral media is incubated with 10 g of washed, chopped and autoclaved *Luffa cylindrica*.

5.1.4. CRUDE EXTRACT PREPARATION Culture in Czapek Dox

Some experiments were performed using only the extracellular crude extract, containing LMEs, obtained from fungal growth.

After growth phase in the rich media (see 5.1.2.) pellets were separated with a strainer, rinsed with deionized water and incubated in flasks together with 100 mL of Czapek Dox. After one week, fungi are separated with the strainer, LMEs are measured and liquid is collected.

Culture in straw

A different type of extracellular crude extract was produced from fungal culture in straw. After fungal growth in liquid culture (as described in 5.1.2.) fungi are separated from liquid media with a strainer, rinsed with deionized water and transferred in 250 mL flasks together with 100 mL of modified Czapek Dox prepared without glucose addition. The mycelium is then homogenized with a blender and incubated in flask together with 7 g of washed, chopped and autoclaved straw and then incubated in agitated conditions (110 rpm) at 26°. After one week, fungi and straw are separated with the strainer, LMEs are measured and liquid is collected.

5.2. KINETIC STUDIES

5.2.1. INTRODUCTION

The performance of every biological process, including the ones dealing with wastewater, depends on the dynamics of microbial growth. An effective design of a biological treatment requires an understanding of the basic principles governing the growth of microorganisms. In particular, one of most useful parameter in designing biological process is the maximum growth rate (expressed commonly in d⁻¹), which is defined as the maximum value of the specific growth rate, which depends on several parameters:

$$\mu = \frac{1}{x} \frac{dX}{dt}$$
(eq 5.1.)

where:

- μ is the specific growth rate of the microorganisms
- *X* is the concentration of microorganisms
- dX/dt represents the growth rate

In order to measure μ_{max} one of the most useful tool is the Monod equation [197]:

$$\mu = \mu_{max} \frac{S}{K_s + S}$$
 (eq 5.2.)

where:

- μ is the specific growth rate of the microorganisms
- μ_{max} is the maximum specific growth rate of the microorganisms
- *S* is the concentration of the limiting substrate for growth
- *K_s* is the "half-velocity constant"

Few considerations on this equation can be done (see Figure 4):

- When S is very low (S << K_s) the equation can be simplified into $\mu = \mu_{max} \cdot S/K_S$, which means that the specific growth rate increase proportionally to the substrate;
- When S = K_S the equation become $\mu = \mu_{max}/2$, which explain the name of K_S, "half velocity constant")
- When S is very high (unlimited substrate conditions, $S >> K_S$), the equation can be simplified as: $\mu = \mu_{max}$, which means that microorganism are growing at their maximum velocity.

Taking into account this last consideration, it is possible to measure the growth rate of microorganisms in unlimited substrate conditions and assume it equal to μ_{max} .



Figure 4. Specific growth rate versus limiting substrate according to Monod equation.

During the years, several kinetic studies were conducted on activated sludge and on single strains of bacteria, but there is little published data on the growth kinetics of fungi [198]. In the present chapter it is presented a preliminary evaluation of the parameter μ_{max} for the two fungi used in this work: *Bjerkandera adusta* and *Pleurotus ostreatus*.

5.2.2. MATERIALS AND METHODS

For the two studied fungi (*Bjerkandera adusta* and *Pleurotus ostreatus*) the maximum specific growth rate was evaluated by studying the growth of mycelium in unlimited substrate conditions.

In particular, for each strain, 24 flasks containing BRM were inoculated as described in 5.1.2., incubated at 26°C and 110 rpm, and, at designed times (0, 1, 2, 3, 5, 7, 9, 12 days) three flasks were sacrificed for mass evaluation. Content of flasks were filtered with preweighted 0.45 μ m filters, dried at 60° until constant weight and then weighted. Specific growth rate was then calculated as follows, considering eq. 5.1.:

$$\mu_{max} = \mu = \frac{1}{X} \frac{dX}{dt}$$

Separating the variables and integrating from t = 0 and generic instant t:

$$\mu_{max}dt = \frac{1}{X}dX \Rightarrow \int_{t=0}^{t} \mu_{max}dt = \int_{X_0}^{X} \frac{1}{X}dx$$

which leads to:

$$\mu_{max} \cdot t = \ln \frac{X}{X_0} \tag{eq 5.3.}$$

In this way μ_{max} can be calculated as the slope of $ln(X/X_0)$ versus time.

5.2.3. RESULTS AND DISCUSSION

As described in 5.2.2. μ_{max} evaluations were made in triple. Figure 5 and Figure 6 show experimental data of one of the described experiments with *Pleurotus ostreatus*. Every experiment showed that fungi, during the 12 days of observation, were in unlimited substrate conditions and grew at their maximum rate (exponential curve fitted satisfactorily the experimental data).



Figure 5. Dry weight of *P. ostreatus* versus time in unlimited substrate conditions.





Table 5 show summarized the results of the experiments.

	Maximum growth rate μ_{max}			
Experiment	Pleurotus ostreatus	Bjerkandera adusta		
1	0.204 d ⁻¹	0.234 d ⁻¹		
2	0.194 d ⁻¹	0.229 d ⁻¹		
3	0.249 d^{-1}	0.181 d ⁻¹		
Mean	0.216 d ⁻¹	$0.215 d^{-1}$		
Standard Deviation	$0.029 \ d^{-1}$	$0.029 \ d^{-1}$		

Table 5. Results of maximum growth rate evaluation for *P. ostreatus* and *B. adusta*.

Obtained results show that experimental data are consistent (standard deviation is relatively low) and that the two fungi grow with very similar rates. These rates, even though significantly lower than the ones of heterotrophic bacteria commonly found in activated sludge plants (which range from 3 to 13 d⁻¹ at 20°C [1]), are compatible with design and operation of a biological treatment plant.

Comparing results with literature, even though every work is made at different temperature (in the range 20° C ÷ 28° C) and with different substrates, it is possible to see that our estimated growth rates are in line with most of published data (see Table 6), which vary between 0.2 an 0.9 d⁻¹. Only one study [199] found a significantly higher growth rate (4.08 d⁻¹), but the studied organism belong to a different division, Ascomycota, while the other studied fungi (*Pleurotus* spp., *Bjerkandera* spp. and *Trametes* spp.) are all Basidiomycota.

Table 6. Comparison of estimated growth rates with literature values.

Fungal strain	μmax	Ref
Trametes pubescens	0.25 d^{-1}	[200]
Trametes spp.	0.472 d ⁻¹	[201]
Aspergillus awamori	4.08 d ⁻¹	[199]
Trametes versicolor	$0.94 d^{-1}$	[198]
Pleurotus ostreatus	0.216 d ⁻¹	This work
Bjerkandera adusta	0.215 d^{-1}	This work

5.2.4. CONCLUSIONS

Experiments performed in this paragraph represent a preliminary evaluation of the maximum growth rate (μ_{max}) for the two fungi used in this work: *Bjerkandera adusta* and *Pleurotus ostreatus*. This parameter is one of the most useful for the design of biological processes, but, despite the increasing interest in using fungal cultures or the enzymes that they produce, there is little published data on the growth kinetics of fungi [198].

For the two studied fungi (*Bjerkandera adusta* and *Pleurotus ostreatus*) the maximum specific growth rate was evaluated by studying the growth of mycelium in unlimited substrate conditions. Obtained results show that the two fungi grow with very similar rates. The estimated growth rates are in line with most of published data and, even though they are significantly lower than the ones of heterotrophic bacteria commonly found in activated sludge plants, they are compatible with design and operation of a biological treatment plant. Further studies are still necessary in order to have a comprehensive knowledge of kinetics of WRF since several useful values such as the specific rates of substrate consumption (e.g., carbon or nitrogen source), oxygen utilization and LMEs production are seldom found.

6. PETROCHEMICAL WASTEWATER TREATMENT

6.1. INTRODUCTION

This chapter describes the research work conducted on fungal treatment of a petrochemical wastewater, collected at the outlet of an Italian factory, containing a mixture of naphthalenesulfonic acid polymers (NSAP) as the largest fraction of organic pollutant. Figure 7 shows the chemical structure of the monomer, 2-naphthalenesulfonic acid (2-NSA), which is formed by two aromatic rings and a sulfonic group.



Figure 7. 2-naphthalenesulfonic acid chemical structure.

The polymers of this acid (NSAP) are commonly employed as dispersants and wetting agents in several industrial sectors, from textile mills and leather tanning operations to the production of pharmaceuticals, azo dyes, pesticides, cosmetics, polymers and construction materials. Technical NSAP mixtures consist of several mono and disulfonated monomers and their condensed oligomers [202]. These chemicals are known to have a highly hydrophilic nature due to the existence of sulpho-groups, but exhibit only limited biodegradability [26]. As already reported in paragraph 2.3., conventional biological processes do not appear effective for treating waters containing NSAP, since most of them are toxic or inhibitory to biological activities and, therefore, present a serious problem in conventional wastewater treatment systems [29]. Among all processes proposed in literature for NSAP treatment, to our knowledge, only one study focused on a fungal degradation (with the fungus *Cunninghamella polymorpha*) [28].

In this chapter several results using the NSAP-containing wastewater are presented: first, a complete chemical-physical characterization, then some preliminary tests such as fungal selection and batch tests at Erlenmeyer scale, then different types of bioreactors.

6.2. ANALYTICAL PROCEDURES

6.2.1. PHYSICO-CHEMICAL PARAMETERS

Total suspended solids (TSS) were determined according to standard methods (APHA, AWWA, WEF, 1998):

- 0.45 μ m filter was dried to constant weight at 105°C and weighted.
- A known volume of sample was vacuum filtered.
- The filter was dried again to constant weight at 105°C and weighted.
- TSS were calculated dividing the difference between the two weight by the volume of the sample.

Density was determined weighing a known volume of sample while pH and the Oxidative-Reductive Potential (ORP) were determined using Hach Lange's probes.

Chemical Oxygen Demand (COD), Total Nitrogen (TN), $N_NO_3^-$, $N_NO_2^-$, $N_NH_4^+$ and $P_PO_4^{3-}$ were all determined using Hach Lange's cuvette tests, in association with Thermostat LT200 and DR 3900 VIS Spectrophotometer.

Soluble COD (sCOD) was determined after 0.45 μ m filtration and particulate COD (pCOD) was obtained by difference.

Metals were determined by an inductively coupled plasma mass spectrometer (ICP-MS) Perkin Elmer Optima 2000 OES DV.

6.2.2. GLUCOSE DETERMINATION AND ENZYMATIC ASSAYS

Glucose concentration was analyzed with an enzymatic-photometric test with Glucose Oxidase (GOD). The glucose is oxidized by GOD in gluconate, with H_2O_2 formation. The gluconate, with peroxide catalysis, oxidizes the chromogenous system constituted by p-hydroxybenzoic acid and 4-aminoantipyrine (Trinder' reaction [203]) with the formation of a colored complex. The color intensity of the sample, proportional to glucose concentration, is read on the spectrophotometer with a wavelength of 510 nm. The glucose concentration (mg/L) is determined in comparison with the intensity of a standard solution. All the chemicals for glucose assay were purchased from SGM Italia.

Manganese Peroxidase (MnP) activity was measured following the oxidation at 590 nm of 3-(dimethylamino)-benzoic acid/3-methyl-2-benzothiazolinone hydrazone (DMAB/MBTH) [204,205]. One milliliter of the reaction mixture contained: 720 μ l of 0.1 M succinic-lactic acid buffer (pH = 4.5), 100 μ l of 25 mM DMAB, 50 μ l of 1 mM MBTH, 100 μ l sample, 20 μ l of 4 mM MnSO₄ and 5 μ l of 20 mM H₂O₂.

Lignin Peroxidase (LiP) was determined following a previously reported assay [206], based on veratryl alcohol oxidation in sodium tartate buffer, pH 3.0.

Laccase (Lac) activity was assayed following the oxidation at 420 nm of 2.2'-azinobis(3ethylbenzothiazoline-6-sulfonic acid) (ABTS), in 0.1 M sodium citrate buffer, pH 3 [207]. One milliliter of the reaction mixture contained: 800 μ l of 100 mM sodium citrate buffer (pH = 3), 25 μ l of 100 mM ABTS and 180 μ l sample.

All the enzyme activities were expressed as International Units (U), where one unit is defined as the amount of enzyme that oxidizes $1 \mu mol$ of substrate per minute.

6.2.3. HPLC-UV DETECTION

Untreated and treated wastewater were analyzed by High performance liquid chromatography (HPLC) coupled to UV-Vis diode array detection (Waters 2690 Separation Module and Waters 996 PDA) using a Novapak column C18 4 μ m, 3.9X150 mm (Waters). A ion-pair reagent, tetrabutylammonium bromide (TBAB), was used to separate polar aromatic sulfonates. The separation method was adapted from a previously reported protocol [208] and consisted of a gradient of water + acetic acid 1% + TBAB 20 mM (A) and acetonitrile + acetic acid 1% + TBAB 20 mM (B), with steps as follows: (A%:B%): 0–35 min linear gradient from 60:40 to 35:65, 36-40 min isocratic 20:80 and 41-45 min isocratic 60:40. The flow rate was 1 mL/min and the injection volume 10 μ L. HPLC-grade water was prepared by purifying demineralized water in a Milli-Q filtration system.

For quantifying the amount of biodegradation and/or biosorption the chromatograms were integrated with Millennium 32 software by Waters corporation in order to estimate the area reduction of each peak.

6.2.4. MASS SPECTROMETRY

Mass spectrometry analyses were performed using a Thermo HPLC-MS system, composed by a HPLC Surveyor coupled to a linear trap quadrupole mass spectrometer through an ESI (electrospray) interface. Analyses were conducted in negative ion mode, recording the m/z range from 200 to 2000, after optimization of the ESI interface parameters and ion optic by using a solution of 2-NSA standard. Samples were analyzed by direct infusion or after ion pairing chromatography, using a Phenomenex Gemini C18 100 x 2 mm, 5 μ m column, operating at a flow rate of 250 μ L/min. Separation was performed using LC-MS grade water (A) and acetonitrile (B), both containing 5 mM dimethylhexylamine (Sigma Aldrich), as the eluents, applying a linear gradient from 30% B to 100% B in 12 minutes.

6.2.5. RESPIROMETRIC TESTS

For the respirometric tests, an open respirometer (static liquid–flowing gas) was used. This respirometer allowed to execute, at constant pH, oxygen uptake rate (OUR) measurements [209], maintaining dissolved oxygen concentrations between 4 and 6 mg/L.

For these respirometric tests, activated sludge was diluted with the WWTP effluent to obtain TSS concentration of 2500 mg/L and aerated for 24 h in order to reach endogenous conditions.

During the tests, conducted at room temperature $(18 \div 22^\circ)$ pH was maintained at 7 by dosing NaOH (0.2 M) or HCl (0.2 M). Nitrifying biomass was inhibited with 30 mg/L of allylthiourea.

A known volume of sample was spiked, and oxygen consumed for substrate degradation was evaluated as the difference (ΔO_2) of the total oxygen consumed and the oxygen consumed for endogenous respiration (area between the total OUR and the OUR related to decay). Tests lasted 24 h and were carried out in duplicates.

The fraction of biodegradable COD (bCOD) was then evaluated as follows:

$$bCOD = \frac{1}{1 - Y_h} \cdot \Delta O_2 \cdot \frac{V_{sam} + V_{res}}{V_{sam}}$$

where Y_{h} is the yield coefficient, assumed equal to 0.49 mgCOD mgCOD⁻¹ on the basis of a previous study [210], V_{sam} is the volume of sample spiked in the respirometer, V_{res} is the total volume of liquid in the respirometer before the addiction of sample and ΔO_2 is the amount of oxygen consumed for substrate degradation.
6.3. WASTEWATER CHARACTERIZATION

Wastewater was sampled at the outlet of the petrochemical factory three times during the period of study (in November 2012, October 2014 and June 2015) and stored at 4°C until use. The wastewater aspect is limpid, pale yellow with some rubber particles in suspension (Figure 8).



Figure 8. Picture of raw wastewater.

The characteristics of wastewater are summarized in Table 7. In particular, we observed a basic pH (about 7.9) and a very low bCOD/COD ratio (<10%), which confirms the recalcitrant nature of NSAP.

Some of the assayed metals, as copper, iron and nickel are essential for fungi, while other metals (such as chromium and lead), are not [211]. In general both essential and non-essential heavy metals could be toxic for fungi, when present in high concentrations [212], but in this case the concentrations measured are compatible with fungal activity.

Parameter	Parameter Mean Value			
Chemical - Physical Parameters				
TSS	18 mg/l	-		
Density	1.013 kg/m ³	-		
pH	7.9	7 – 10		
ORP	180 mV	-		
Total COD	4900 mg/L	3300 - 6200 mg/L		
Soluble COD	87%	-		
Particulate COD	13%	-		
Biodegradable COD	9%	1% - 15%		
Non biodegradable COD	91%	85% - 99%		
Nutrients				
TN	120 mg/L	80 – 170 mg/L		
N_NO ₃ -	6 mg/L	5.5 – 7 mg/L		
N_NO ₂ ⁻	Under detection limit	-		
$N_NH_4^+$	5 mg/L	0 – 15 mg/L		
P_PO ₄ ³⁻	Under detection limit	-		
	Others			
Cadmium	2.46 µg/L	0.8 - 4 µg/L		
Calcium	511 μg/L	-		
Chrome	0.7 μg/L	-		
Cobalt	Under detection limit	-		
Copper	5.28 µg/L	$5.1-5.4\mu g/L$		
Iron	6.93 mg/L	1.5 – 12 mg/L		
Lead	5.46 µg/L	$3.8-7\mu g/L$		
Magnesium	47 mg/L	-		
Manganese	5.01 µg/L	-		
Nichel	2.36 µg/L	$0.9 - 3.8 \mu g/L$		
Potassium	363 mg/L	-		
Sodium	105 mg/L	-		
Zinc	9 μg/L	$0.\overline{1-19 \ \mu g/L}$		

Table 7. Wastewater characteristics.

*Values are referred to three samples taken from November 2012 to June 2015.

For what concerns NSAP characterization in the wastewater, the HPLC chromatogram shows at least ten fractions, named F1 - F10 (Figure 9).



Figure 9. Raw wastewater chromatogram. Ten fractions F1-F10 are visible.

A clear correspondence between fraction 1 chromatogram and 2-NSA standard chromatogram was evident (data not shown).

Since no NSAP HPLC standards seem to be commercially available, but 2-NSA monomer, every fraction was then individually analyzed by LC-MS.

LC-MS tests confirmed that fraction 1 (F1) contains only 2-NSA monomer and fractions from F2 to F10 contain a mixture of naphtalene-sulphonate polymers. Table 8 shows only the main ions obtained in the different fractions, but we observed that every spectrum was characterized by mono and/or bi-charged molecules and by the co-elution of oligomers of different grade of polymerization, until dodecamers. Anyway, a clear increase in the molecular weight of the oligomers is evident, going from F2 to F10. Similar results were obtained by other authors [208], which analyzed a mixture of naphtalene-sulphonated polymers used as tanning agent.

Fraction	Retention Time (min)	UV λ max (nm)	m/z	Oligomers	Relative Area %
F1	2.1 ÷ 4	233	207,01	monomers	4.38
F2	4 ÷ 7.6	230 ÷ 234	427,03	dimer	11.11
F3	7,6 ÷ 12.2	228 ÷ 234	647,05	trimer	18.51
F4	12.2 ÷ 17.2	229 ÷ 232	867,05	tetramer	17.61
F5	17.2 ÷ 22	230 ÷ 231	1087,05	pentamer	14.86
F6	22 ÷ 26	229 ÷ 231	1307,05	examer	11.31
F7	26 ÷ 29	228 ÷ 231	1527,05	eptamer	8.46
F8	29 ÷ 31.5	229 ÷ 230	1747,05	octamer	6.18
F9	31.5 ÷ 33.7	229	1967,05	nonamer	4.43
F10	33.7 ÷ 35.7	229	2187,05	decamer	3.07

Table 8. Main ions obtained in HPLC-MS test.





6.4. PRELIMINARY TESTS

6.4.1. FUNGAL SELECTION

One of the first steps of the research work has been a initial screening of several fungal strains, in order to find the ones able to degrade 2-NSA and its condensed polymers.

From this screening, which was performed before the beginning of the present work [210,213], a *Pleurotus ostreatus* (NCBI KJ02093) was chosen as one of the best degrader of NSAP. It has been therefore decided to use this strain for the present PhD research.

6.4.2. BATCH DEGRADATION EXPERIMENTS

Firstly, batch biodegradation experiments in Erlenmeyer flasks were performed in order to assess the ability of the selected fungus to degrade NSAP in liquid culture.

In particular, three different tests were performed:

- 1. Biosorption tests, with heat-killed mycelium;
- 2. Biodegradation tests, with alive mycelium;
- 3. Cytochrome P450 inhibition studies.

Biosorption tests

It is known that fungal biomass have a great sorption capacity [214]. To better understand the modifications of the chromatograms and to determine in which proportion the loss of NSAP is due to degradation or to biosorption, this latter was measured in inactivated fungal biomass. 1g of *P. ostreatus* was grown as described in paragraph 5.1.3., and then heat-killed by autoclaving at 120°C for 20 min together with 100 mL of wastewater (pH 6) in 250 mL Erlenmeyer flask.

After 24 h the samples were analyzed by HPLC and the comparison with the initial wastewater elution profile showed a reduction of all fraction areas (Figure 11). We observed that $20 \div 24\%$ of the total mixture were adsorbed to fungi and after this first loss we did not observe any further chromatogram variations. Other authors [28] observed that about 10% of the total polymers were adsorbed to fungal biomass and that the higher weight oligomers were adsorbed to a greater extent than the lower ones. Contrary to what they have stated, we observed a greater sorption rate in the first three fractions (55%, 39% and 24%, respectively) and almost the same sorption rate (19%) in the others fractions.



Figure 11. Wastewater chromatogram before (solid line) and after (dotted line) biosorption on dead biomass.

Biodegradation tests

These experiments were performed with *Pleurotus ostreatus* immobilized on PUF (see paragraph 5.1.3.). After growth, an average mass of 1.0 g (dry weight) of fungi was transferred to 250 mL flask, containing 100 mL of autoclaved wastewater and 5 g/L of glucose, as additional carbon source. Wastewater pH was lowered to 6 using 0.1M H_2SO_4 in order to make the environment more suitable for fungal growth and enzyme activity (see paragraph 4.2.3.) Further flasks without glucose addition were prepared as control.

After 15 days of treatment, all flasks chromatograms, except for controls, showed a reduction of area in correspondence of fractions from 3 to 10 (Figure 12); on the contrary, in the firsts 6 - 7 min (corresponding to fraction 1 and 2) new peaks appeared, probably associated with the formation of new compounds with lower molecular weight, suggesting that the mechanism in place is a depolymerization of NSAP.

Experiments led to an NSAP removal (calculated considering the variation of areas from fraction 3 to 10) on average of 70% after 15 days (see Figure 13). Glucose took from 10 to 15 days to be completely consumed (see Figure 13), and after this period of time the degradation process reaches a plateau. As regards the monitoring of pH, this was substantially unaltered on 6 (data not shown). Regarding enzymatic activities, among the ones tested (LiP, MnP and Lac), *P. ostreatus* expressed only Lac, variable in the range of 1 \div 8 U/L (see Figure 13).



Figure 12. Chromatogram of raw wastewater (solid line) and after 15 days of treatment with *P. ostreatus* immobilized on PUF.



Figure 13. Time course of laccase production (solid line), glucose consumption (dotted line) and degradation of NSAP (dashed line) with *P. ostreatus* immobilized on PUF.

Control flasks (with no glucose added) showed a constant NSAP removal (about 24% of area related to fractions $3 \div 10$), comparable to the one observed in biosorption experiments (see above). According to these results, the addition of a carbon source was necessary to obtain NSAP biodegradation by *P. ostreatus*. At the end of the experiments the treated wastewater was also analyzed in terms of COD and bCOD according to methods described in 6.2. Even though COD value was not sensitively reduced after fungal treatment, respirometric tests results showed a clear increase in the bCOD/COD ratio, which rose from 9% (raw wastewater) to 40-55%. This variation was clearly correlated with the increase in oligomers removal, and this meant that the fungal action was a transformation of high molecular weight NSAP into more biodegradable molecules. Those results suggested the possibility to use fungi as a pretreatment for an activated sludge process since, according to the respirometric tests, the combined treatment allowed to remove up to 60% of the original COD.

Cytochrome P450 Inhibition studies

Some intracellular enzymes, such as cytochrome P450 (cytP450), can be involved in different degradation pathways, such as removal of trace organic contaminants (TrOC), pharmaceuticals and personal care products (PPCPs) and EDCs [61]. Specific inhibitors can be used for assessing the involvement of the cytP450 in degradation processes. 1-Aminobenzotriazole (ABT) is the most common suicide inhibitor, used widely in *in vitro* and *in vivo* experiments [63].

In order to evaluate a possible involvement of the cytP450 family in the degradation process of NSAP, specific tests in presence of this inhibitor were performed. These tests were carried out following the same procedure as the previous (biodegradation experiments), with the addition of ABT. In particular, ABT was added to *P. ostreatus* on PUF as follows: fungus was incubated for 30 minutes in 50 mL of aqueous ABT solution (10 mM), and afterwards wastewater (50 mL) was added. ABT thus reached a final concentration of 5 mM while wastewater was also diluted 2-fold. Control cultures without cytP450 inhibitor were conducted in parallel with 2-fold diluted wastewater.

As reported in Figure 14, flasks with ABT showed a clear and unexpected increase in polymer removal, from 65% to 90%, ruling out a possible role of cytP450 in the degradation process. The increase in degradation observed in presence of ABT is typical of a laccase redox mediator. In order to verify this hypothesis, further experiments with purified laccase and with ABT as redox mediator were performed (see paragraph 6.4.3.).



Figure 14. Time course of polymer degradation with (\bullet) or without ABT (0).

6.4.3. ENZYMATIC TREATMENT

In the present paragraph, several experiments are presented, with the aim of better clarify the conditions in which NSAP degradation occurs, working either with purified laccase or using the extracellular crude extracts of fungal growth.

Experiments conducted are summarized in Figure 15.



Figure 15. Schematic summary of in-vitro experiments presented in this paragraph.

In vitro degradation of NSAP by purified laccases

In order to better understand the NSAP degradation mechanism by *P. ostreatus*, experiments with purified laccase with or without a redox mediator were performed.

Three types of purified laccases were used: from *Funalia trogii* [215], from *Lentinus tigrinus* [216] and a commercial laccase from *Pleurotus ostreatus* (Sigma Aldrich). Considering that optimum pH for laccase activity is between 3 and 5 [51], the wastewater pH was lowered to 4.5 (with H_2SO_4 0.1M). In these experiments 10 mL of wastewater was used and laccase concentration was 500 U/L. Flasks were maintained at 28 °C under shaking conditions (130 rpm) for 24 hours. Samples were collected at 0, 2, 4, 7 and 24 hours and analyzed by HPLC. In addition the same three laccases were tested in presence of a synthetic mediator, 1-hydroxybenzotriazole (HBT) 4 mM.

Laccase from *P. ostreatus* was also tested in presence of a natural mediator, *p*-coumaric acid, in two different concentrations, 4 and 8 mM. Further experiments with laccase from *P. ostreatus* were conducted in presence of ABT, in the same conditions used for HBT.

Experiments conducted with laccase alone did not show any significant NSAP removal (on average 2.5%, see Figure 16), while when HBT was added high removal percentages (on average 60%) were achieved in only 24 hours. These results clearly show that laccases alone are not able to degrade the polymers, probably because of their non-phenolic nature and of their steric hindrance (see paragraph 3.2.1).



Figure 16. Removal percentages for the three purified laccase: *Lentinus tigrinus* (solid line), *Funalia trogii* (dotted line) and *Pleurotus ostreatus* (dashed line), with (▲) or without a redox mediator, HBT (●).

It is interesting to notice that, even if with different rates, at 24 h the final percentage is similar for all the laccases tested. The removal percentages were similar to the ones obtained, in 15 days, in presence of the fungus where no mediators were added (paragraph 6.4.2). The presence of a mediator appears therefore essential for *in vitro* NSAP degradation by laccase. It is necessary to consider that HBT is, in the concentration used, potentially toxic and too expensive to be used in a full scale-plant [217]. Despite this, these results indicates that a possible way *P. ostreatus* uses to degrade NSAP is through a laccase-mediated process.

It is known that, when lignin is degraded, natural mediators, such as acetosyringone and pcoumaric acid, are produced [49]. In order to verify if a natural mediator gave the same results obtained with HBT, experiments with commercial p-coumaric acid and purified laccase from P. ostreatus were performed. After 24 h polymer removal in presence of pcoumaric acid 4 mM was 37%, versus 62% with HBT. When the concentration of the mediator was increased to 8 mM the biodegradation reached a similar amount (61%). It is known that different mediators have different behavior towards pollutants, depending on chemical properties of both mediator and pollutant [218]. Despite this, it is interesting to consider that a natural mediator, which can be produced from natural lignin degradation can be, in specific concentrations, as effective as a synthetic one.

Further experiments were conducted with purified laccase from *P. ostreatus* and ABT, the inhibitor of cytP450 activity, and these showed that ABT acted like a laccase mediator. Indeed Figure 17 shows that purified laccase in presence of ABT 5 mM is able to degrade NSAP up to 22%, and this would explain the increase in degradation observed when ABT was added to *P. ostreatus* (as shown in 6.4.2.).



Figure 17. Removal percentages for laccase from *P. ostreatus*, with (solid line) or without ABT (dashed line).

Extracellular crude extract experiments

Considering the results reported previously, a different approach of NSAP degradation could be the use of extracellular crude extract, containing laccase and natural mediators produced by the fungus. In this way the process is carried out in two steps: first the production of the laccase-containing extract and then the use of this latter for NSAP degradation. This strategy do not involve expensive and/or toxic chemicals addition and, therefore, it has potential for full-scale applications [151].

For the extracellular fungal extract production *P. ostreatus* was cultivated as reported in 5.1.4. The extracts were collected from Czapek Dox culture after 12 days of growth, corresponding with complete consumption of glucose and maximum laccase production. Each flask contained 5 mL of fungal extract, on average 50 U/L of laccase and 50 μ L of concentrated NSAP mixture, provided by the factory, and was maintained at 28 °C, 130 rpm, for 3 days.

NSAP degradation was followed by HPLC for 3 days, and at the end of the experiment, we did not observe any removal of polymers. Therefore, it seems the NSAP degradation can be achieved only in presence of the fungus. Possible explanation are:

- 1. Crude extracts contain proteases, which rapidly denaturate proteins (such as laccases), while, in the presence of the fungus, a continuous production of enzyme compensate this effect;
- 2. Since *P. ostreatus* own several isoforms of laccases, produced in different phases of growth [219], it is possible that the isoform produced in Czapek Dox is not effective as the one produced in presence of NSAP for its degradation;
- 3. The degradation process happens on the cell bound [32];
- 4. Some intracellular enzyme is involved in the process, maybe producing some molecule which cooperate with ligninolytic enzyme.

Considering the good results obtained with purified laccase and redox mediator, and considering that waste lignocellulosic materials like straw are suitable and inexpensive carbon sources for fungal growth [220], with the further property that during their degradation natural mediators are produced [49], a different type of extracellular crude extract was produced. As already reported in 5.1.4., extracellular liquid from fungal culture in straw was produced, the concentrated polymer mixture was added and the pH was adjusted to 5. Also in this case, NSAP degradation was evaluated through HPLC and, as

shown in Figure 18, 41% of NSAP removal was achieved after 24 h. It is important to underline that in these experimental conditions more laccase units were obtained, 500 U/L versus 50 U/L obtained in presence of glucose. Moreover, this extract probably contained different mediators than the former one, coming from lignin degradation [49].



Figure 18. Comparison between raw wastewater chromatogram (solid line) and after treatment with extracellular crude extract from straw (dotted line).

These data indicate a possible approach to an industrial application of the process, because straw is a good laccase-inducer and it is a source of natural mediators. The advantages of using straw for fungal growth lie also in its low cost and easy availability. Straw can be used for long periods of time for fungal growth and when it is exhausted it can be sent to incineration or anaerobic digestion for energy recovery. For these reasons the production of an extracellular extract containing laccases and natural mediators coming from fungal growth on lignocellulosic material seems to be a promising strategy to degrade NSAP.

6.4.4. EFFECT OF DIFFERENT CARBON SOURCES

Studying the literature of fungal wastewater treatment (see paragraph 4.2.1) it becomes evident that in most cases target pollutants are not directly used by fungi as carbon and energy source, but they are degraded in co-metabolism, making their degradation feasible only in presence of another carbon source. In particular, glucose is the most used, although variable concentrations in the range 100 mg/L – 10 g/L have been reported in literature [156,157,221]. Unfortunately, glucose not only may causes bacterial growth, which often compromises fungal biodegradation [160,222], but is also an expensive carbon source.

The experiments reported in the present paragraph investigate the effect of alternative carbon sources on the degradation process, a topic little studied so far.

In particular, these experiments allowed to study the effect of replacing glucose with 5 g/L of three selected carbon sources (corn starch, cellulose and lignin) on NSAP removal. It has been decided to use the same weight of the different carbon sources even if in terms of C/N ratio probably they provide different nutrition for the fungus.

Experiments were performed in the same experimental conditions used in the batch biodegradation tests (paragraph 6.4.2.), using *P. ostreatus* attached on PUF, replacing glucose with the other carbon sources. Flasks with no external carbon source were added as control. These flasks were daily monitored in terms of enzymatic activities, glucose concentration and area reduction of chromatograms.

After 24 h treatment with *P. ostreatus* we observed in all cases, including control, a decrease in all fraction areas, probably due to NSAP fungal biosorption as already described (see biosorption tests, paragraph 6.4.2.). While the control chromatograms remained stable during all the experiment, for the other carbon sources chromatograms showed an area reduction in correspondence of fractions from 3 to 10 (Figure 19).

As showed in Figure 19 we observed, after 40 days, an average area reduction of 70% for all the different carbon sources. During the study we observed very low Lac activity $(2 \div 8 \text{ U/L})$ in all cases, including control, except in the flasks with lignin, where we registered 20 \div 50 U/L. This is not surprising since lignin is a well-know laccase inducer [223]. Also in this case no MnP or LiP were detected. As we can see in Figure 19, the flasks with lignin showed a more rapid action on the oligomers at the beginning of the experiment, maybe due to the higher Lac concentration, but after the first days the loss remained lower than other C sources. On the contrary, glucose gave the more rapid action on NSAP, followed by corn starch and cellulose.



Figure 19. Time course of NSAP removal with the addition of different carbon sources. In control flasks no carbon source was added. Error bars are referred to minimum and maximum observed.

This behavior can be correlated with the structural complexity of the used carbon source: simpler molecules (e.g. glucose) required less time to be metabolized and, consequently, the simultaneous NSAP depolymerization is faster. On the contrary, lignin is a complex polymer and required much more time to be hydrolyzed and used as carbon source. It is interesting to consider that, despite the different velocity, all the carbon sources allowed to remove, as a combination of biosorption and biodegradation, about 70% of the oligomers. This suggests the possibility of using ligno-cellulosic materials, such as low cost waste products, for carrying on the process. As reported by other authors [156,182,224], our experimental data did not show any direct relationship between laccase activity and the degree of depolymerization. Possible explanations could be: the presence of other enzymes, not tested in the experiments, the involvement of intracellular laccase or an indirect role of the enzyme in the biodegradation process, e.g. in cooperation with other compounds (mediators or other enzymes), which are produced by *P. ostreatus* only in presence of an additional carbon source, different from NSAP.

6.5. BATCH REACTORS

6.5.1. INTRODUCTION

One of the main objectives of the work has been the scale-up of the process observed in flasks into lab scale bio-reactors, studying the influence of different parameters and investigating the passage from sterile to non-sterile conditions.

In this stage it has been decided to work with *Pleurotus ostreatus* (NCBI KJ02093) and with another strain, a *Bjerkandera adusta* (MUT 1236), which has been selected together with *P. ostreatus* in previous works [210,213] and it is capable of expressing MnPs, which have higher redox potential that *P. ostreatus* laccases [32].

The first designed reactors were the following batch reactors:

- Continuous Stirred Tank Reactors (CSTR) with free fungi;
- Moving Bed Biofilm Reactors (MBBR) with fungi attached on PUF;
- Fixed Bed Bioreactors (FBBR) with fungi attached on PUF.

6.5.2. MATERIALS AND METHODS

Continuous Stirred Tank Reactors (CSTR)

The CSTRs were Plexiglas rectangular reactors (20x8x30 cm), with a working volume (at the free surface level) of 4 L (see Figure 20). After the growth phase (see paragraph 5.1.2.) these reactors were filled with free cells of *Bjerkandera adusta* and raw non-sterile wastewater. Glucose was added in concentration of 500 mg/L every 5 days. Air was provided continuously with an air stone and the pH was controlled automatically by a pH controller (during experiments, various pH values were tested, see later). COD, polymer removal and glucose concentration were evaluated three times per week according to methods reported in 6.2.



Figure 20. Picture of a CSTR with *B. adusta*.

Moving Bed Biofilm Reactors (MBBR)

The MBBRs were similar Plexiglas rectangular reactors (20x8x30 cm), but with a central partition and two inclined walls (see Figure 21) in order to guarantee a correct circulation of the carriers, which rise up because of the air flow (in the right part of the reactor, see Figure 21) and settle in the left part. In this case the working volume (at the free surface level) is 3.6 L. These reactors were filled with attached *B. adusta* on PUF (see paragraph 5.1.3.) and raw non-sterile wastewater.

Also in this case glucose was added in concentration of 500 mg/L every 5 days, air was provided continuously with an air stone and the pH was controlled automatically by a pH

controller. COD, polymer removal and glucose concentration were evaluated three times per week according to methods reported in 6.2.



Figure 21. Picture of a MBBR with *B. adusta*.

Fixed Bed Bioreactor (FBBR)

The fixed bed bioreactors were glass cylinders (25 cm height, 5 cm diameter), with a working volume of 400 mL. A gas outlet with a 0,2 μ m filter was provided in order to avoid possible harmful emissions from the reactor.

These reactors were filled with immobilized *Bjerkandera adusta* and *Pleurotus ostreatus* on PUF, prepared with the procedure described in 5.1.3. Main operational parameters are summarized in Table 9.

When reactors started, non-sterile wastewater (which pH was adjusted to 6) with 5 g/L of glucose was continuously recirculated from a reservoir to the reactor, with a flow of 50 mL/min. Air was supplied continuously inside the reservoir with an air stone. Reactors samples were taken daily and monitored in terms of enzymes activities, pH, dissolved oxygen, temperature, polymer removal, COD and bCOD.

Table 9. Main operational parameters of F DDRs.			
	P. ostreatus	B. adusta	
Volume	400 mL	400 mL	
Fungal biomass (dry weight)	1,44 g	1,44 g	
Biomass concentration	3,60 g/L	3,60 g/L	
рН	6	6	
Additional carbon source	Glucose 5 g/L	Glucose 5 g/L	

Table 9. Main operational parameters of FBBRs.

6.5.3. RESULTS AND DISCUSSION

Continuous Stirred Tank Reactors (CSTR)

During the conduction of this first plant, lasted three months, we encountered several operational problems, which led to few start-ups of the system. During time several conditions were tested: different pH values (between 5 and 7), pellets dimensions and biomass concentrations (between 500 and 2000 mg/L).

In particular, every reactor showed signs of bacterial growth (e.g. increase in turbidity, see Figure 22), which led to poor or none NSAP removal, as already reported by other authors [160,222]. It has been largely demonstrated that the presence of bacteria very often compromise fungal activities [156,160,176]. In particular, it has been shown that bacteria 1) affect fungus morphology [3,194], 2) lead to cessation of fungal growth [129], 3) inhibit enzyme secretion capacity [129], 4) denaturate the secreted fungal enzymes [129] and 5) outperform fungi in the competition for the substrate [3].



Figure 22. Increase in turbidity in CSTR reactor. From left to right: reactor at day 1, day 6, day 8.

To sum up the results obtained in this first phase it is possible to say that lower pH (5 instead of 7) and/or higher amount of biomass (2000 mg/L instead of 500 mg/L) gave better results that the ones with higher pH and/or less amount of biomass. These two results are not surprising because it is well known that pH play a key role in maintaining fungal biomass active [165,192] and that the optimum pH for growth and enzyme secretion of many fungal species is in the interval $3.5 \div 5.5$ [183,192]. Moreover, more fungal biomass in the beginning is an advantage for fungi in the competition with bacteria, at least in the first phase of operation.

Moving Bed Biofilm Reactors (MBBR)

These reactors were conducted in parallel with the previous ones and they encountered the same problems of CSTRs. Moreover, one of the main issue was the correct circulation of the carriers inside the reactors: in order to obtain the same amount of biomass used in CSTRs the number of carriers had to be so high that they entangled in each other and were not able to move inside the reactors as designed. For this reason, tests were conducted with lower biomass concentrations (between 150 mg/L and 1000 mg/L), at different pH values (between 5 and 7).

Also in this case, every reactor showed signs of bacterial growth (see Figure 23), which, again, led to poor or none NSAP removal, even though PUF seemed to be still colonized by fungi.





While for CSTRs the amount of fungal biomass seems to be a key factor together with pH value, for MBBR only this latter seems to influence the obtained results: reactor with lower pH (5) gave better results in comparison with the ones with higher pH (7).

The fact that these reactors showed almost the same results as the previous ones, despite the little amount of biomass, may means that this type of reactor create more favorable conditions for the process. In fact, some authors reported that a strategy to enhance fungal biodegradation is the use of attached biomasses [161,188,190], because, as already pointed out in paragraph 4.2.2., it has been shown that degradation capacity and tolerance to toxic pollutant concentrations can be increased when fungal biomass is immobilized [190,225]. Moreover, cell immobilization reduces protease activity [191] and make fungal biomass more effective in suppressing bacterial growth [192].

Fixed Bed Bioreactors (FBBRs)

This third set of reactors were set up after the first two, and it was aimed at increasing biomass concentration, which was more than triple in comparison with the firsts, using attached biomass on PUF, for the reasons explained above.

Reactor filled with *B. adusta* exhibited limited enzymatic activity and polymer removal (data not shown), while the reactor filled with *P. ostreatus* showed low but stable enzymatic activity ($2 \div 16$ U/L) and 22% of polymer removal after 15 days of work. This different behavior is probably due to the different bacterial contamination: the reactor filled with *B. adusta* showed a great bacterial growth since the first days of operation, while in the reactor with *P. ostreatus* this growth remained (in appearance) contained.

6.6. STRAW-BED BIOREACTOR

6.6.1. INTRODUCTION

As already observed previously in the present thesis, NSAP degradation by fungi is achieved only in presence of an additional carbon source. In the previous paragraph (6.5.) several experiments in bioreactors, using glucose as additional carbon source, were presented. Almost every experiment led to bacterial growth inside the reactor, poor or none enzymatic activity and NSAP removal. Glucose is an easily assimilated carbon source, which both bacteria and fungi can metabolize and, therefore, it is not surprising that it fostered bacterial growth. Since this growth compromised fungal biodegradation, it is important to use a more selective carbon source for ligninolytic fungi, when working under non-sterile conditions.

In paragraph 6.4.4. we evaluated the possibility of using alternative carbon sources (corn starch, cellulose and lignin) and we find out that all these sources led to the same final NSAP removal, even if with different velocities.

Lignocellulosic materials can enhance the expression of LMEs and, as a consequence, the biodegradation of xenobiotic [165]. Examples of lignocellulosic compounds already used as carbon source for fungi are pinewood chips [164], hay, rye, spelt grains, straw and peanut shells [165]. Moreover, it is also possible to obtain lignocellulosic materials also from wastes [226]. On the other hand, these materials carriers present some operational problems (due to the fact that their structural behavior change during time [168]) and can release some soluble by-products in the liquid which can be non biodegradable, toxic and give rise to a colored solution.

Taking all of this into consideration, together with the fact that attached biomass seems to be a more favorable condition for the process (see 6.5), it has been decided to test a packed bed bioreactor (PBR) with a bed made of straw. In this way straw can act as selective carbon source for fungi, physical support and source of natural mediators (see paragraph 6.4.3). Moreover, its negligible cost and easily availability make the process potentially feasible for industrial applications.

6.6.2. MATERIALS AND METHODS

The reactors used in this test were the same glass cylinders used previously as FBBRs (see paragraph 6.5.). These reactors were 25 cm height, 5 cm diameter and had a working volume of 450 mL. The system is represented in a schematic overview in Figure 24. Also in this case a gas outlet with a 0,2 μ m filter was provided in order to avoid possible harmful emissions from the reactor.



Figure 24. PBR schematic overview.

The two reactors were filled with *Bjerkandera adusta* and *Pleurotus ostreatus* immobilized on 18 g straw (see paragraph 5.1.3.).

When reactors started Czapek Dox media (without glucose, see paragraph 5.1.3.) was continuously recirculated from a reservoir to the reactor, trickling from the top with a flow of 50 mL/min. After a week of adaptation, when the liquid was continuously recirculated with no inflow or outflow, the continuous process was started. In order to evaluate the contribution of straw degradation metabolites to the final COD the bioreactors were fed again with new Czapek Dox (without glucose) for 15 days and then fed with the wastewater, which pH was adjusted to 7.

Both Czapek Dox and wastewater flew into the system with an HRT of 3 days and flew out through an outlet located at the free surface level (Figure 24). The hydraulic retention time

was calculated by dividing the total volume of the system (measured by filling the whole system before starting the experiment) for the provided flow.

The provided HRT was in line with the ones utilized in fungal processes [176,227]. These long HRTs are required in order to maintain a good level of removal during continuous operation, due to the typical slow rate of degradation that fungi show towards most pollutants. Reactor effluents were monitored in terms of enzymatic activities, pH, temperature, polymer removal, COD and bCOD. This latter was measured using samples of activated sludge (AS) taken from the oxidation tank of two WWTPs: the municipal plant of the city of Florence, managed by Publiacqua SpA, and an industrial one, managed by Consorzio Cuoiodepur Spa (Pisa, Italy), which treats tannery wastewater.

6.6.3. RESULTS AND DISCUSSION

As described in paragraph 6.6.2., in order to study the release of by-products in the medium (in terms of COD and bCOD), for the firsts 15 days reactors were fed with the mineral medium with the same HRT (3 d) used afterwards for NSAP biodegradation. During this phase reactor with *B. adusta* showed a decreasing enzymatic activity (going from 60 U/L of MnP in day 1 to 3 U/L in day 15) while reactor with *P. ostreatus* was able to maintain stable laccase activity (ranging from 315 U/L to 1500 U/L). As shown in Table 10 the effluent in this phase had a high value of not biodegradable COD (1200 mg/L) for both municipal and industrial sludge. This COD is probably due to by-products or metabolites released during fungal straw degradation, since feeding medium COD is zero.

After this first period bioreactors were fed with real wastewater continuously for three months. Enzymatic activities and polymer removal were evaluated as reported in paragraph 6.2. From the beginning *B. adusta* exhibited a limited enzymatic activity and no significant polymer removal (data not shown) and it was decided to stop the reactor.

On the contrary, reactor inoculated with *P. ostreatus* showed stable Lac activity, varying in the range 100 - 500 U/L during the three months of work.

Noticeable NSAP biodegradation was achieved after two weeks of work and remains until the end of the experiment; the mean polymer removal was $30 \pm 5\%$.

Figure 25 shows a comparison between chromatograms of raw wastewater and effluent from P. ostreatus reactor, where a decrease in high molecular weight polymers and an increase of the first fractions are evident.



Figure 25. Wastewater chromatogram before (solid line) and after (bold line) treatment in PBR inoculated with *P. ostreatus*.

Although COD was not reduced after fungal treatment, the results of the respirometric tests showed a clear increase in the bCOD/COD ratio (see Table 10). Also Anastasi et al. [161] found an increase in the COD value after fungal treatment, may due to secondary metabolites secretes by the fungi. In our case this increase can be due to the same metabolites or, probably, to by-products released from straw degradation.

 Table 10. COD and bCOD of raw wastewater and effluent from PBR with P. ostreatus (mean values).

 In parenthesis bCOD/COD values (%).

	COD	bCOD (mg/L)		
	(mg/L)	Measured with municipal AS	Measured with industrial AS	
Czapek Dox	0	0	0	
Effluent of PBR fed with Czapek Dox	1200	0	0	
Raw wastewater	3480	20 (1%)	315 (9%)	
Effluent of PBR fed with wastewater	3560	450 (13%)	1413 (40%)	

A clear difference exists between the results obtained with municipal and industrial sludge: when the tests were conducted with the first, bCOD values were lower. This is not surprising since the industrial plant, which treats tannery wastewater, owns a biomass acclimated to natural and synthetic tannins, including sulfonated naphthalene-formaldehyde condensates [8]. According to these results, bCOD obtained using a even more acclimated sludge, for example a sludge taken from a plant that treats these specific compounds, should be even higher.

These results suggest the idea of using fungi as a pre-treatment for the activated sludge (AS) process since, according to the respirometric tests, the combined treatment would enable the removal of up to 40% of the original COD. This approach has already been proposed by other authors who created a synergy between fungi and bacteria for different pollutants degradation [161,194,195].

The contribution of metabolites from the straw degradation significantly affected the results of the bioreactor. Indeed, the by-products released by the PBR, when fed with mineral medium, significantly contribute to the COD, but not to the bCOD. Taking this contribution into account, the combined treatment of PBR and AS could theoretically be able to reduce the COD by up to 73%.

6.7. BIOTRICKLING FILTER WITH LUFFA CYLINDRICA

6.7.1. INTRODUCTION

The results obtained with the straw-bed bioreactors were interesting, since these reactors were the firsts to give stable results in non-sterile environment. Trickling filter with fungal biomass can enhance biodegradation with submerged, partially submerged and non-submerged carriers (semi and solid state fermentation), increasing the contact between compounds and mycelia [164].

Using ligno-cellulosic materials helped suppressing bacterial growth, enhanced the fungal one, induced LMEs and, very likely, produced natural lignin-derived phenols that could have acted as laccase mediators (as already hypothesized in paragraph 6.4.3.), which are necessary for non-phenolic compound degradation by laccases [49]. Moreover, very often these materials are easily available and have a negligible cost. For all these reasons the idea of using ligno-cellulosic materials as carbon source and physical support for fungi appears very promising for treatment of NSAP-containing wastewaters.

Nevertheless, degradation of straw released by-products in the effluent of the reactor which seriously affected the results. It has therefore decided to set up other reactors, using another type of material, richer in cellulose and poorer in lignin. These reactors have several important differences and improvements, such as pH control, temperature control, better evaluation of by-products releases, and better evaluation of the combination process (fungi/activated sludge). These aspects are better highlighted in the following paragraph.

6.7.2. MATERIALS AND METHODS

Reactors and filling materials

The reactors, specifically designed for this experiments, were two trickling filters. The reactors were realized entirely in glass, cylinder-shaped with a working volume of 3L each. The dimensional drawing of a reactor and a picture are reported in Figure 26.





Figure 26. Dimensional drawing of the reactor (left) and a picture (right).

These reactors were filled with *Pleurotus ostreatus* attached on a carrier made of a natural sponge obtained from the internal stroma of *Luffa cylindrica*, a subtropical vine in the cucumber family (Figure 27).



Figure 27. *Luffa cylindrica*: raw sponge (left), detail of porosity (centre), colonized sponge where the white mycelium of *P. ostreatus* can be seen (right).

This material was chosen because it has a mechanical resistance and a porosity suitable for fungal growth, a low content in lignin, which can cause the release of non-biodegradable compounds, and high content in cellulose, which represent a good carbon source for fungi.

In comparison with straw, lignin content of *Luffa* is half, 11% instead of 21% and cellulose content is double, 63% instead of 34% [228,229].

Reactor inoculums were prepared according to methods presented in paragraph 5.1.3.

This system worked similarly to the straw-bed bioreactor: inside the reactor the wastewater enter from a sprinkler on the top (which had the purpose of evenly distributing the flow), trickled down throughout the filling materials to a reservoir bottle. The wastewater was continuously recirculated from this reservoir to the reactor and, for the continuous process, the feeding wastewater flew into the reservoir at a designed flow rate and flew out through an outlet located at the free surface level (Figure 28). The hydraulic retention time was calculated by dividing the total volume of the system for the provided flow. This volume was measured by filling the whole system before starting the experiment and it includes the volume of the reservoir, the tubes and the liquid absorbed by fungi and *Luffa*.

The plant was then completed with an air supply system inside the reactors, an external heating jacket for temperature control and automatic pH-controllers.



Figure 28. Scheme (left) and picture (right) of the pilot plant. Reactors, reservoir bottles, pumps, pH controllers are visible.

For the start-up of the system, Czapek Dox media prepared without glucose addition (the same media used for the colonization phase, see paragraph 5.1.3.) was used as feeding liquid for the first week, in order to adapt the biomass to the new environment. After this adaptation period, the reactors were fed with the wastewater and the experiment started.

Experimental set-up

Three experiments and a control reactor were conducted, that differ in few operational parameters (see Table 11). Thanks to these experiments it has been possible to analyze two different hydraulic retention times (2 days and 3 days) by comparing experiments 1 and 2 and two different pH values by comparing experiments 1 and 3.

Control reactor was fed with tap water, in order to evaluate the release of compounds coming from *Luffa* degradation in the reactor effluent, since these seems to play an important role in COD determination (see straw-bed bioreactor, 6.6.3.).

	Control	Experiment 1	Experiment 2	Experiment 3
рН	6	6	6	5
Т	27° C	27° C	27°C	27°C
HRT	3 d	3 d	2 d	3 d
Luffa cylindrica (Dry weight)	55 g	55g	55g	55g
Pleurotus ostreatus (Dry Weight)	4.7 g	4.7g	8.8g	8.8g

Table 11. Experiments conducted with trickling filters with Luffa cylindrica.

As already observed, these long HRTs were in line with the ones utilized in fungal processes [176,227], required in order to maintain a good level of removal during continuous operations, due to the typical slow rate of degradation that fungi show towards most pollutants.

Reactor effluents samples were taken daily and several parameters were measured: DO and T with portable probe, pH with pH-controller, soluble and total COD (three times per weeks), N_{TOT} , $N_NH_4^+$, $N_NO_3^-$, $N_NO_2^-$, PO_4^{3-} (two times per week), Laccase and Manganese Peroxidase (daily) and polymer removal with HPLC (three times per week). For analytical procedures see paragraph 6.2. Moreover, once a week, evaporation rate and actual HRT were controlled by measuring volumes added to the alimentation tank, to acid/base solutions, withdrawn for sampling and collected at the outlet. Evaporation rates (data not shown) was used to correct evaluated parameters (COD and polymer removal).

Effluent biodegradability evaluation

In addition to polymer and COD removal, the process was monitored by evaluating the biodegradability of the effluent. For control reactor and experiment 1, respirometric tests (see paragraph 6.2.) were performed on effluent samples for bCOD measurement.

For experiments 2 and 3 two activated sludge (AS) reactors were provided, fed with the effluent of the fungal reactors. These reactors were the same described in paragraph 6.5. and worked as CSTR with no biomass retention system (Figure 29); in this way sludge retention time (SRT) and hydraulic retention time (HRT) were the same. Given the flow rates and the fixed volume of these reactors these times were 6.5 days for experiment 2 and 10 days for experiment 3, considered sufficient for biodegradable COD removal.

These reactors were inoculated with activated sludge taken from the oxidation tank of an industrial WWTP, managed by Consorzio Cuoiodepur Spa (Pisa, Italy), which treats tannery wastewater and owns a biomass acclimated to natural and synthetic tannins, including sulfonated naphthalene-formaldehyde condensates [8].



Figure 29. Picture of complete pilot plant: fungal bioreactors on the top and AS reactors at the bottom.

This system has been designed in order to evaluate total COD removal in a two-stage process: the first stage with fungi, in which polymers are depolymerized and transformed into more biodegradable compounds (such as monomers and dimers) and the second stage with AS, where these compounds are mineralized. Sample of AS were taken daily and monitored in terms of pH, soluble COD, TSS and polymer removal.

A third AS reactor was added as control. This latter reactor was fed with raw wastewater with HRT 6.5 d and pH 6.5 (equal to the values observed in AS reactor fed with effluent of experiment 2).

6.7.3. RESULTS AND DISCUSSION

Fungal control reactor

One of the first results is represented by data collected from fungal control reactor. This reactor, fed with tap water, showed how fungal growth on *Luffa* sponges give rise to organic compounds releases in the solution. This is demonstrated by effluent COD, N and P content (see Figure 30). As expected, these releases are higher in the initial part of the process and then decrease almost to zero, probably because of *Luffa* consumption by the fungus. N and P releases could be due to some Czapek Dox used in the adaptation period (see 6.7.2.) absorbed into *Luffa* sponges, but COD is due only to fungi metabolism on *Luffa*, since Czapek Dox COD is zero. However, this COD value does not represent a problem, because it is at least one order of magnitude lower than wastewater COD (which vary between 3500 and 5000 mg/L). Moreover, respirometric tests conducted on the effluents of this reactor showed that this COD is 100% biodegradable by activated sludge. This means that, in a two-stage process, even if this COD is created in the first stage is then consumed in the second one.





Activated sludge control reactor

The other control reactor was the activated sludge reactor, fed with raw wastewater. This reactor showed a steep decrease in the TSS value and an increase in COD up about to the value of the raw wastewater (see Figure 31). These data, confirmed by HPLC scans (data not shown) show that activated sludge are not able to depolymerize NSAP and/or to consume the COD of the wastewater (coherently with respirometric tests results, see paragraph 6.6.3.).



Figure 31. Time course of COD and TSS of the activated sludge control reactor. Yellow line represents raw wastewater COD.

Experiment 1

The experiment 1 was the first to be conducted and it suffered from some operational problems regarding feeding pumps and acid/base dosing pumps. For this reason the experiment, especially in the firsts weeks of works had suffered from a couple of pH-shocks (up to pH 2). These problems may explain the dispersion of data shown in Figure 33; this experiment lasted 70 days, during which both COD removal and polymer removal were highly fluctuating. At the beginning of the experiment (first 20 days) COD removal was negative, which means that the COD of the effluent was higher than the influent COD. This behavior may be due to some organic compounds release in the effluent (from *Luffa* degradation), as confirmed by the release of nitrogen and phosphorus in the same period (see Figure 34). On the other hand these values are too high if compared to the ones of the control reactor, leading to think that the pH-shocks mentioned above may have enhanced *Luffa* and/or mycelium breakdown with subsequent by-products release.

Mean polymer removal during second period of observation (from day 30 to the end) was 19% while COD removal was 15%. Respirometric tests conducted on the effluent of this reactor showed bCOD/COD values between 40% and 60%, while the raw wastewater value was 9%. This increase in biodegradability is probably associated with the obtained depolymerization, as already observed (see paragraph 6.6.3.).

Another important point to be addressed is the sudden and unexpected increase in Lac production, from few units to more than 700 U/L (see Figure 35). This peak is associated to the growth of a green and unidentified mycelium inside the reactor (see Figure 32), which may be responsible of this massive production of laccase. Another possibility is that laccases are secreted by both fungi as defense against each other.



Figure 32. Green mycelium grown inside the reactor.



Figure 33. Time course of COD and polymer removal (%) in experiment 1.



Figure 34. Time course of N and P content in effluent of experiment 1.




Experiment 2

Experiment 2 differs from the first for the shorter value of the HRT (2 d instead of 3 d).

Regarding COD and polymer removal (reported in Figure 36) three separate phases can be observed. The first phase, from day 1 to day 15, characterized by low COD and polymer concentration, is the non-steady state, where the liquid inside the reactor (mineral medium) is slowly replaced by the wastewater. In the intermediate phase, from day 15 to day 45, the process appears to work as designed: polymers are removed in the fungal reactor (on average 35%), together with a part of the COD (on average 31%), while in the second stage activated sludge removed further COD, for a total COD removal (after combined treatment) of 49% on average. From day 45 to the end of the experiment some differences are evident:

- Polymer removal is sensitively lower, 10% on average (Figure 36).
- COD removal after fungi is higher, 46% (Figure 36).
- COD removal after combined treatment is not higher than the previous (Figure 36). This means that no increase in biodegradability is obtained in the fungal reactor.
- Ammonium nitrogen in the effluent is suddenly increasing, from less than 1 mg/L up to 77 mg/L, while total nitrogen does not change sensitively (Figure 37).
- Laccase completely disappears in the effluent (Figure 38).

These data probably indicate an ammonification process of the organic nitrogen of the wastewater (80 - 110 mg/L, almost 100% of total nitrogen, see Table 7), which is not bonded to the polymers. This could explain the COD removal in the fungal reactor (without polymer removal) and the ammonium formation. The absence of enzymes is consistent with the low polymer removal and consequent absence of COD removal in the activated sludge reactor. Our theory is that *Pleurotus ostreatus* was not active in this phase, maybe because of the depletion of *Luffa* (data not shown), and another organism overgrew inside the reactor, carrying on the above-mentioned process, which does not require other carbon sources.

Comparing these results with the ones obtained in experiment 1, COD and polymer removal (in the intermediate phase) was higher in this case, despite the shorter contact time. This may means that long HRTs are not necessary for carry on the process.



Figure 36. Time course of COD and polymer removal (%) in experiment 2.



Figure 37. Time course of N and P content in effluent of experiment 2.



Figure 38. Time course of Laccase and Manganese Peroxidase production in effluent of experiment 2.

Experiment 3

Results of experiment 3 are reported in Figure 39 (COD and polymer removal), in Figure 40 (nitrogen and phosphorus content) and in Figure 41 (enzyme activities). The behavior of this set of reactors is similar to the previous one; it is indeed possible to observe the same three phenomena described for experiment 2:

- First phase (day 1 day 15): non-steady state with low polymer and COD concentration.
- Second phase (day 15 day 50) with low ammonium content, presence of laccase activity, depolymerization of NSAP (on average 26%) and COD removal (on average 14%) in the fungal reactor, further COD removal in the activated sludge reactor (on average 41%).
- Third phase (day 50 end) with lower polymer removal (7%), higher COD removal in the fungi reactor (32%) with low further COD removal in the activated sludge reactor (total mean COD removal 34%), increase in ammonium nitrogen concentration (up to 80 mg/L) and complete depletion of laccase.

It is therefore possible to assume that the same processes described for the previous reactor took places also in this one.

Comparing data of experiment 1 and 3 (which differ only for pH value, 6 for experiment 1 and 5 for experiment 3) some consideration regarding pH effect can be done. NSAP removal was higher in this experiment (26% instead of 19%), while total COD removal was almost the same. Lower pH values seems therefore to enhance the process.



Figure 39. Time course of COD and polymer removal (%) in experiment 3.



Figure 40. Time course of N and P content in effluent of experiment 3.





Generally, it is difficult to compare the performance of these bioreactors with those in literature since, to our knowledge, there are no papers reporting fungal degradation of NSAP in non-sterile environment.

However, Novotný et al. [155] tested a fungal trickling filter with *Irpex latus* immobilized on straw carriers for the treatment of textile wastewater followed by a bacterial treatment. Sequential two-step application of fungal and bacterial reactors resulted in efficient decolorization in first step (90–99%) and TOC reduction of 95–97% in the two steps. Also Anastasi et al. [161] tested a two-steps process for textile wastewater treatment. In the fungal reactor (first step) they obtained decolorization but also COD increase, while in the AS reactor (second step) they obtained reduction of COD and toxicity. On the other hand, Pant & Adholeya [168] obtained 61.5% color and 65.4% COD removal using a fungal consortium immobilized on straw for treatment of molasses distillery wastewater.

Besides the COD removal, the treatment with fungi can also result in the formation of colored metabolites or toxic ones [172,230,231], not measured during this study. It is therefore important to underline that such evaluations need to be done before further applications.

6.8. CONCLUSIONS

The present chapter described the research conducted about the treatment of a petrochemical wastewater with fungi, containing a mixture of naphthalenesulfonic acid polymers (NSAP) as the largest fraction of organic pollutants.

The wastewater had an high COD (between 3300 and 6200 mg/L) and a very low bCOD/COD ratio (<10%), which confirms the recalcitrant nature of NSAP.

Early experiments in Erlenmeyer flasks showed that *Pleurotus ostreatus* was able to depolymerize NSAP in presence of glucose, while no degradation occurred with no external carbon source addition. Other carbon sources (corn starch, cellulose and lignin) were tested and it is interesting to consider that, despite the different velocity, all the carbon sources allowed to remove about 70% of the oligomers. In every case this biodegradation led to an overall increase in wastewater biodegradability (the bCOD/COD ratio rose from 9% to 40÷55%). These results suggested the possibility to use fungi as a pretreatment for an activated sludge process.

Further tests conducted *in vitro* using purified laccases and fungal crude extracts showed that laccases alone are not able to degrade NSAP, but the presence of a mediator (synthetic or natural) appears essential. Crude extracellular extracts containing laccase from *P*. *ostreatus* was unable to degrade NSAP mixture. On the contrary, *P. ostreatus* growing on lignocellulosic materials, like straw, produces extracellular extracts containing laccase and natural mediators, that can be used directly for the degradation of NSAP. These data indicate a possible approach to an industrial application of the process, because straw is a good laccase-inducer and it is a source of natural mediators coming from lignin degradation. For these reasons the production of extracellular extracts containing laccases and natural mediators coming from fungal growth on lignocellulosic materials seems to be a promising strategy to degrade NSAP.

A different strategy tested during the research work was the use *in vivo* of fungi in bioreactors for direct wastewater treatment.

In the first phase of work with fungal bioreactors running under non-sterile conditions, several problems were encountered regarding bacterial contamination. This contamination, very often, led to poor or none enzymatic activity and, consequently, poor or none NSAP removal. These problems led to the conclusion that simple and easily biodegradable carbon sources, such as glucose, do not bring to acceptable degradation in non-sterile conditions,

and that a possible strategy to enhance fungal biodegradation could be the use of attached biomasses.

Another generation of reactors were the straw-bed bioreactors, which joined the use of attached biomasses and the use of straw as carbon source. These reactors, using complex lignocellulosic materials as carbon and energy source for fungi gave stable results in non-sterile environment in terms of enzymatic activity and degradation capacity. The conducted respirometric tests allowed to assess the potential of a two-phase process. In fact the combination of fungal treatment and an AS process is able to remove up to 40% of the initial COD. Nevertheless the use of straw as co-substrate caused the release of by-products, which contribute to non-biodegradable COD.

For this reason, the last set of reactors was set up using another biodegradable carrier, Luffa cylindrica, with lower content in lignin, which can cause the release of non-biodegradable compounds, and higher content in cellulose, which represent a good carbon source for fungi. Nevertheless, fungal growth on Luffa sponges give rise to COD release in the solution, probably due to soluble organic compounds production during fungal metabolism. However, this COD value does not represent a problem, because it is at least one order of magnitude lower than wastewater COD and moreover, respirometric tests showed that this COD is 100% biodegradable by activated sludge. This means that, in a two-stage process, even if this COD is created in the first stage is then consumed in the second one. Reactors treating wastewater showed stable enzymatic activity, polymer removal (20-35%), COD removal after combined treatment with AS (40-50%) and allowed to conclude that long HRT are not necessary for carry on the process, while, on the other hand, lower pH values seems to enhance the process. Nevertheless, in the final period of operation, a lack of performance of the reactors were observed, probably due to the depletion of Luffa. It is therefore reasonable to think that the initial amount of Luffa was not sufficient to allow P. ostreatus to degrade NSAP for all the period of observation.

The research work conducted so far showed clearly that *Pleurotus ostreatus* is able to degrade NSAP both in sterile and in non-sterile environment, in presence of an additional carbon source, increasing the biodegradability of the wastewater. The two-stage process (fungi and activated sludge) worked as designed and allowed to remove about 50% of the initial COD. The use of biodegradable carriers allowed the fungal reactor to limit bacterial contamination and enhanced enzymatic production. However the choice of the support is a

delicate passage since it may give rise to by-product release (as it happened with straw). Moreover it is necessary to provide sufficient amount of supports (meaning carbon source) in every moment of the process, because otherwise other microorganisms can overgrow in the system. It is important to underline that some important parameters such as color and toxicity of the effluent were not measured during this study, but such evaluations need to be done before further applications.

7. PHARMACEUTICAL BIODEGRADATION

7.1. INTRODUCTION

The present chapter describes the research work conducted during an abroad stay at the Universitat Autònoma de Barcelona (UAB) during the second year of the PhD course.

In particular, the capability of *Pleurotus ostreatus* to biodegrade selected pharmaceuticals (Atenolol, Diclofenac and Ketoprofen) in synthetic and real wastewater was evaluated. Experiments were conducted firstly in flasks, with the purpose of identify the enzymatic system involved in the degradation process and then in bioreactor, where it was possible to study the behavior of the fungus in more realistic conditions.

Pollution from pharmaceuticals in surface and ground-waters is becoming recognized as an environmental concern in many countries [232] and for this reason it is gaining increasing attention from both the public and the scientific community [124,233,234]. Pharmaceuticals are considered as contaminants of emerging concern and many journal articles addressing their possible adverse effects have been published during the past 20 years [235].

These compounds are released into the environment mainly via patient excretion and, once into the sewage system, they reach wastewater treatment plants, where often they are not completely degraded [236]. As a result, a significant portion of drugs and/or their metabolites are released into the aquatic system and they might reach drinking water plants [237].

These pharmaceutical compounds include antipyretics, analgesics, blood lipid regulators, antibiotics, antidepressants, chemotherapy agents, contraceptive drugs and much more [238].

The compounds selected for this study are widely used pharmaceuticals: Atenolol (a β -blocker), Diclofenac and Ketoprofen (two non-steroidal anti-inflammatory drugs, NSAIDs).

 β -blockers are extensively used for the treatment of disorders such as hypertension, angina and arrhythmias [239] and were frequently detected in sewage treatment plant effluents and even in surface waters with a concentration ranging from ng/L to μ g/L [240]. Among the β blockers, Atenolol (ATL) is most toxic for humans and aquatic organisms [241].

NSAIDs represent instead a remarkable group of pharmaceuticals, which exhibits antiinflammatory, analgesic and antipyretic activities [242]. In particular, Diclofenac (DCF) and Ketoprofen (KTP) are two of the most used non-prescription drugs, and they both have been detected in surface waters in concentrations ranging from ng/L up to μ g/L [242,243]. A promising approach for biodegradation of drugs in waters is based on the use of white rot fungi; in fact there are many evidences that WRF are capable of degrade a wide range of pharmaceutical compounds, such as antibiotics [244], analgesics [245], lipid regulators [125], antiviral drugs [233] and estrogens [246].

7.2. MATERIALS AND METHODS

7.2.1. PREPARATION OF FUNGAL INOCULUM

Pellets of *P. ostreatus* were produced using an adaptation of a previously reported method [247]: a mycelia suspension was obtained by inoculating four 1 cm² plugs taken from the growing zone of fungus on MEA plate in 500 mL Erlenmeyer flasks containing 150 mL of BRM (see paragraph 5.1.2.). Flasks were incubated at 25°C in agitated condition (135 rpm). After 5 days, a dense mycelia mass had been formed which was then separated from the culture medium and disrupted with a homogenizer. The resulting mycelia suspension was used to produce pellets inoculating 1 mL of suspension in 500 mL Erlenmeyer flasks containing 150 mL of BRM, which were then incubated at 25°C in agitated condition (135 rpm) for 5 days. The formed pellets were then separated from the medium with the help of a strainer and stored in sterile saline solution (0.85% NaCl) at 4°C. Pellets were then rinsed with sterile water and used in the degradation experiments.

7.2.2. HOSPITAL WASTEWATER

Hospital wastewater was collected from the main sewer manifold of Dr. Josep Trueta Universitary Hospital (Girona, Spain). The main characteristics of the wastewater (in mg/L) are: COD (350-500), TOC (150-260), N_NH₄⁺ (48-70), TSS (6.1-11.1). The conductivity was in the range 1195 – 1455 μ g/cm and the pH 8.2 \pm 0.1 [175]. The wastewater pH was adjusted to 4.5 before sterilization at 121°C for 30 min. Before using the wastewater, it was spiked with the selected pharmaceuticals (ATL, DCF and KTP) to give the final concentration of about 10 mg/L each and to allow its detection by HPLC.

7.2.3. ANALYTICAL PROCEDURES

Analysis of pharmaceutical compounds

Pharmaceuticals analyses were performed using a Dionex 3000 Ultimate HPLC equipped with a UV detector at 230 nm. The column temperature was 30°C and a sample volume of 20 μ L was injected from a Dionex autosampler. Chromatographic separation was achieved on a GraceSmart RP 18 column (250mm x 4 mm, particle size 5 μ m). For the quantification of DCF and KTP, the mobile phase consisted of acetic acid 6.9 mM adjusted to pH 4 (by NaOH) with 35% v/v acetonitrile, delivered isocratically at 1 mL/min. For the quantification of ATL, the mobile phase (A) of 10 mM ammonium acetate (pH 7) and mobile phase (B) acetonitrile were delivered at flow rate of 1.2 mL/min in a gradient elution (t=0min A=95%, t=20 min A=80%). The detection limit of ATL, DCF and KTP was calculated to be 125 μ g/L, 118 μ g/L and 125 μ g/L respectively.

Enzymatic assays

Laccase activity was assayed using a modified version of the method for the determination of MnP as described by Kaal et al. [248], based on the measure of absorbance variance at 468 nm at 30°C during 2 minutes in a Varian Cary 3 UV/VIS spectrophotometer. Reaction volumes were: 600 μ l of sample, 200 μ l of 250 mM sodium malonate at 4,5 pH and 50 μ l of 20 mM 2,6-dimethoxyphenol (DMP). The molar extinction coefficient of DMP was considered as 24.8 mM⁻¹cm⁻¹.

Manganese peroxidase activity was assayed using the method described by Kaal et al. [248].

Other analysis

Glucose concentration was determined using YSI 2700 D SELECT biochemical analyzer from Yellow Springs Instruments and Co. (Yellow Springs, OH, USA).

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

7.2.4. EXPERIMENTAL PROCEDURES

In the present chapter are presented results of different types of experiments, summarized in Figure 42.



Figure 42. Scheme of conducted pharmaceutical degradation experiments.

Degradation experiments in Erlenmeyer flasks

Degradation of the selected pharmaceuticals were carried out in 250 mL flasks containing about 200 mg of pellets (dry weight) and 50 mL of synthetic wastewater (8 g/L of glucose, 3.3 g/L of ammonium tartrate, 1.168 g/L of 2,2-dimethylsuccinate buffer, 10 and 100 mL, respectively, of a micro and macronutrient solution from Kirk medium [249]), which pH was adjusted to 4.5. ATL, DCF and KTP were added to the medium from a stock solution in ethanol (1000 mg/L) to give the final concentration of 10 mg/L each. The flasks were maintained in the dark, under orbital shaking (130 rpm) and at controlled temperature (25°C). Flasks inoculated with autoclaved pellets of *P. ostreatus* were used as heat killed control to measure the drug adsorption to fungal biomass. Every experiment was performed in triplicate and under sterile conditions. During experiments, 1 mL sample was withdrawn at different time points, filtered through a Millipore 0.45 μ m nylon filters and subsequently analyzed by HPLC.

Experiments with cytochrome P450 inhibitor

In order to study the involvement of cytochrome P450 system (cytP450) in the degradation process of the pharmaceuticals, these experiments were conducted as the previous, but with the addition of 1-aminobenzotriazole (ABT) 5mM as final concentration, a well-known cytochrome P450 inhibitor [250]. Every experiment was performed in triplicate and under sterile conditions.

Experiments with purified laccase

Laccase-mediated degradation experiments were performed in 100 mL Erlenmeyer flasks containing 20 mL of synthetic wastewater (see above) and commercial purified laccase (Lac) at a final enzyme activity of 500 U/L (pH 4.5). Also in this case pharmaceutical compounds were added to the final concentration of 10 mg/L each and the flasks were incubated in the dark on orbital shaker (130 rpm, 25°C). Experiments were conducted with and without the addition of 1-Hydroxybenzotriazole (HBT) 1mM as laccase mediator [251]. At designed times, samples were taken and 10% of acetic acid was added to stop the reaction before the analysis by HPLC. Experiments were performed in triplicate.

Degradation experiments in fluidized bioreactor

A glass fluidized bed bioreactor with a working volume of 1500 mL was used for the batch and continuous pharmaceuticals degradation process. Fluidized conditions were maintained by air pulses generated by an electrovalve [174], with an air flow of 12 L/h. The bioreactor was equipped with a pH controller in order to maintain the pH at 4.5 by adding NaOH or HCl (1M) and the temperature was maintained at 25°C. Bioreactors were autoclaved at 121°C for 30 min.

Two experiments in bioreactors were performed. In the case of Reactor 1, it was operated in discontinuous (fed-batch) mode with synthetic wastewater (as described for experiments in flasks, see above) during 7 days, and inoculated with approximately 158 g of pellets, equivalent to a biomass concentration of 3.5 g dry weight/L. On the contrary, in Reactor 2, fed with a continuous flow of real autoclaved hospital wastewater (see 7.2.2.) for 32 days, this value was slightly higher (205 g of pellets, equivalent to 3.8 g dry weight/L). Glucose and ammonium tartrate were fed continuously from a stock solution (at concentrations of 100 g/L and 1 g/L, respectively) to maintain the biomass, at an approximated rate of 100 $mg \cdot g^{-1}DW$ biomass $\cdot d^{-1}$ in the case of glucose and at 10 $mg \cdot g^{-1}DW$ biomass $\cdot d^{-1}$ in the case of ammonium tartrate. These rates were evaluated in separate tests as the minimum rate required for maintenance (data not shown). In continuous treatment the sterilized wastewater (spiked with 10 mg/L of each drug) was fed continuously, and influent flow rate was adjusted to provide a hydraulic retention time (HRT) of 1.63 d or 3 d, depending on the experiment stage. The biomass, in pellet form, was retained in the bioreactor throughout the experiment with no loss in the effluent. Samples from the liquid phase in both treatments were collected and filtered through Millipore 0.45 µm nylon filters for further analyses.

7.3. RESULTS AND DISCUSSION

7.3.1. DEGRADATION EXPERIMENTS IN ERLENMEYER FLASKS

The involvement of two of the main enzymatic systems of *P. ostreatus* on the degradation of the three selected pharmaceuticals was investigated. These are the cytochrome P450 monooxygenase system and the laccase system.

To test whether cytP450 played a role on the degradation, an inhibitor (1-ABT) was added to the synthetic wastewate in order to reduce its activity. Heat killed control was also added to assess the pharmaceuticals removal by adsorption on the biomass. Time-course of the pharmaceuticals concentration in the experiments is shown in Figure 43.



Figure 43. Time course of ATL (A), DCF (B), KTP (C) concentrations during degradation experiment. Symbols: heat-killed control (□), inhibitor-free controls (●), and cultures containing ABT 5mM (△).

The results show that about 30% of DCF is removed by adsorption on the biomass while for ATL and KTP, this phenomenon is negligible.

Published works reported that DCF and KTP were highly stable in uninoculated experiments performed in similar conditions [242,243]. These authors also reported that the adsorption of DCF and KTP on *Trametes versicolor* biomass were 47% and 15%, respectively, obtained from the heat killed control, but these percentages were reduced to 10% and 0%, respectively, when sodium azide was used to inactivate biomass by blocking active transport across membrane or vesicular pathways.

For what concerns ATL our tests demonstrated that, in presence of the fungus, a partial transformation was observed (40%), with a similar behavior in both inhibited and inhibitor-free flasks. This probably means that degradation of ATL does not involve cytP450. Similar results are obtained for KTP, whereas a little difference exists between inhibited and inhibitor-free cultures, may indicating some involvement of cytP450 family.

A rapid and almost complete degradation (> 98% in three days) is observed instead for DCF in inhibitor-free flasks, and a slower decrease is obtained in inhibited flasks. These results are slightly different from the ones obtained in a previous study with *T. versicolor* [243], in which it was observed a steep decrease in both inhibited and inhibitor-free flasks.

This behavior indicates that cytP450 may play a role in degradation of DCF by *P*. *ostreatus*.

7.3.2. DEGRADATION EXPERIMENTS WITH PURE LACCASE

These experiments were conducted on synthetic wastewater with the purpose of studying the role of extracellular laccase commonly expressed by *P. ostreatus* [83,252] in the degradation process. As can be observed in Figure 44 ATL is degraded up to complete removal in 1h, both with and without the redox mediator. In the case of DCF, 4h were necessary to reach full degradation, but this time could be extremely reduced by adding a mediator. On the contrary Lac seems to be not able to degrade KTP even in presence of the mediator. Since in vivo experiments led to a removal of this compound, probably the degradation pathway involves other enzymatic systems.

It is necessary to consider that HBT is, in the concentration used, potentially toxic and too expensive [217] to be used in a full scale-plant. Despite this, these results indicates that a possible way *P. ostreatus* uses to degrade ATL and DCF is through a laccase-mediated process.



Figure 44. Time course of pharmaceuticals concentration during experiments with purified laccase without (ATL: ■; DCF: •; KTP: ▲)and with HBT 1mM (ATL:□;DCF: •; KTP:△).

7.3.3. DEGRADATION EXPERIMENTS IN BIOREACTOR

To assess DCF, KTP and ATL degradation by pellets of *P. ostreatus* in a bioreactor, two degradation experiments in air-pulsed fluidized bed bioreactors were performed. The air pulses guaranteed the homogeneous distribution of the pellets within the liquid phase without mechanical stress.

In the first experiment, the reactor worked with synthetic wastewater (see 7.2.4.) for 7 days. As observed in Figure 45, DCF was completely removed in few hours while KTP and ATL were removed only of 36% and 8%, respectively, after 2 days. Similar results were obtained when a new pulse of pharmaceuticals (vertical line in Figure 45) were added to the bioreactor at day 2 in order to increase at 10 mg/L the pharmaceuticals concentration. The ATL degradation was negligible during the 2 first days but it increased up to 25% 5 days after the addition. On the contrary, DCF was completely removed after 1d, while 39% of the KTP was removed after 2 days, and up to 50% at the end of experiment. Enzymatic assays evidenced very low extracellular laccase activity (< 2 U/L), although a slight improvement (5 U/L) after the second addition of pharmaceuticals was detected. No MnP activity was detected. The glucose concentration was always below 20 mg/L, since it was dosed at the same rate of consumption, as already mentioned.



Figure 45. Time course of pharmaceuticals concentrations and laccase activity in the first reactor operating in fedbatch mode. Symbols: ATN (O),DCF (△),KTP (▽), and laccase activity (■)

In the second reactor, after 7 days of batch process, a continuous feeding of wastewater (see 7.2.2.) was shifted to operate at 1.63 d of HRT. Figure 46 depicts the pharmaceuticals concentration profiles in the bioreactor. During the batch stage, DCF was removed completely after 2 days, KTP was removed up to 90% after 7 d while ATL concentration was scarcely reduced (< 20%). In addition a peak of laccase was detected after 6 days. During the continuous treatment the DCF was fully removed while the KTP removal was about 70% after day 15, when the hydraulic steady state had been reached. Again no ATL degradation was achieved. Although the laccase activity oscillated significantly, a level of approximately 7 U/L was reached in the steady state. In order to improve the degradation level of KTP and ATL, the HRT was increased to 3 days at day 20. The KTP removal was increased up to 85% and also the ATL starts to be removed, reaching levels higher than 80% at the new hydraulic steady state (day 32). During this period the laccase activity was maintained at high level but the biomass concentration was 8.75 g DW/L, which means an increase of 130%, comparing to the initial inoculum. With such high level of biomass too severe operational problems were came across, such as lack of pellets dispersion, blockage of the out ports, accumulation on the bottom and upper part of the bioreactor, which forced the end of the experiment. The observed growth must be caused by a lack of accurate control on the nutrient feeding pumps. Such problems can be very common in air-lift reactors [188].



Figure 46. Time course of pharmaceuticals concentration and laccase activity during the second reactor (operating in continuous mode). Symbols: ATL (O), KTP (∇), DCF (△), and laccase activity (■).

7.4. CONCLUSIONS

The present chapter described the research conducted about fungal biodegradation of three pharmaceutical compounds: a β -blocker, Atenolol and two non-steroidal anti-inflammatory drugs, Ketoprofen and Diclofenac. Experiments were performed in flasks, in order to investigate the involvement of two of the main enzymatic systems of *P. ostreatus* (the cytochrome P450 monooxygenase system and the ligninolytic system of the white-rot fungi), and then in sterile lab-bioreactors.

Erlenmeyer tests pointed out that Diclofenac is degraded very fast by alive fungus, while, when the cytP450 system was inhibited the removal rate was slower. This behavior indicates that cytP450 may play a role in degradation of DCF by *P. ostreatus*. Also laccase alone is able to degrade DCF, even faster if in presence of a chemical mediator.

On the contrary, KTP cannot be degraded by pure laccase, not even with the addition of a redox mediator, while, in presence of the fungus, a little difference exists between inhibited and inhibitor-free cultures, may indicating some involvement of cytP450 family.

For what concerns ATL our tests demonstrated that, in presence of the fungus, a partial degradation was observed, with a similar behavior in both inhibited and inhibitor-free flasks. This probably means that degradation of ATL does not involve cytP450, whereas probably involves laccase, which is able to efficiently degrade ATL, even without a mediator. Therefore, in fungal cultures and/or in bioreactors, it is necessary to maintain the fungus under conditions promoting laccase production in order to reach high levels of ATL degradation.

Experiments in bioreactors showed that *Pleurotus ostreatus* is able to degrade ATL, KTP and DCF in a real hospital wastewater in a fluidized bed bioreactor. During the batch stage, DCF was removed completely, KTP was removed up to 90% while ATL concentration was scarcely reduced (< 20%). During the continuous treatment the DCF was fully removed, the KTP removal was about 70%, while again no ATL degradation was detected. When the HRT of the system was increased from 1.63 d to 3 d, DCF was still completely removed, KTP removal was increased up to 85% and also the ATL was removed up to 80%. Both HRTs tested are relatively low if compared to the ones generally employed in fungal processes [176,227] and in line with the ones used for the treatment of petrochemical wastewater shown in paragraphs 6.6 and 6.7.

It is important to underline that the fungus was maintained active without operational problems for almost 30 days, when nutrient pumps disarrangement caused excess biomass growth and consequently the system collapsed. This represents a weakness of the bioreactor configuration: indeed, the absence of a biomass removal system can cause severe operational problems in case of fungal growth. However, the tested configuration was able to obtain good removal rate of the three selected drugs.

Further researches could involve the study of the behavior in non-sterile environment, the degradation abilities towards more realistic pharmaceutical concentrations, cost evaluation of the process and toxicity assays on the effluent.

SECTION III CONCLUSIONS AND FUTURE PERSPECTIVES

8. CONCLUDING REMARKS

In the present thesis the potential of some fungal processes in the field of wastewater treatment has been studied.

In particular, the ability of the white-rot fungus *Pleurotus ostreatus* to degrade naphthalene sulphonated polymers contained in a petrochemical wastewater has been demonstrated. Erlenmeyer flasks experiments showed that *P. ostreatus* was able to depolymerize high molecular weight polymers into simpler and more biodegradable molecules (such as monomers and dimers) in a co-metabolic process. The process was then scaled-up into lab trickling filters with fungi attached to biodegradable supports, in non-sterile conditions. These reactors was coupled with an activated sludge process and the combined treatment allowed to remove polymers and COD from wastewater. It is important to underline that nowadays very few cases of long-term operations with fungi on real wastewaters in non-sterile environment can be found in literature and, therefore, this work contributes to the development of fungal processes suitable for full-scale application.

Another successfully tested process was the use of extracellular crude extract, produced by *P. ostreatus* attached on straw, containing laccase and natural mediators, for the degradation of NSAP.

P. ostreatus was also successfully employed for the degradation of selected pharmaceuticals in a sterile fluidized bed bioreactor during a stay at the Autonomous University of Barcelona.

Research conducted so far allowed to evaluate two different approaches to wastewater treatment with fungi: the use of whole mycelia cells (as in trickling filters and fluidized bed bioreactors) and the use of extracellular crude extracts. It is important to underline that every tested process can be used for the treatment of theoretically every contaminated stream (e.g. textile wastewater, landfill leachate, etc.), adapting the conditions case by case (e.g. fungal strain, presence of additional carbon source, etc.).

The use of crude extracellular extracts present several advantages: by producing the extracts in a side-stream reactor and adding them into the main-stream, no significant changes in the main line must be done in order to implement this technology in an existing plant. Moreover, if the side reactor is designed to maximize enzyme expression, its volume can be considerably smaller and easier to manage than a fungal reactor treating the whole flow rate of the plant. On the other hand, the cost of this solution must be evaluated carefully, because it is strictly dependent on the amount of enzyme needed and on which

growth substrate is used in the side stream fungal reactor. Moreover, the presence of nonbiodegradable compounds in the extracellular extracts must be carefully evaluated.

Among reactors using whole fungal cells, fluidized bed reactors were used in the present work for the degradation of some selected drugs. These reactors are easy to design and manage, and are highly resistant to shocks, due to the complete mixing, but, as evidenced in the experimental work, often present problems of clogging, due to the morphology that filamentous fungi assume growing as free cells. The other type of reactor tested, the trickling filter, is more complicated to design and manage and also have problems of clogging, but presents the advantage of having submerged, partially submerged and nonsubmerged supports, which can enhance the biodegradation process. In particular, the tested trickling filters had biodegradable supports, which acted at the same time as growth substrate and physical support for fungi, enhanced enzymatic and natural mediator production and helped prevent bacterial overgrowth inside the reactor. On the other hand, biodegradable supports can cause operational problems due to clogging (their structural behavior change during time), and if they are not completely biodegradable by the fungus the process will generate some solid residuals to dispose of and/or non-biodegradable byproducts in the effluent (as in the case of straw). Moreover new supports must be periodically added to the reactor and evaluation of addition techniques and costs must be done in order to scale up the process.

For what concerns interactions with bacteria, one of the main issue of every fungal reactor is how to design an ecosystem populated mainly by fungi. In the present work this goal was achieved by the use of selective carbon source (lignocellulosic materials), but some other solutions could be found, for example by employing a fungal strain which can use the recalcitrant compound as carbon source (avoiding possible competitions), or maintaining low concentrations of soluble carbon source for which fungi may have higher affinity than bacteria.

Finally, a possible way to integrate fungal treatment with existing activated sludge processes has been tested. The synergy between fungi and activated sludge is widely reported in literature and in the present work allowed to remove COD and polymers that could not be removed by one process only. In the present work fungi were used as a pre-treatment of activated sludge. This strategy presents several advantages because if a suitable carbon source is present in the wastewater it can be directly used by fungi and the

need of external addition could be neglected. Moreover, if fungi partially degrade the pollutants into simpler molecules and/or release biodegradable by-products in the effluent, these can be easily degraded in the second stage by activated sludge. On the other hand, using fungi as a post-treatment of activated sludge could reduce bacterial competition in the fungal stage, because all the biodegradable COD has already been removed and only the recalcitrant compounds are left in the stream.

To conclude, it is possible to state that, even if several aspects still need to be properly set up, the research conducted so far indicate fungal processes as a promising alternative to physical-chemical treatments for the removal of toxic recalcitrant contaminants from wastewater and thus for the reduction of their releases into the environment.

9. FUTURE PERSPECTIVES

In order to promote the use of fungal processes at full-scale, further researches should focus on:

- Process optimization in terms of process parameters such as T, HRT, pH, C/N ratio.
- Design and production of completely biodegradable carriers, which would not release non-biodegradable by-products in the effluent and would not give rise to solid residuals.
- Understanding the mutual interactions between fungi and other organisms present in the reactor and their possible synergy in the degradation of pollutants.
- Studying kinetics of selected fungi, since several useful values such as the specific rates of substrate consumption, oxygen utilization and enzyme production are seldom found in literature.
- Comparing the use of fungal processes as pre- or post- treatment of activated sludge, in terms of effluent quality and treatment cost.
- Evaluating the fungal process effluent in terms of color, toxicity, and, where possible, process by-products.
- Scaling up the process into pilot plants in order to investigate the behavior of the process in more realistic conditions and thus to better evaluate the overall treatment cost.

NOMENCLATURE

- 2,4,5-T: 2,4,5-trichlorophenoxyacetic acid
- 2,4-D: 2,4-dichlorophenoxyacetic acid
- ABT: Aminobenzotriazole
- ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
- AOP: Advanced Oxidation Process
- APO: Aromatic Peroxygenase
- AS: Activated Sludge
- ATL: Atenolol
- bCOD: Biodegradable COD
- BOD: Biochemical Oxygen Demand
- BRM: Basidiomycete Rich Medium
- CDH: Cellobiose dehydrogenase
- COD: Chemical Oxygen Demand
- CSTR: Continuous Stirred Tank Reactor
- cytP450: Cytochrome P450
- DCF: Diclofenac
- DDT: 1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane
- DEET: Diethyltoluamide
- DMAB: 3-(dimethylamino)-benzoic acid
- DMP: 2,6-dimethoxyphenol
- DO: Dissolved Oxygen
- DW: Dry Weight
- DyP: Dye Peroxidase
- EDC: Endocrine Disrupting Chemical
- FBBR: Fixed Bed Bioreactor
- GAC: Granular Activated Carbon
- GOD: Glucose Oxidase
- HBT: 1-Hydroxybenzotriazole
- HPLC: High Performance Liquid Chromatography
- HRT: Hydraulic Retention Time
- KTP: Ketoprofen

- Lac: Laccase
- LC-MS: Liquid Chromatography Mass Spectrometry
- LiP: Lignin Peroxidase
- LME: Lignin-Modifying Enzyme
- LTQ: Linear Trap Quadrupole
- MBBR: Moving Bed Biofilm Reactor
- MBfR: Membrane Biofilm Reactor
- MBR: Membrane Bioreactor
- MBTH: 3-methyl-2-benzothiazolinone hydrazone
- MEA: Malt Extract Agar
- MnP: Manganese Peroxidase
- MPs: Micro-Pollutants
- nbCOD: Non-Biodegradable COD
- NSA: Naphthalene-Sulfonic Acid
- NSAID: Non-Steroidal Anti-Inflammatory Drug
- NSAP: Naphthalene-Sulfonic Acid Polymer
- OMW: Olive mill wastewater
- ORP: Oxidative-Reductive Potential
- OUR: Oxygen Uptake Rate
- PAH: Polycyclic Aromatic Hydrocarbon
- PBR: Packed Bed Reactor
- PCB: Polychlorinated Biphenyl
- PCDD: Polychlorinated dibenzo-*p*-dioxin
- PCDF: Polychlorinated dibenzofuran
- PCE: Perchloroethylene
- pCOD: Particulate COD
- PPCP: Pharmaceuticals and personal care products
- PU-DSCM: Polyurethane-Dye Sludge Carbonaceous Material
- PUF: Polyurethane Foam
- PVAL: Polyvinyl alcohol
- RBC: Rotating Biological Contactor
- SBR: Sequencing Batch Reactor

- sCOD: Soluble COD
- SRT: Sludge Retention Time
- T: Temperature
- TBAB: Tetrabutylammonium bromide
- TCE: Trichloroethylene
- TEMPO: 2,2,6,6-tetramethyl-1-piperidinyloxyl
- TN: Total Nitrogen
- TNT: 2,4,6 Trinitrotoluene
- TOC: Total Organic Carbon
- TrOC: Trace Organic Compound
- TSS: Total Suspended Solids
- UASB: Upflow Anaerobic Sludge Blanket
- VA: Veratryl alcohol, 3,4-dimethoxy benzyl alcohol
- WRF: White-Rot Fungi
- WWTP: Wastewater Treatment Plant

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