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Involvement of Lignin-Modifying Enzymes in the Degradation of Herbicides

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1. Introduction

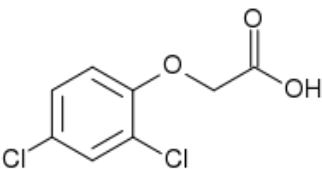
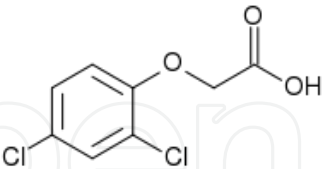
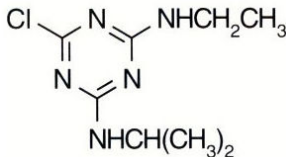
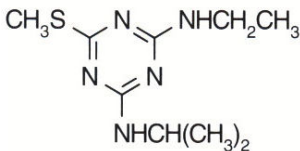
The high demand for food due to the increase in the world population has led to an increasing use of plant protection products, also known as pesticides, in order to improve productivity. However, along with the success in food production, the accumulation of these persistent chemicals in soil and water is harmful to the environmental and human health [1]. In recent years, agricultural pesticide application has increased all over the world. A total of 5,197 mil lbs of pesticides were used worldwide in 2007 [2]. There are many different types of pesticides; each is meant to be effective against specific pests. The term "-cide" comes from the Latin word "to kill." Among them, herbicides account for the largest market (around 40%) share, followed by insecticides (17%) and fungicides (10%). Herbicides are chemicals used to kill undesired plants, such as weeds, and they are used extensively in home gardens and in agriculture. Due to the relative predominance of herbicides, the present review will focus on this class of compounds.

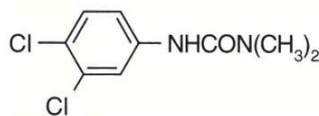
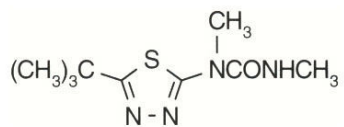
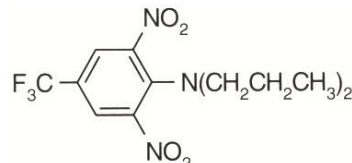
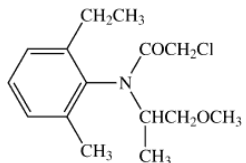
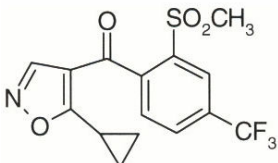
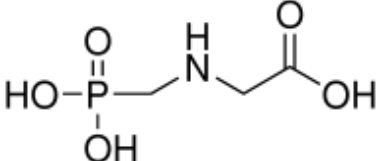

Herbicides have well defined pros and cons associated with their use. Their use tends to increase yields, and thus makes a significant difference in food production, particularly in countries that struggle periodically against famines. On the other hand, they can cause water pollution when erosion and/or rainwater carry the chemicals off the farms together with the eroded soils after each rainfall. Herbicides vary in their potential to persist in the soil. They are chemically heterogeneous and their structure is one of the main features that determine persistence. For example, some substitutions on aromatic rings (-F, -Cl, -NO₂, -NH₂, -CF₃, -

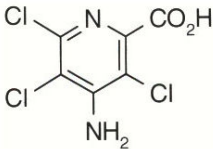
SO₃H) [1] in picloram, atrazine, flazasulfuron and other herbicides (Table 1), have a marked influence on persistence. Furthermore, water insoluble molecules are less susceptible to microbial attack because they may sorb tightly to particles and thus stay less available [3].

The herbicide persistence in soil is also affected by the properties of soil such as organic matter content, moisture, pH, microbial activity, among other particularities. Generally, soils with high contents in organic matter have low loss of chemicals through volatilization as a consequence of a stimulated adsorption to soil particles [3]. On the other hand, herbicides that sorb tightly to soil particles have low mobility which decreases the possibility of groundwater contamination. The organic matter also serves as an energy source for microorganisms that degrade pesticides. Furthermore, the soil pH may affect the adsorption processes, changing the mobility and bioavailability of the herbicide [1].

The presence of native microorganisms (fungi, bacteria, protozoans, etc) can greatly influence the toxicity and persistence of herbicides. The degradation process by native microorganisms produces less toxic products and it is probably the most important pathway for the breakdown of herbicides. However, microorganisms require special environmental conditions such as adequate temperature, oxygen and nutrient supply for growth and herbicide degradation [1].

Chemical group	Common name	Water solubility and persistence	Chemical structure
Phenoxy acetic	2,4 D	900 ppm 1 month	
	2,4,5 T	278 ppm 1 month	
Triazines	Atrazine	33 ppm (22°C) 5-7 months	
	Ametryne	185 ppm (20°C) 3-6 months	

Chemical group	Common name	Water solubility and persistence	Chemical structure
Ureas	Diuron	42 ppm 4-8 months	
	tebuthiuron	2,500 ppm 12-15 months	
dinitroanilines	Trifluralin	0.3 ppm 6 months	
acetanilides	metolachlor	488 ppm (20°C) 50 days	
isoxalone	isoxaflutole	6 ppm (20°C) 38 days	
Glycine derivative	Glyphosate	15,700 ppm 30-90 days	
imidazolinones	Imazapyr	11,272 ppm 3-6 months	

Chemical group	Common name	Water solubility and persistence	Chemical structure
Picolinic acid	Picloram	Soluble 3 months	

* Water solubility at 25°C. ** Soil average persistence at recommended rates [4]

Table 1. Chemical properties of the most common herbicides

2. General bioremediation processes

The impact of hazardous herbicide residues on the environment has led to the necessity of finding feasible technologies to remediate these sites. The conventional remediation methods use physical and chemical processes, such as incineration, adsorption on resins and UV irradiation. These methods generally generate excellent contaminant removal; however, from an ecological viewpoint, they are not friendly since they produce unwanted by-products and hazardous residues, besides the danger of human exposure to contaminants.

An innovative technology for complementing or substituting the conventional methods and which presents the same or even improved efficiency is bioremediation. By definition, bioremediation is the use of biological processes to clean up polluted sites. Such biological methods have the potential of being less expensive and more eco-friendly than the physical and chemical treatments [5]. Bioremediation is based on biological systems such as living organisms (bacteria, fungi and plant) and enzymes. They are effective systems to treat a polluted site because they are able to modify the chemical structure of the contaminant into less hazardous end-products [5]. Among these possibilities, microorganisms have become the most attractive option in bioremediation strategies [1].

The naturally occurring bacteria, fungi or plants in a contaminated site are commonly used in bioremediation but microorganisms may be isolated elsewhere and inoculated into the site with the goal of accelerating the remediation. This process is called bioaugmentation [6]. Other terms are commonly used in bioremediation studies. When a chemical compound is broken down into water and CO₂, the biodegradation is completed and the process is called mineralization. On the other hand, the term biotransformation usually refers to the process whereby a molecule is converted into a different molecule which is not always less toxic than the precursor. Another term usually used is bioaccumulation or biosorption. This process does not involve any transformation of the molecule and refers to the concentrative transfer of substances into living or dead biomass. The microorganisms can still transform the molecule but are unable to grow on it, in other words, the compound is not used as an energy source [7].

Bioremediation can be developed by two main methods: in situ and ex situ. In situ methods treat the materials in place, while ex situ methods are based on excavation or removal of the polluted materials for processing in another place, such as in bioreactors and biopiles, where optimal environmental conditions for rapid biodegradation may be provided. The in situ bioremediation is more advantageous because it avoids the transport of hazardous materials and is more cost-effective. On the other hand, this process can be limited in some cases since the remediation is not effective in low-permeability soils [1].

3. Herbicide bioremediation studies

Bacteria and fungi are the main microorganisms capable of degrading herbicides. Most bioremediation studies have been focused on bacteria. Among those, several species of *Pseudomonas* have been found to degrade a wide range of herbicide groups [8-10]. Generally, the bacteria strains utilize the herbicide as the sole carbon and/or nitrogen source. In order to metabolize pollutants, bacteria must take the molecule inside the cell where the enzymes are located [11].

Although bacteria are the main agents used in most commercial bioremediation processes, filamentous fungi have received much attention in recent years, especially white rot fungi (WRF) or ligninolytic fungi [12]. Most WRF belong to the Basidiomycete group and comprise the organisms that are capable of degrading lignin, a polymer found in wood [13]. These fungi have been extensively investigated since the mid-1980s as bioremediation agents because of some particular characteristics. First, as filamentous fungi, WRF present extensive branching and mycelial growth, hence they are able to spread out in the environment reaching the pollutants more efficiently than bacteria. Second, they possess non-specific and radical-based enzymatic systems for degrading lignin. These oxidative enzymes allow them to degrade a broad range of pollutants, including low-solubility compounds, without the necessity of incorporating them into the cells. Third, WRF prefers to grow on substrates such as agricultural crop wastes (straw, corn cobs, sawdust, peanut shells) which are inexpensive and may be used as additional nutrient sources [5,14]. In this context, the primary focus of this review is on the main white-rot species which have been studied for herbicide transformation.

4. White-rot fungi and their lignin-modifying enzymes

White-rot fungi are known as the most efficient lignin degraders. Lignin is a polymer found in wood and vascular tissues [15]. In the cell wall, lignin is covalently associated with carbohydrate components (cellulose and hemicelluloses) forming a matrix that protects the cell wall against microbial attack [16]. Unlike most natural polymers, lignin is irregular and non-repeating. Its biosynthesis is the result of oxidative polymerization of several phenylpropanoid precursors, such as coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol. The polymerization occurs at random by various carbon-carbon and ether bonds resulting in an irregular structure (Fig. 1)

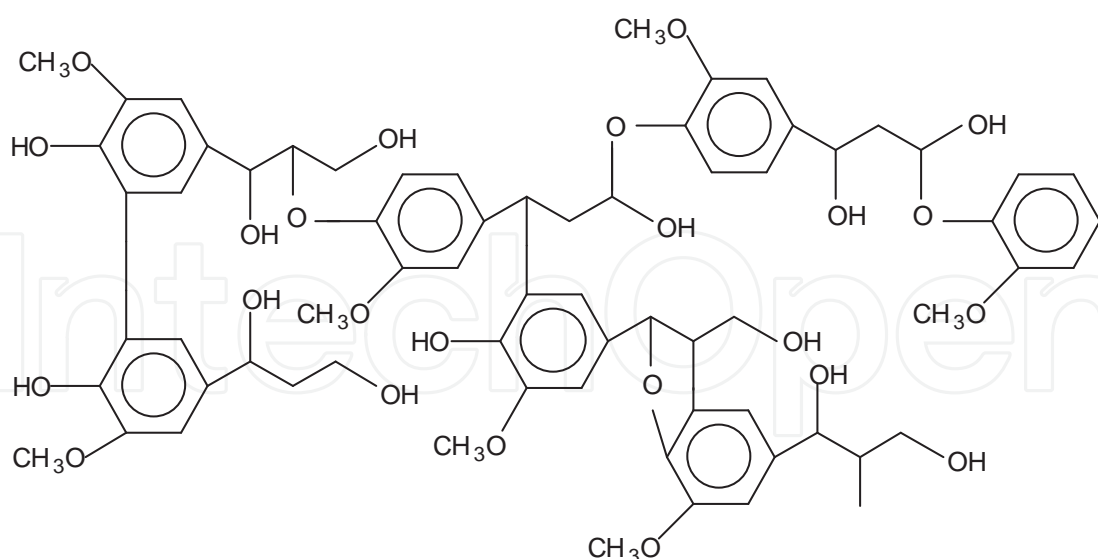


Figure 1. General structure of lignin

Lignin resists attacks by most microorganisms; anaerobic processes tend not to attack the aromatic rings at all and the aerobic breakdown of lignin is slow. In nature, only basidiomyceteous white-rot fungi (WRF) are able to degrade lignin efficiently. Wood decayed by white-rot fungi is pale in color because of oxidative bleaching and loss of lignin and often retains a fibrous texture.

In order to become the most effective wood degraders in nature, WRF developed non-specific and radical-based mechanisms for degrading lignin. WRF variously secrete one or more of four extracellular enzymes that are involved not only in the lignin degradation but also in the degradation of several pollutants. The four major lignin-modifying enzymes (LMEs) are: lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16) and laccase (Lac, EC 1.10.3.2) [17]. All these enzymes act via generation of free radicals which represent an efficient way to reach their substrates.

The LMEs are highly non-specific and, thus, capable of degrading a wide range of recalcitrant compounds structurally similar to lignin. White-rot fungi usually secrete one or more of the LMEs in different combinations. Distribution of white-rot fungi into groups according to their enzymatic systems have been undertaken [18-20]. General classification is based on the capacity of different fungi to produce one or a combination of three ligninolytic enzymes: LiP, laccase and MnP. However, the absence or non-detection of these enzymes in some white-rot fungi, the sequencing of white-rot fungal genomes and the discovery of new enzymes has led to the distinction of different groups [21]. Generally, white-rot fungi can be distributed into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP and VP):

- a. Laccase, MnP and LiP (*Trametes versicolor*, *Bjerkandera adusta*)
- b. laccase and at least one of the peroxidases (*Lentinus edodes*, *Pleurotus eryngii*, *Ceriporiopsis subvermispora*)

- c. only laccase (*Schizophyllum commune*)
- d. only peroxidases (*Phanerochaete chrysosporium*)

The most frequently observed LMEs among the white-rot fungi species are laccases and MnP and the least are LiP and VP.

A considerable number of reviews detailing the numerous characteristics and applications of ligninolytic enzymes have been published in past years [22-24]. A general description of the mechanisms and functions of the main ligninolytic enzymes of the white-rot fungi is given in Panel 1.

Enzyme: LACCASE (EC 1.10.3.2)

Active site/prosthetic group: four copper atoms.

Catalytic mechanism: catalysis of the one electron oxidation of phenolics, aromatic amines and other aromatic hydrogen donors with simultaneous reduction of O₂ to H₂O.

General reaction: 4 benzenediol + O₂ → 4 benzosemiquinone + 2 H₂O

Mediators: 3-HAA, ABTS, HBT, RBB

Function: Removal of polyphenols, degradation of pesticides and textile dyes, bleaching, removal of lignin, synthesis of melanin, formation of basidiomes, generation of hydroxyl radicals, stress defense

Main laccase producer fungi: *Ganoderma lucidum*, *Pleurotus eryngii*, *Pleurotus ostreatus*, *Rigidoporus lignosus*, *Trametes versicolor*

Enzyme: LIGNIN PEROXIDASE (LiP, EC 1.11.1.14)

Active site/prosthetic group: Heme (Iron protoporphyrin IX)

Catalytic mechanism: oxidation of lignin side chains by one electron abstraction in the presence of H₂O₂, generating reactive radicals. Cleavage of C-C and ether bonds in lignin. Cleavage of aromatic rings.

General reaction: 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H₂O₂ → 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H₂O

Mediators: VA, 2Cl-14DMB

Function: Degrades lignin (non-phenolic lignin units), mineralizes recalcitrant aromatic compounds.

Main LiP producer fungi: *Bjerkandera adusta*, *Phanerochaete chrysosporium*, *Trametes cervina*, *Trametes versicolor*

Enzyme: MANGANESE PEROXIDASE (MnP, EC 1.11.1.13)

Active site/prosthetic group: Heme (Iron protoporphyrin IX)

Catalytic mechanism: Oxidize Mn²⁺ into Mn³⁺. Mn³⁺ acts as a diffusible oxidizer on phenolic or non-phenolic lignin units and amino-aromatic compounds

General reaction: 2 Mn²⁺ + 2 H⁺ + H₂O₂ → 2 Mn³⁺ + 2 H₂O

Mediators: Chelated Mn³⁺

Co-oxidants: unsaturated fatty acids and thiols

Function: Oxidation and depolymerization of lignin, degradation of dyes, phenols and other recalcitrant compounds

Main MnP producer fungi: *Ceriporiopsis subvermispota*, *Irpex lacteus*, *Lentinus edodes*, *Pleurotus ostreatus*, *Trametes versicolor*

Enzyme: VERSATILE PEROXIDASE (VP, EC 1.11.1.16)

Active site/prosthetic group: Heme (Iron protoporphyrin IX). Hybrid molecular architecture with multiple binding sites
 Catalytic mechanism: Combination of the catalytic properties of LiP and MnP; Oxidation of Mn^{2+} to Mn^{3+} and also phenolic and nonphenolic aromatic compounds.

General reaction: donor + $H_2O_2 \rightarrow$ oxidized donor + 2 H_2O

Mediator: Chelated Mn^{3+}

Function: Oxidizes phenols, hydroquinones, dyes, amines, aromatic alcohols and xenobiotics.

Main VP producer fungi: *Bjerkandera adusta*, *Pleurotus eryngii*

3-HAA (3-Hydroxyanthranilic acid), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)), HBT (1-hydroxybenzotriazole), RBB (Remazol brilliant blue), VA (veratryl alcohol), 2Cl-14DMB (2-Chloro-1,4-dimethoxybenzene).

Panel 1. Lignin-modifying enzymes of the white-rot fungi [17]

The LMEs may function cooperatively or separately from each other, however, auxiliary enzymes (unable to degrade lignin on their own) are necessary to complete the process of lignin and/or xenobiotics degradation: aryl alcohol oxidase (AAO, EC 1.1.3.7), aryl alcohol dehydrogenase (AAD, EC 1.1.1.90), glyoxal oxidase (GLOX, EC 1.2.3.5), quinone reductase (QR, EC 1.1.5.1), cellobiose dehydrogenase (CDH, EC 1.1.99.18), superoxide dismutase (SOD, EC 1.15.1.1), glucose 1-oxidase (GOX, EC 1.1.3.4), pyranose 2-oxidase (P2Ox, EC 1.1.3.4) and methanol oxidase (EC 1.1.3.13). These are mostly oxidases generating H_2O_2 and dehydrogenases. Cytochrome P450 monooxygenases are also significant components involved in the degradation of lignin and many xenobiotics.

Recent additions to the enzymatic system of white-rot fungi include dye-decolorizing peroxidases or DyP involved in the oxidation of synthetic high redox-potential dyes and nonphenolic lignin model compounds [30] and aromatic peroxygenases (APOs) that catalyze diverse oxygen transfer reactions which can result in the cleavage of ethers [31-32].

Other important components of the lignin degradative system of white-rot fungi are low molecular mass oxidants such as hydroxyl radicals ($\bullet OH$) and chelated Mn^{3+} (Mn^{3+} mainly chelated by oxalic acid), produced through the action of the ligninolytic enzymes. These are particularly important during the early stages of wood decay and can also act on the degradation of xenobiotics [24,33-35].

5. Environmental applications of ligninolytic enzymes

Ligninolytic enzymes are highly non-specific and, thus, capable of degrading a wide range of recalcitrant compounds structurally similar to lignin. In recent years, the capability of white-rot fungi and their enzymes to biodegrade several xenobiotics and recalcitrant pollutants has generated considerable research interest in the area of industrial/environmental microbiology. As a consequence, a considerable number of reviews detailing the numerous characteristics and applications of ligninolytic enzymes have been published [22-27,36-40]. Among the industrial applications of the ligninolytic enzymes found in WRF are: a). paper whitening, b). degradation of industrial dyes from the textile industry and c). degradation of xenobiotic

compounds, including polycyclic aromatic hydrocarbons, phenolics, herbicides and other pesticides. In this review, we focused on the degradation of herbicides by white-rot fungi with emphasis on work done in the last decade.

6. Isolation and laboratory maintenance of white-rot fungi

There are about 10,000 species of white-rot fungi in the world. However, not more than 3 dozen have been more properly studied. Among these, the majority of studies have focused on *Phanerochaete chrysosporium*, followed by *Trametes versicolor*, *Bjerkandera adusta* and *Pleurotus sp* [40]. White-rot fungi have great potential for biotechnological applications. For this reason, there has been a growing interest in screening for new white-rot fungi species and strains. A classical strategy is to collect basidioma or mycelia of white-rot fungi in forests, dead trees, and lignocellulosic crop residues showing signs of attack by fungi. Samples of basidioma or mycelia are aseptically transferred onto potato dextrose agar or malt extract agar and sub-cultured until the obtainment of pure mycelia. Identification is based on morphological, physiological, biochemical and genetic characteristics of the basidioma, hyphae and spores. More recently, molecular biology techniques are being used in the identification of new isolates. Advancements in molecular methods have permitted a more rational study of the phylogenetic relationships within the various organisms. Non-coding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA seem to be one of the most frequently employed analytical tools [41].

There are several options for the maintenance of white-rot fungi in the laboratory. Continuous growth methods of preservation in which white-rot fungi are grown on agar (e.g., malt extract agar, potato dextrose agar and yeast extract agar) are typically used for short-term storage. For long-term storage, preservation in distilled water (method of Castellani [42]), mineral oil and anhydrous silica gel are some of the indicated methods. These are low cost methods but none is considered permanent. Lyophilization and liquid nitrogen refrigeration (cryo-preservation) are expensive methods but are considered permanent.

7. Herbicide biotransformation and detoxification by white-rot fungi

Phanerochaete chrysosporium is the main white-rot fungus used in herbicide biodegradation studies. The lignin-degrading system of *P. chrysosporium* comprises the peroxidases LiP and MnP. Studies have demonstrated that these enzymes are responsible, at least in part, for the transformation of several persistent pollutant molecules including toxic herbicides [43]. The biotransformation of triazine by *P. chrysosporium* was studied in liquid cultures [44]. The herbicide atrazine was partly transformed into polar dechlorinated and/or N-dealkylated metabolites. The removal of the ethyl side chain was the preferred reaction and produced metabolites which were not further transformed by the fungus. They found that the N-dealkylation was supported by the mycelium and not by purified peroxidases. Thus, the authors suggested that cytochrome-P450 could have been involved in the degradation.

Using non-sterile substrates, *P. chrysosporium* demonstrated a satisfactory growth and significant effect on the degradation of several herbicides such as simazine and trifluralin [45], bentazon and 2-methyl-4-chlorophenoxyacetic acid (MCPA) [46-47]. Thus, the systems based on non-sterile substrates offer good perspectives of practical application since the preparation of large amounts of sterile substrates is expensive.

The herbicide diuron has also been degraded by *P. chrysosporium* in several culture conditions. Higher degradation efficiency (95%) was found in ashwood culture as compared to the liquid culture (75%) [48]. The authors suggested that MnP could be responsible for the degradation since it was the dominant enzyme detected in the cultures. In liquid medium, the degradation performance of *P. chrysosporium* is not very good when compared with other white-rot species in the same experiments. Within 7 days the depletion of diuron, linuron and propanil was not higher than 34% [49]. In another work, no *Phanerochaete* strain was able to degrade more than 42% of the herbicides chlortoluron, isoproturon and diuron during 5 days [50]. However, *P. chrysosporium* was more efficient than *Trametes versicolor* in cleaving diketonitrile, which is derived from the herbicide isoxaflotole [51]. Enzyme preparations derived from these organisms, namely LiP and MnP, were unable to catalyze the transformation even in the presence of mediators.

Solid state cultures (SSC) seem to be great systems for cultivation of ligninolytic fungi as well as for herbicide degradation. Isoproturon [52] and bentazon [46-47] were extensively degraded by *P. chrysosporium* in wheat straw cultures. In vitro tests suggest the action of LiP and MnP in the degradation of both herbicides although the cytochrome P450 activity can not be ruled out. For isoproturon, non-identified hydroxylated metabolites were formed in SSC as well as in pure enzyme tests. Furthermore, a second degradation reaction seems to occur with isoproturon since N-demethylated metabolites were also formed in both systems.

Bjerkandera adusta is one of the most promising strains of white-rot fungi for bioremediation. This fungus possesses high ligninolytic activity and produces several extracellular lignin-degrading enzymes, including LiP, MnP and laccase [53]. Also, it is one of the few species to produce VP [54]. *B. adusta* has demonstrated a great ability to degrade pollutant molecules, especially polycyclic aromatic hydrocarbons [55] and synthetic dyes [56-57]. The removal of the herbicides chlortoluron, isoproturon and diuron by *B. adusta* and other fungi was reported in shaking liquid cultures [50], *B. adusta* was the most efficient WRF able to degrade the three herbicides. Removal of chlortoluron, diuron and isoproturon reached 98%, 92% and 88%, respectively, after two weeks. On the other hand, it was reported that *B. adusta* was able to transform efficiently the herbicide linuron but to lower extents than propanil and diuron [49]. The authors suggested that the low depletion of some molecules may be due to the absence of phenolic or aromatic amine groups. Unfortunately, no efforts were made to evaluate which enzymatic system from *B. adusta* could be involved in these herbicide transformations. Nevertheless, in vitro experiments using purified versatile peroxidase from *B. adusta* proved that this enzyme is able to transform halogenated phenolic pesticides including the herbicide bromoxynil [54]. This enzyme polymerized bromoxynil molecules producing a dimer and a trimer as the main products with the loss of bromide atoms and hydroxyl groups. Nevertheless, VP from *B. adusta* was able to oxidize only three of 13 halogenated pesticides tested. It was

suggested that the peroxidase-mediated reactions are limited by the presence of phenoxy groups and by the electronic properties of the substituents on the aromatic ring.

The genus *Pleurotus* includes edible and medicinal species which are found both in temperate and tropical climates. They are one of the most important cultivated mushrooms in the world and are commonly referred to as "oyster mushrooms". *Pleurotus ostreatus* and *P. pulmonarius* are the main species applied in bioremediation studies. These species are able to degrade PAH, chlorinated biphenyls and industrial dyes [36, 58-59]. *Pleurotus* species are known to be good producers of ligninolytic enzymes, mainly laccases [59], versatile peroxidase (VP) and manganese peroxidase (MnP) [24]. *P. pulmonarius*, cultured in pasteurized straw solid medium in association with bacteria, was able to degrade the highly persistent herbicide atrazine [60]. This study demonstrated that *P. pulmonarius* acts on the atrazine molecule producing chloro-metabolites which were further dechlorinated by bacteria, producing non-toxic metabolites. In liquid cultures, *P. ostreatus* was able to reduce the concentration of several herbicides. During five days, the herbicides chlortoluron, isoproturon and diuron were depleted 35, 42 and 33%, respectively [50]. Another study demonstrated maximum depletions of 42, 81 and 94% for diuron, linuron and propanil, respectively, during seven days [49]. In the latter studies no investigation about the enzymatic system involved in herbicide transformations was carried out.

Trametes versicolor seems to be highly promising for herbicide bioremediation. It is a good lignin degrader and produces Lac, MnP and LiP under certain culture conditions [61], being one of the most-studied laccase-producing fungus. Direct involvement of its laccase in herbicide transformation has been demonstrated. Recently, in vitro degradation experiments of glyphosate using several ligninolytic purified enzymes in combination with different mediators was carried out [62]. Laccase from *T. versicolor* was able to rapidly degrade the herbicide, but only in the presence of mediators such as ABTS, MnSO₄ and Tween 80. The best glyphosate degradation by laccase (90.1%) was obtained in the presence of all these mediators in the same reaction. The laccase-mediator reactions produced and accumulated the metabolite AMPA, a compound frequently found in soil exposed to glyphosate. Another work demonstrated that the compound diketonitrile, the active principle of the herbicide isoxaflutole, was transformed by laccase, purified from *T. versicolor* cultures, in the presence of the mediator ABTS [51]. The main metabolite produced in the reaction was benzoic acid, an inactive compound also produced by tolerant plants. Laccase from this white-rot species also has been reported to degrade metabolites from phenylurea herbicides commonly found in the fields. These are hydroxylated metabolites produced by fungi and plants as part of their phenylurea herbicide metabolization. The hydroxylation of their aromatic rings makes phenylureas substrates for laccases and thus ready for further degradation [63]. Purified laccases were able to transform the metabolites 2-hydroxyphenyl-urea (2-HF) and 4-hydroxyphenyl-urea (4-HF) into several products without mediator addition. The results suggested that these enzymes from *T. versicolor* allow the formation of polymers between the hydroxyphenylurea products, as deduced from the formation of insoluble compounds in the reaction medium [64].

In vivo studies have also been carried out. *T. versicolor* strains were able to deplete diuron, linuron and propanil from the culture medium after seven days, reaching 37%, 47% and 94%

pesticide transformation, respectively [49]. It was demonstrated that the herbicide diuron was the more extensively removed phenylurea in cultures of the *Trametes* species, with 72% transformation by *Trametes sp* and 33% by *T. versicolor* [50]. *T. versicolor* was able to degrade diuron and atrazine [65], chlornitrofen (CNP) and nitrofen (NIP) [66]. In the last work, the authors suggest that extracellular ligninolytic enzymes are not involved in the initial step of CNP and NIP degradation. However, the possibility of an effective participation of cytochrome P450 in the degradation of CNP and NIP has been emphasized.

Trametes versicolor has also been monitored for soil bioremediation in microcosms of non-sterile soils under water stress. Under this condition, this species was able to decompose atrazine without correlation with the production of ligninolytic enzymes [67] and to act on a pesticide mixture containing herbicides (simazine and trifluralin) and an insecticide (dieldrin) [45]. This type of study is very important because the capability of a given fungus to act in a soil microcosm can be strongly affected by competition with the native soil microflora and by the adverse conditions such as water stress.

Coriopsis fulvocenerea, *Cerrena maxima* and *Coriolus hirsutus* were investigated as atrazine decomposers in liquid cultures [68]. Among the several fungi strains, the authors found that the best degraders were those that produce laccases. The results showed that these fungi degrade at least 80-92% of atrazine after 40 days. Further, cultures with addition of laccase inducers such as guaiacol and syringaldazine, were more effective in decreasing the atrazine concentration, suggesting an important role of these enzymes in atrazine degradation [69]. Recently, it was studied the effect of laccase from *C. hirsutus* on atrazine binding to soil [70]. Laccase introduction into the soil caused a rapid increase in irreversibly bound atrazine. This mechanism probably decreases the herbicide toxicity and thus contributes to atrazine detoxification in soil.

Recently, the decrease of the concentration of the herbicides bentazon and diuron in *Ganoderma lucidum* cultures was reported [71-72]. The action of the ligninolytic enzymes on bentazon was confirmed by in vitro experiments with the crude enzyme filtrates. After 24 hours, bentazon was completely degraded by the liquid culture filtrate. Also, solid state cultures using corn cobs as substrates were a good medium for the production of laccase and MnP as well as for the transformation of bentazon.

The white-rot fungus *Phlebia brevispora* metabolizes the herbicide CNP (chlornitrofen) by nearly 80% within 28 days of incubation with the production of five metabolites [73]. *P. vispora* was also able to degrade a highly toxic compound, a dibenzo-*p*-dioxin, present as a contaminant of commercially produced CNP herbicide [73]. The transformation of phenylurea herbicides by *Phlebia radiata* after five days has also been reported [50].

Hypholoma fasciculare is another white-rot fungus capable of degrading different herbicide classes [65]. This fungus possesses a great ability to degrade the triazine herbicides terbuthylazine and atrazine and the phenylurea herbicide diuron in liquid cultures and also in biobed bioremediation system. No relationship between the ligninolytic activity and the fungi capacity to degrade pesticides was found, although similar mechanisms could be involved in degradation of all the compounds.

Lentinus subnudus was highly efficient in removing atrazine (78%) and metalachlor (94%) from contaminated soils after 25 days [74]. No attempts were done to identify the participation of the ligninolytic enzymes in the process.

8. Are ligninolytic enzymes actually involved in herbicide degradation?

WRF have been demonstrated to be capable of transforming and/or degrading a wide range of herbicide classes. Two mechanisms or systems have been proposed. The first is transformation in the extracellular space and it involves lignin-degrading enzymes. These powerful capabilities of WRF reside in the fact that many pollutants have structural similarities to lignin and because ligninolytic enzymes are non-specific they can also act on the pollutant molecules. Furthermore, the transformation of some compounds can be enhanced with the use of small molecules, called mediators, which can extend the enzymatic reactivity of enzymes towards the substrates; this process is often referred to as the enzyme-mediator system. The participation of extracellular enzymes in the transformation of several herbicides by WRF was conclusively demonstrated by studies performed with purified enzymes [46,47,49,51,54,62,63].

The second system of WRF involved in herbicide transformation is an intracellular enzymatic mechanism, represented mainly by cytochrome P450. Purification of fungal cytochrome P450, in order to obtain conclusive data, has been accomplished in only a few studies, due to the difficulties in keeping the activation of the enzymes during microsome preparation. Hence, most conclusions were drawn from the results of indirect experiments consisting in the addition of specific cytochrome P450 inhibitors to the culture medium, such as piperonyl butoxide and 1-aminobenzotriazole [66,75-76] (Fig. 2). Direct evidence is also available. Some experiments were carried out with the microsomal fraction isolated from *Pleurotus ostreatus* [77]. The authors found that the microsomes transformed the pesticides in vitro in a NADPH-dependent reaction. In Table 2 and 3, representative studies were listed in which ligninolytic enzymes of different WRF were used for removing herbicides. These tables show only those studies in which direct or strong indirect evidence is presented about the participation of enzymes (laccases, peroxidases and cytochrome P450 monooxygenases) for the removal of herbicides by WRF.

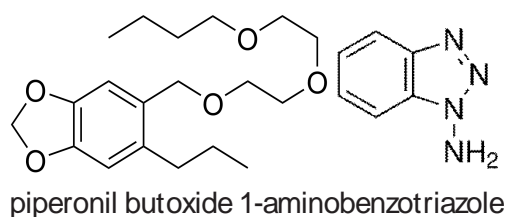


Figure 2. Specific cytochrome P450 inhibitors

Pesticide/metabolite(s)	Treatment conditions	Fungal source	Ref.
Glyphosate/AMPA	In vitro assay. The presence of ABTS, Mn ²⁺ and Tween 80 resulted in the removal of 90.1% of glyphosate.	<i>Trametes versicolor</i>	[62]
2,4-dichlorophenol (DCP): metabolite of 2,4-D	In vitro assay. Laccase/mediator system. Laccase was immobilized on chitosan.	<i>Coriolus versicolor</i> (syn. <i>Trametes versicolor</i>)	[78]
Dymron	In vitro assay. Laccase/mediator system. Over 90% of the herbicide was degraded by the addition of ABTS as mediator.	<i>Trametes sp.</i>	[79]
Bromofenoxim; Bromoxynil; Dichlorophen; Niclosamide	In vitro assay. Laccase/mediator system. Addition of syringaldehyde or acetosyringone resulted in higher rates of enzymatic degradation.	<i>Corioliopsis gallica</i>	[49]
Bentazon	In vitro assay. Crude enzymes from liquid and solid state cultures were applied. A synergistic effect of ABTS, Mn ²⁺ , H ₂ O ₂ , and Tween 80 was observed.	<i>Ganoderma lucidum</i>	[72]
Isoxaflutole. In plants and soils, isoxaflutole is rapidly converted to a diketonitrile derivative (DKN)	The WRF are able to convert the DKN to inactive benzoic acid analogue when cultured in liquid media. The DKN derivative was incubated with fungal extracellular oxidases, without or with their specific redox mediator. Both LiPs and MnPs presenting high specific activities were unable to catalyze the enzymatic transformation of DKN, with or without mediators, during 12-h incubations. Laccase transformed the herbicide when incubated with 1 mM ABTS.	<i>Phanerochaete chrysosporium</i> and <i>Trametes versicolor</i>	[51]
Pentachlorophenol (PCP)	Submerged cultures in the presence and absence of laccase inducers. Cultures actively producing laccase removed 70% of initial PCP. The removal of PCP was less than 20 percent in the cultures with low laccase activity.	<i>Pleurotus pulmonarius</i>	[80]

ABTS = 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate), AMPA ((aminomethyl)phosphonic acid).

Table 2. Studies where strong evidences of participation of laccase in the biodegradation of herbicides were found

Enzymes	Pesticide/ metabolite(s)	Treatment conditions	Fungal source	Ref.
MnP Manganese peroxidase	Glyphosate/AMPA	In vitro assay. Addition of Mn ²⁺ was essential for the oxidative activity of the enzyme. A reaction mixture containing Mn ²⁺ and Tween 80 with or without H ₂ O ₂ resulted in total degradation of the herbicide.	<i>Nematoloma frowardii</i>	[62]
	Pentachlorophenol (PCP)	Fungal inoculation in soil microcosms contaminated with PCP. Addition of wheat straw increased enzyme production and PCP degradation.	<i>Anthracoxyllum discolor</i>	[82]
	Bentazon	In vitro assay. Crude enzymes from liquid and solid state cultures were applied. A synergistic effect of ABTS, Mn ²⁺ , H ₂ O ₂ , and Tween 80 was observed.	<i>Ganoderma lucidum</i>	[72]
VP Versatile peroxidase	Bromoxynil; Dichlorophen; PCP	In vitro assay. Purified versatile peroxidase (VP) able to transform several pesticides. Analysis of reaction products by GC-MS showed the presence of 2,3,5,6-tetrachloroquinone among the products from pentachlorophenol oxidation, while the main product from dichlorophen was 4-chlorophenol-2,2-methylenequinone. Several polymers were obtained from the peroxidase oxidation of bromoxynil. In all cases, VP mediated dehalogenation reactions.	<i>Bjerkandera adusta</i>	[54]
CYP450 enzymes / Cytochrome P450 monooxygenase	Azinphos-methyl; Phosmet; Terbufos	In vitro assay. Pesticide transformation by microsomal fraction. Addition of NADPH in the reaction mixture was required.	<i>Pleurotus ostreatus</i>	[77]
	<i>trans</i> -Chlordane	Static liquid cultures. Addition of cytochrome P450 inhibitors reduced degradation activity and demonstrated involvement of CYP450 enzymes in the pesticide metabolism.	<i>Phlebia lindtneri</i> , <i>Phlebia brevispora</i> <i>Phlebia aurea</i>	[76]
	Cloronitrofen (CNP) and nitrofen (NIP)	Extracellular LiP, MnP and Laccase did not catalyze the oxidation of either CNP or NIP. Piperonyl butoxide, an inhibitor of cytochrome P450, suppressed fungal oxidation of CNP and NIP to their hydroxylated products. The inhibition resulted in increasing the amount of reductively dechlorinated and nitro-reduced products.	<i>Trametes (Coriolus) versicolor</i>	[66]

Table 3. Studies where evidence of participation of peroxidases and CYP450 enzymes in the biodegradation of herbicides

9. Concluding remarks

The ability of WRF to transform a wide variety of herbicide classes makes them suitable candidates to detoxify contaminated water and soils. Nevertheless, numerous WRF species and herbicide classes are still not explored. Ligninolytic enzymes, such as laccases and peroxidases, have shown to be responsible for many herbicide transformations, although their action remains to be confirmed in most studies. In this context, also the cytochrome P450 system has demonstrated to have a very important role in bioremediation of numerous types of pollutants including herbicides, although further studies must be done in order to understand the action of this system in transformation of herbicides by WRF.

The employment of ligninolytic enzyme preparations could be advantageous over the direct use of white-rot fungi, considering that these preparations can be more easily standardized, facilitating accurate dosage. Enzyme application is simpler than the use of microorganisms and can be rapidly modified according to the characteristics of the herbicide to be removed. In addition to this, analysis of metabolite compounds produced by enzyme preparations is easier than analysis of the metabolites produced by the whole white-rot fungi.

There is a need for introducing new methodologies for the elucidation of the chemical structures of the metabolites produced by the action of ligninolytic enzymes. This should also facilitate the elucidation of the metabolic routes used by the white-rot fungi in herbicide degradation. Furthermore, the proof that the process of degradation corresponds to a real detoxification is a crucial step that must be overcome before its practical use.

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