

Chapter 2

Degradation of Chloro-organic Pollutants by White Rot Fungi

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2.1 Introduction

Ligno-cellulosic plant biomass is the most abundant renewable organic resource on earth and contains cellulose, hemicellulose, and lignin polymers as its key components. Lignin, the most abundant aromatic polymer in the biosphere, is a highly complex, three dimensional, branched, recalcitrant polymer. Because of its recalcitrance, biodegradation of lignin is the rate-limiting step in the degradation of lignocellulosic biomass and thus lignin plays a pivotal role in global carbon cycling. Bacteria and most fungi are unable to mineralize lignin, but white rot fungi, a group of basidiomycetes that cause white rot decay of wood materials, are considered the most efficient organisms in mineralizing lignin in nature (Buswell and Odier 1987).

Three families of extracellular lignin modifying enzymes (LME) consisting of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are key components of the lignin-degrading enzyme system of white rot fungi. These enzymes are relatively non-specific and provide white rot fungi the unique ability to degrade a broad array of environmental pollutants such as dioxins, polychlorinated biphenyls (PCBs), petroleum hydrocarbons, munitions wastes (such as trinitrotoluene), industrial dye effluents, herbicides and pesticides (Aust 1990; Reddy 1995; Pointing 2001; Reddy and Mathew 2001). Of these, chloro-organic pollutants are some of the

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most common and most toxic pollutants encountered in the environment. Similar to ligninolysis, a feature of the secondary metabolism of white rot fungi, degradation of a number of environmental pollutants (but not all) by these organisms is triggered by limitation for nutrients, such as N and C and is also temporally correlated to lignin mineralization (Bumpus et al. 1985; Baldrian 2008). Moreover, many white rot fungi do not utilize environmental pollutants as sources of carbon and energy; instead, they utilize other available sources of energy in their environment, such as sugars and polysaccharides and gratuitously breakdown various pollutant chemicals, which are usually present in minute amounts (ppm or ppb) (Aust 1990; Reddy and Mathew 2001). Furthermore, although wood is their natural substrate, some white rot fungi have the ability to survive in soil and effectively compete with soil microflora (Baldrian 2008).

What are the characteristics of white rot fungi that make them attractive candidates for use in bioremediation applications? There are several reasons as mentioned by various workers (Aust 1990; Reddy 1995; Pointing 2001; Reddy and Mathew 2001; Baldrian 2008). 1. They are widely distributed in nature worldwide, particularly in forest soils where woody materials are undergoing decay, and thus are readily available for isolation and utilization in bioremediation studies. 2. White rot fungi are extremely versatile as they are able to degrade a long list of commonly used chloro-organic pollutants individually or in mixtures. 3. Constitutive nature of key biodegradation enzymes in white rot fungi eliminates or reduces the need for adaptation to the pollutant of interest. 4. White rot fungi can oxidatively breakdown pollutant substrates with low solubility because peroxidases and laccases, the key enzymes involved in lignin degradation as well as in pollutant degradation, are extracellular obviating the need for internalizing the substrates. 5. White rot fungi do not use organo-pollutants as sources of carbon and energy to any significant extent; therefore, relatively inexpensive lignocellulose sources, such as saw dust, peanut hulls, corn cobs, straw and other such materials can be provided for effective fungal colonization and biomass production at the contaminated sites. 6. White rot fungi (and filamentous fungi in general) grow by hyphal extension through the soil and have an advantage in gaining better access to some of the pollutant chemicals that accumulate in tiny pores in soil (Baldrian 2008; Pinedo-Rilla et al. 2009).

A number of reviews dealing with degradation of environmental pollutants by white rot fungi have been published (Bumpus et al. 1985; Reddy 1995; Raghukumar 2000; Pointing 2001; Reddy and Mathew 2001; Wesenberg et al. 2003; Chang 2008; Pinedo-Rilla et al. 2009; Majeau et al. 2010). In this review, we focused on degradation of selected classes of chloro-organic pollutants by white rot fungi with emphasis on work done in the last decade. Also, we focused on enzyme mechanisms and pathways including identification of metabolic intermediates involved in degradation of chloro-organics. *Phanerochaete chrysosporium* is the most intensively studied model white rot fungus in investigations on enzymology, molecular biology, and genetics of lignin degradation as well as on biodegradation of chloro-organics (Gold and Alic 1993; Reddy and D'Souza 1994; Hofrichter et al. 2010; Lundell et al. 2010) and hence received greater focus in this review. *P. chrysosporium* is also the

first white rot fungus for which whole genome sequence is available (Martinez et al. 2004; Vanden Wymelenberg et al. 2006, 2009).

2.2 Enzymology

2.2.1 Extracellular Peroxidases and Laccases

The unique ability of white rot fungi to degrade lignin is largely attributable to the non-specific free radical mediated oxidizing reactions carried out by their extracellular LMEs, peroxidases and laccases (Dosoretz and Reddy 2007; Wong 2009; Hofrichter et al. 2010; Lundell et al. 2010; Majeau et al. 2010). These enzymes are believed to have evolved to give white rot fungi (and a few other groups of fungi) the ability to breakdown lignin in plant biomass and obtain a better access to the cellulose and hemicellulose, which are not efficiently accessed by bacteria. LMEs cleave the C–C and C–O bonds of lignin regardless of the chiral conformations of the lignin molecule (Dosoretz and Reddy 2007). This manner of bond fission is partially contributed by the free radical mechanism employed by white rot fungi in lignin degradation (Reddy and D'Souza 1994; Dosoretz and Reddy 2007; Hofrichter et al. 2010). In addition, free radical species generated during the degradation process (of either lignin or organo-pollutants) may serve as secondary oxidants that may, in turn, mediate oxidation of other compounds away from the active sites of the enzymes.

Extracellular peroxidases of white rot fungi include lignin peroxidase (LiP; EC.1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), and versatile peroxidase (VP; EC 1.11.1.16). These enzymes belong to Class II fungal heme peroxidases (Lundell et al. 2010). Both LiPs and MnPs belong to a family of multiple isozymes coded by multiple genes and are produced during idiophasic growth (Gold and Alic 1993; Reddy and D'Souza 1994; Lundell et al. 2010).

LiP, MnP, and VP have similar catalytic cycles based on two electron oxidation of the enzyme by using H_2O_2 as primary oxidant to yield Compound I (Fig. 2.1; Table 2.1). Two consecutive one electron reduction steps of Compound I via Compound II by electron donor substrates return the enzyme to the initial ferric oxidation state. The H_2O_2 required by ligninolytic peroxidases is mainly generated by direct reduction of O_2 to H_2O_2 catalyzed by extracellular enzymes of the fungi, such as glyoxal oxidase, pyranose oxidase and aryl alcohol oxidase (Dosoretz and Reddy 2007).

LiP (Class II heme peroxidases, group A.1) possesses a higher redox potential and lower pH optimum than most other isolated peroxidases and oxidases and is able to oxidize non-phenolic aromatic substrates, typically not oxidized by MnPs (Table 2.1). It attacks nonphenolic phenyl propanoid units of the lignin polymer and the stable cation-centered radicals formed during these oxidations may serve as redox mediators for LiP-catalyzed reactions effectively extending the substrate

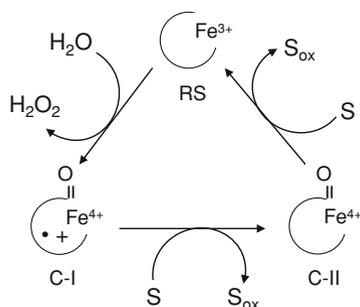


Fig. 2.1 Catalytic cycle of ligninolytic peroxidases (LiP, MnP, and VP). The enzyme resting state (RS, containing Fe^{3+}) undergoes oxidation by hydrogen peroxide to yield Compound I (C-I, containing Fe^{4+} -oxo and porphyrin cation radical). Reduction of C-I in two one electron steps results in the formation of Compound II (C-II, containing $\text{Fe}^{4+} = \text{O}$ after porphyrin reduction) which is then converted to RS. During the catalytic cycle, two substrate (S) molecules are oxidized which could be low redox potential phenols or Mn^{2+} in the cases of MnP and VP

range (Barr et al. 1992; Dosoretz and Reddy 2007; Hofrichter et al. 2010; Lundell et al. 2010). Similar to other peroxidases, LiP is also capable of oxidizing most phenolic compounds through the generation of phenoxy radicals. Besides the direct catalytic action of the enzyme, *P. chrysosporium* produces veratryl alcohol (VA), that temporally parallels LiP production, and VA is suspected to have the role of a redox mediator in the oxidation of lignin. Veratryl alcohol is oxidized by LiP to veratryl alcohol radical cation intermediate and the latter can initiate free radical reactions oxidizing oxalate, a natural substrate present in wood, but also produced by white rot fungi, to carboxylate anion radical ($\text{CO}_2^{\bullet-}$). This anion radical possesses a reduction potential (-1.9 V vs. NHE) that was shown sufficient to reductively dechlorinate some chloro-organics (Shah et al. 1993; Khindaria et al. 1995). Reactions catalyzed by LiP include ring opening and side chain cleavage reactions, benzyl alcohol oxidations, oxidative dechlorination reactions, and methoxylations.

MnP, unlike LiP, belongs to Class II heme peroxidases, group B (Lundell et al. 2010). MnPs of different fungal species have strikingly similar sequence homology. MnP, unlike LiP, oxidizes Mn(II) via Compound I and Compound II to yield Mn(III) and it is the later that is responsible for the oxidation of organic compounds catalyzed by MnP (Fig. 2.1 and Table 2.1) (Dosoretz and Reddy 2007; Hofrichter et al. 2010). Mn(III) is a diffusible oxidant that is able to penetrate the small molecular pores between cellulose microfibrils, which preclude the action of LiP because of steric hindrances. Organic acids, such as oxalate, malate, and fumarate, which are produced in cultures of white rot fungi, chelate Mn(III) and these stable complexes are involved in oxidation of the substrate. Although MnP does not normally oxidize non-phenolic lignin substructures, the latter have been shown to be slowly co-oxidized when MnP peroxidatively oxidizes unsaturated

Table 2.1 Comparison of the main lignin modifying enzymes produced by white rot fungi

Enzyme	Production	Mechanism and specificity	Fungus producing	References ^a
Laccase	Extracellular, most basidiomycetes	One electron oxidation of organic substrates coupled to 4-electron reduction of molecular oxygen to water. Broad specificity. Oxidation of phenolic compounds. Oxidation of non-phenolic compounds in the presence of mediators	<i>T. versicolor</i> , <i>B. adusta</i> , <i>G. lucidum</i> , <i>C. maxima</i> , <i>P. ostreatus</i> , <i>T. pubescens</i>	Cañas and Camarero (2010), Thurston (1994), Hildén et al. (1994)
Lignin peroxidase	Extracellular, most basidiomycetes	Two electron oxidation of the enzyme by H ₂ O ₂ to yield Compound I which undergoes two consecutive one electron reduction steps by oxidizing organic substrates. Broad specificity. Oxidation of phenolic and nonphenolic compounds	<i>P. chrysosporium</i> , <i>P. sordida</i> , <i>P. radiata</i> , <i>P. tremellosa</i> , <i>T. versicolor</i> , <i>B. adusta</i>	Hofrichter et al. (2010), ten Have et al. (1998), Sugiura et al. (2009)
Manganese peroxidase	Extracellular, most basidiomycetes	Two electron oxidation of the enzyme by H ₂ O ₂ to yield Compound I which undergoes two consecutive one electron reduction steps by oxidizing Mn ²⁺ into Mn ³⁺ that in turn oxidizes phenolic compounds	<i>P. chrysosporium</i> , <i>P. sordida</i> , <i>C. subvermispora</i> , <i>P. radiata</i> , <i>D. squalens</i> , <i>P. rivulosus</i>	Gold et al. (2000), Hakala et al. (2006)
Versatile peroxidase	Extracellular, most basidiomycetes	VP oxidizes Mn ²⁺ into Mn ³⁺ as MnP does, and also high redox potential aromatic compounds, as LiP does; has broad specificity and oxidizes nonphenolic, phenolic and dye substrates	<i>P. eryngii</i> , <i>P. ostreatus</i> , <i>B. adusta</i> , <i>Trametes</i> spp.	Ruiz-Dueñas et al. (2009), Moreira et al. (2005)

^a Includes mainly more recent reviews when available

fatty acids. MnP mediates oxidation of phenols, dyes, chlorophenols and other organopollutants (Pointing 2001; Reddy and Mathew 2001).

Versatile peroxidase (VP) belongs to fungal class II heme peroxidases, group A.3. It has been reported from genera *Pleurotus*, *Bjerkandera*, and *Trametes* and shares catalytic properties of both LiP and MnP (Dosoretz and Reddy 2007; Hofrichter et al. 2010). Similar to MnP, it exhibits high affinity for Mn(II) and catalyzes oxidation of Mn(II) to Mn(III), and also oxidizes both phenolic and non-phenolic substrates that is typical for LiP in the absence of Mn(II) (Table 2.1). VP oxidizes dimethoxybenzene, lignin dimers, phenols, amines, dyes, and aromatic alcohols (Dosoretz and Reddy 2007; Hofrichter et al. 2010). In the absence of Mn(II), VP oxidizes phenolic and non-phenolic substrates similar to LiP.

Another enzyme designated hybrid manganese peroxidase (hMnP) belongs to fungal class II heme peroxidases, group A.2. It has been isolated from *Phlebia radiata*, *Trametes versicolor* and other species and shares catalytic characteristics of VP and MnP (Hofrichter et al. 2010).

Laccases (EC1.10.3.2) are blue multicopper oxidases that catalyze the four-electron reduction of O₂ to water coupled with the oxidation of various organic substrates (Table 2.1). These are perhaps the most widely distributed oxidases in white rot fungi. These four-copper metalloenzymes catalyze O₂-dependent oxidation of a variety of phenolic compounds and do not require H₂O₂ or Mn(II) for activity. Similar to peroxidases, laccases catalyze subtraction of one electron from phenolic hydroxyl groups of phenolic compounds to form phenoxy radicals as intermediates. They also oxidize non-phenolic substrates in the presence of mediators which are oxidized to reactive radical or cation substrates by laccase and undergo further oxidation of non-phenolic targets (Dosoretz and Reddy 2007; Hofrichter et al. 2010; Rodgers et al. 2010).

With regard to the oxidation potential of LMEs, LiP possess the highest redox potential ($E'_{o} \sim 1.2$ V, pH 3), followed by MnP ($E'_{o} \sim 0.8$ V, pH 4.5) and laccase ($E'_{o} \sim 0.79$ V, pH 5.5) enzymes (Wong 2009). A comparison of LiP, MnP and laccase in the oxidation of a homologous series of various methoxybenzenes (ranged from 0.81 to 1.76 V at pH 3) showed a correlation between the redox potential of the enzymes and the compound substrate (Kersten et al. 1990; Popp and Kirk 1991). However, it should be noted that the affinity of LMEs to degrade chloro-organic pollutants varies from one compound to the other and should be tested on a case by case basis.

Another extracellular fungal class II heme peroxidase (EC 1:11:1:7; CiP), originally described from *Coprinopsis cinerius*, has low redox potential and is unable to oxidize veratryl alcohol. It lacks the Mn(II) binding site of MnP and VP (Hofrichter et al. 2010). It is commercially available from Novozyme (Baylase®) and has been used to clean up phenolics from waste water streams. Yet another peroxidase, designated dye-decolorizing peroxidase (EC 1:1:1:x) from *Agaricus* type fungi, was reported to catalyze the oxidation of dyes and phenolic compounds (Hofrichter et al. 2010).

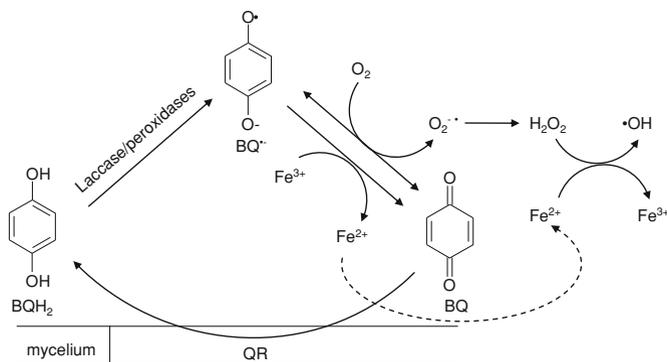


Fig. 2.2 Reaction scheme involved in the production of hydroxyl radical by white rot fungi via quinone redox cycling (Gomez-Toribio et al. 2009a, b). 1,4-benzoquinone (BQ) is reduced by quinone reductase (QR) producing hydroquinone (BQH_2), which is oxidized by any of the lignin modifying enzymes to semiquinones ($BQ^{\cdot-}$). The production of superoxide anion radicals ($O_2^{\cdot-}$) by $BQ^{\cdot-}$ autooxidation is mainly catalyzed by Fe^{3+} , that is reduced to Fe^{2+} . Fenton's reagent formation is accomplished by $O_2^{\cdot-}$ dismutation to H_2O_2

2.2.2 Induction of Hydroxyl Radicals

Hydroxyl radicals are highly reactive, non-specific, and potent oxidants described in cultures of white rot fungi during degradation of pollutants. Redox potential of hydroxyl radicals is estimated to be 2.8 V (Lawton and Robertson 1999) which allows degradation of some environmental pollutants that are not susceptible to peroxidases and laccases.

A simple strategy for the induction of extracellular hydroxyl radicals in white rot fungi, via Fenton's reaction employing the quinone redox cycling, has been described (Gomez-Toribio et al. 2009a, b). Quinone redox cycling involves cell-bound reduction of quinones to hydroquinones. Hydroquinones are known substrates of all LMEs, and undergo further extracellular oxidation to semiquinones. In the presence of O_2 , semiquinones are autooxidized to regenerate the quinone, while O_2 is reduced to superoxide anion radical ($O_2^{\cdot-}$) (Guillen et al. 1997). In this strategy, the incubation of the mycelium with quinones and ferric ion (Fe^{3+}) generates the required Fenton's reagent (Fe^{2+} and H_2O_2) to produce hydroxyl radical via reduction of Fe^{3+} to Fe^{2+} by semiquinone radical and spontaneous dismutation of $O_2^{\cdot-}$ to H_2O_2 (Fig. 2.2). H_2O_2 and Fe^{2+} react to produce $\cdot OH$ and Fe^{3+} . Involvement of hydroxyl radicals in the degradation of chloro-organics, petroleum hydrocarbons, pharmaceuticals, and dyes has recently been reported (Gomez-Toribio et al. 2009a; Marco-Urrea et al. 2009a, 2010; Aranda et al. 2010).

White rot fungi produce cellobiose dehydrogenase (CDH) when grown on cellulose as the carbon source. CDH catalyzes the two electron oxidation of cellobiose to cellobionolactone coupled to the reduction of quinones, phenolic intermediates, O_2 or Fe^{3+} and catalyzes the formation of Fenton's reagent (Kremer

and Wood 1992; Mason et al. 2003). It has been reported that hydroxyl radicals produced by CDH oxidize oxalate to carboxylate anion radical ($\text{CO}_2^{\bullet-}$), which was shown to dechlorinate chloro-organic compounds, similar to that described above for LiP, when incubated with veratryl alcohol and $\text{CO}_2^{\bullet-}$ (Cameron and Aust 1999). Also, LiP has been shown to act as an indirect source of hydroxyl radicals in a cascade of reactions mediated by veratryl alcohol, ferric ion and oxalate, although this mechanism is apparently not employed for degradation of chloroaromatic pollutants (Barr et al. 1992).

2.2.3 Cytochrome P450 System

Originally, the ability of ligninolytic (nitrogen-limited) cultures of *P. chrysosporium* to degrade pollutants was attributed to the action of LMEs, particularly to LiP and MnP. Nevertheless, several chloro-organic pollutants were found to be degraded and even mineralized by non-ligninolytic (nitrogen-rich) cultures of *P. chrysosporium* that were not expressing LiP and MnP (Yadav and Reddy 1992, 1993). This indicated that enzyme system(s), other than peroxidases were involved in the degradation of the pollutants studied by these investigators. Recent studies indicate that cytochrome P450 system is important in catalyzing the detoxification of several organic pollutants, including chloro-organics, by white rot fungi. The evidence for this is based on marked decrease in degradation of chloro-organics in the presence of cytochrome P450 inhibitors as well as the induction pattern of cytochrome P450-encoding genes in response to several of the chloro-organics (Doddapaneni and Yadav 2004). Furthermore, the whole genome sequence of *P. chrysosporium* revealed an estimated number of 148 P450 monooxygenase genes, the highest number known until that time among the fungal genomes (Martinez et al. 2004; Yadav et al. 2006). The main reactions catalyzed by these intracellular cytochrome P450 enzymes include epoxidation of C=C double bonds and hydroxylation of aromatic compounds. It has been suggested that cytochrome P450 enzyme system in nature plays an important role in the mineralization of lignin metabolites resulting from peroxidase-depolymerized lignin polymer (Subramanian and Yadav 2008).

2.2.4 Phase II Conjugation Reactions

Biotransformation of xenobiotics by higher animals is usually carried out in two steps known as phase I and phase II reactions. Phase I reactions are generally reactions which modify the chemical by adding a functional group. These reactions typically involve oxidation by the cytochrome P450 system leading to a new intermediate that contains a reactive chemical group (such as the addition of hydroxyl, carboxyl, or an amine group). Besides oxidations, reductions and hydrolyses are also common phase I reactions. Phase II reactions are conjugation

reactions, in which the phase I metabolite is conjugated to form sulfates, glucuronides, glucosides, or glutathione conjugates. In general, a conjugated metabolite is more water-soluble, less toxic, and more easily excreted from the body than the original xenobiotic or phase I metabolite. The whole genome sequence of the white rot fungus *P. chrysosporium* revealed a high diversity of glutathione *S*-transferases, a class of phase II detoxifying enzymes found mainly in cytosol. Some phase II enzymes have peroxidase activities with a probable role in protecting cells against H₂O₂-induced cell death. It has been hypothesized that phase II enzymes are related to a large number of cytochrome P450 sequences identified in the whole genome sequence of *P. chrysosporium* (Morel et al. 2009). However, little definitive data are available on the likely role of phase II enzymes of white rot fungi in detoxification processes. Some phase II enzymes, such as glucosidases and xylosidases of white rot fungi have been reported to catalyze the conversion of some chlorinated organic compounds to the corresponding conjugated derivatives, that showed markedly lower cytotoxicity than the parent compound (Reddy et al. 1997; Hundt et al. 2000).

2.3 Biodegradation of Chloro-organic Compounds by White Rot Fungi

2.3.1 Chlorinated Alkanes and Alkenes

Contamination of soils and aquifers by the aliphatic halocarbons trichloroethylene (TCE) and perchloroethylene (PCE), widely used as degreasing solvents, is a serious environmental pollution problem. TCE and PCE are among the most frequently detected chemicals at hazardous waste sites and are on the EPA's list of priority pollutants. Khindaria et al. (1995) reported that TCE is mineralized by *P. chrysosporium* cultures grown aerobically. These investigators proposed that TCE is subject to in vitro reductive dehalogenation catalyzed by LiP of *P. chrysosporium* in the presence of veratryl alcohol, H₂O₂, and EDTA (or oxalate) leading to the production of the corresponding reduced chlorinated radicals (Fig. 2.3a). Later research by Yadav et al. (2000) showed TCE mineralization by *P. chrysosporium* in malt extract medium and in a nitrogen-rich defined medium, in which the LiP and MnP production by the fungus is known to be suppressed, as well as in nitrogen-limited medium in which normal levels of Lip and MnP are known to be produced. Comparison of values for total degradation (46.2%) and mineralization (38.5%) using [¹⁴C] TCE as the substrates showed that most of the TCE was transformed to ¹⁴CO₂. Therefore, contrary to the results of Khindaria et al. (1995), an alternate enzyme system that does not involve LiP or MnP appeared to be important in TCE degradation by *P. chrysosporium*. More recently, Marco-Urrea et al. (2008a) showed that three other white rot fungi including *T. versicolor*, *Irpex lacteus* and *Ganoderma lucidum* degraded TCE. Their results

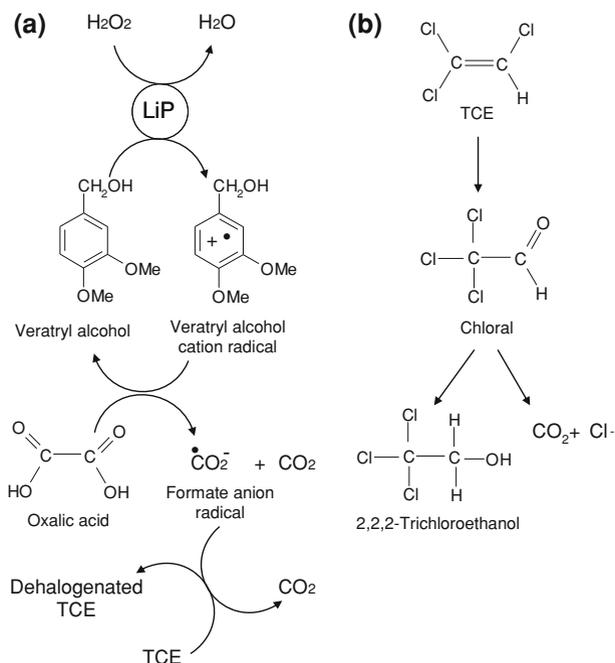


Fig. 2.3 Mechanisms of trichloroethylene (TCE) degradation by white rot fungi. **a** Proposed reductive dechlorination of TCE by LiP of *P. chrysosporium* using oxalate as the electron donor (Khindaria et al. 1995). **b** Oxidation of TCE catalyzed by cytochrome P450 system in *T. versicolor* (Marco-Urrea et al. 2008b)

further indicated that TCE degradation by *T. versicolor* involves cytochrome P450 system, as suggested by inhibition of TCE degradation in the presence of 1-aminobenzotriazole (a known inhibitor of cyt. P450). Also, *T. versicolor* was shown to mineralize 53% of the added [^{13}C] TCE, while the rest accumulated in the form of [^{13}C] 2,2,2-trichloroethanol, a common intermediate in [^{13}C]TCE degradation mediated by mammalian cytochrome P450 (Fig. 2.3b).

For many years, PCE was considered to be non-biodegradable under aerobic conditions, attributable to the high oxidation state of the molecule. However, Marco-Urrea et al. (2006) reported for the first time aerobic PCE degradation by *T. versicolor*. Their results indicated that PCE is first transformed to trichloroacetyl chloride, which was rapidly hydrolyzed in water (abiotically) to trichloroacetic acid. The PCE conversion to trichloroacetic acid appears to be catalyzed by cytochrome P450 system, as evidenced by marked inhibition of this conversion in the presence of cytochrome P450 inhibitor, 1-aminobenzotriazole.

Extracellular hydroxyl radicals produced by *T. versicolor*, via quinone redox cycling, were also shown to catalyze degradation of PCE and TCE (Marco-Urrea et al. 2009a). The advantage of using this strategy lies in the higher dechlorination

ratio obtained, in comparison to the cytochrome P450 mediated system. Using isotopic labeled [^{13}C] TCE, these investigators showed that mineralization of TCE by hydroxyl radicals produced by *T. versicolor* does not involve 2,2,2-trichloroethanol as a product. CDH produced by *P. chrysosporium* was also able to catalyze dechlorination of bromotrichloromethane to trichloromethyl radical after the production of carboxylate anion radical (Cameron and Aust 1999). Also, *P. chrysosporium* was shown to use the gaseous natural product chloromethane as methyl donor for veratryl alcohol biosynthesis (Harper et al. 1990).

2.3.2 Polychlorinated Biphenyls

Polychlorinated Biphenyls (PCBs) is a generic name for a family of compounds with multiple chlorines (usually 2–8) per biphenyl molecule. Because of their environmental persistence and toxicity, they have been banned now, but in the past, they had a wide range of industrial applications, such as heat transfer, dielectric, and hydraulic fluids, solvent extenders, flame retardants, and plasticizers. Commercial PCBs were manufactured as mixtures under the trade names Aroclor, Clophen, and Declor and consist of a mixture of congeners which differ in the number and positions of chlorines on the biphenyl nucleus (Table 2.2).

About 150 congeners of PCBs have been found in the environment. *P. chrysosporium* was the first white rot fungus that was shown to degrade a wide range of PCB congeners (Bumpus et al. 1985; Yadav et al. 1995a, b). In general, the extent of degradation/mineralization of PCBs decreases with increase in the number of chlorines on the biphenyl nucleus. For instance, negligible levels of mineralization for individual hexa- and tetrachlorobiphenyls were reported for several of the white rot fungi studied (Bumpus et al. 1985; Thomas et al. 1992; Vyas et al. 1994), but the level of degradation dramatically increased when PCBs with three or less chlorine substitutions were used (Dietrich et al. 1995; Beaudette et al. 1998). Mineralization of commercial PCB mixtures by *P. chrysosporium* also appears to follow the trend expected on the basis of PCB chlorination levels. Thus, mineralization of [^{14}C]Aroclor 1242 (42% chlorine by weight) was about 20%, while that of [^{14}C]Aroclor 1254 (54% chlorine by weight) ranged from 10 to 14% (Eaton 1985; Bumpus and Aust 1987; Yadav et al. 1995b). Furthermore, Yadav et al. (1995b) showed that PCB degradation decreased with an increase in chlorine content (Aroclor 1242 > Aroclor 1254 > Aroclor 1260). Also, commercial PCB mixtures with varying number of ortho, meta, and para substitutions on the biphenyl ring were degraded extensively (Yadav et al. 1995b). In more recent studies, Kamei et al. (2006b) reported undetectable levels of degradation when a chlorine was changed from an *ortho* position to *meta* position in 2,3',4,4',5,5'-hexachlorobiphenyl and 3,3,4,4,5,5'-hexachlorobiphenyl in cultures of *P. brevispora*. Other investigators showed lack of mineralization of 3,3',4,4'-tetrachlorobiphenyl as compared to 10% mineralization observed with 2,2',4,4'-tetrachloro biphenyl in *P. chrysosporium* cultures (Thomas et al. 1992; Dietrich et al. 1995).

Table 2.2. Degradation of individual chlorinated biphenyl (CBP) congeners by white rot fungi

Compound	Fungus/enzyme	Comments on mineralization and metabolic intermediates	Reference
2-CBP	<i>P. chrysosporium</i>	Mineralization of [¹⁴ C]2-CBP.	Thomas et al. (1992)
2,5-diCBP	<i>T. versicolor</i>	Identification of dichlorobenzenes, chlorophenols and alkylated benzenes as intermediates.	Koller et al. (2000)
4,4'-diCBP	<i>P. chrysosporium</i> <i>Phanerochaete</i> sp. MZ142	Identification of metabolites 2-OH- and 3-OH-diCBP in <i>Phanerochaete</i> sp. MZ142 and 3-OH- and 4-OH-diCBP in <i>P. chrysosporium</i> ; and 3-methoxy-4,4'-diCBP, 4-CBZ, 4-CBZH, and 4-CBZOH in both.	Kamei et al. (2006a)
2,4',5-triCBP	<i>P. chrysosporium</i> <i>P. chrysosporium</i> , <i>B. adusta</i> <i>T. versicolor</i> , <i>P. ostreatus</i>	Mineralization of [¹⁴ C]4,4'-diCBP. Identification of CBZ and CBZOH as metabolites. Mineralization of [¹⁴ C]2,4',5-triCBP: <i>T. versicolor</i> > <i>B. adusta</i> > <i>P. ostreatus</i> > <i>P. chrysosporium</i> . No correlation established between degradation, mineralization, and peroxidase production.	Dietrich et al. (1995) Beaudette et al. (1998)
2,2',4,4'-tetraCBP	<i>T. versicolor</i>	Mineralization of [¹⁴ C]2,4,5-triCBP was stimulated by the surfactant Triton X-100.	Beaudette et al. (2000)
3,3',4,4'-tetraCBP	<i>P. chrysosporium</i> <i>P. brevispora</i>	Mineralization of [¹⁴ C]2,2',4,4'-tetraCBP. Identification of 5-methoxy-3,3',4,4'-tetraCBP as metabolite.	Thomas et al. (1992) Kamei et al. (2006b)
	<i>P. chrysosporium</i> <i>P. chrysosporium</i> <i>P. chrysosporium</i>	Low mineralization of [¹⁴ C]3,3',4,4'-tetraCBP (0.8%). Low mineralization of [¹⁴ C]3,3',4,4'-tetraCBP (1.1%). Low mineralization of [¹⁴ C]3,3',4,4'-tetraCBP (1.4%).	Dietrich et al. (1995) Bumpus et al. (1985) Vyas et al. (1994)

(continued)

Table 2.2. (continued)

Compound	Fungus/enzyme	Comments on mineralization and metabolic intermediates	Reference
2,3',4,4',5-pentaCBP	<i>P. brevispora</i>	3-methoxy, 5'-methoxy-pentaCBP, 4-methoxy-2,3',4',5-tetraCBP, and as intermediates	Kamei et al. (2006b)
2,3,3',4,4'-pentaCBP	<i>P. brevispora</i>	Three metabolites identified: 5-methoxy-, 5'-methoxy-pentaCBP and 4-methoxy-tetraCBP.	Kamei et al. (2006b)
3,3',4,4',5-pentaCBP	<i>P. brevispora</i>	Identification of 5'-methoxy-pentaCBP as metabolite.	Kamei et al. (2006b)
3,3',4,4',5,5'-hexaCBP	<i>P. brevispora</i>	Identification of 5'-methoxy-pentaCBP.	Kamei et al. (2006b)
2,2',4,4',5,5'-hexaCBP	<i>P. chrysosporium</i>	Low mineralization of [¹⁴ C]2,2',4,4',5,5'-hexaCBP (0.9%).	Bumpus et al. (1985)

CBP chlorinated biphenyl, OH hydroxyl group, CBZ chlorobenzoic acid, CBZH chlorobenzaldehyde, CBZOH chlorobenzyl alcohol

P. ostreatus strains showed selective PCB degradation with preference for congeners with chlorine atoms in *ortho* > *meta* > *para* position in the commercial mixture Declor 103 (Kubatova et al. 2001). On the contrary, *P. chrysosporium* did not show any noticeable specificity for the position of chlorine substitutions in Aroclor 1242, 1242 and 1260 (Yadav et al. 1995b). However, additional studies are required to better understand the apparent inconsistencies in the observed results.

The effect of addition of surfactants was studied to see if the hydrophobic PCBs would become more bioavailable leading to increased rates of PCB degradation (Ruiz-Aguilar et al. 2002). Addition of Triton X-100 and Dowfax 8390 at low concentration did not affect levels of total biodegradation of PCB mixtures, but mineralization of 2,4',5-trichlorobiphenyl increased by 12%, when Triton X-100 was used (Beaudette et al. 2000).

In further studies on the nature of degradation of PCBs by white rot fungi, it was reported that hexa-, penta- and tetrachloro-PCB congeners were methoxylated, leading to some dechlorination. Transformation products derived from ring fission are only reported for dichlorobiphenyls. *P. brevispora* transformed 3,3,4,4'-tetra-, 2,3,3',4,4'-penta-, 2,3',4,4',5-penta-, 3,3',4,4',5-penta-chlorobiphenyl, and 2,3',4,4',5,5-hexachlorobiphenyl (HCB) to *meta*-monomethoxylated PCBs (Kamei et al. 2006b). Dechlorination was detected in the degradation of 2,3,3',4,4'-penta-, 2,3',4,4',5-penta-, and 2,3',4,4',5,5'-hexachlorobiphenyls and occurred exclusively at the *para* position (Kamei et al. 2006b). Therefore, it appears that orthochlorines in monochlorobiphenyls are more likely to undergo dechlorination and that lack of chlorine in the meta position favors ring fission. Kamei et al. (2006a) demonstrated that 4,4'-dichlorobiphenyl (DCB) was hydroxylated by *Phanerochaete* sp. MZ142 and *P. chrysosporium* at different positions. In the case of strain MZ142, 4,4'-DCB oxidation produced 2-hydroxyl-4,4'-DCB and 3-hydroxyl-4,4'-DCB. The metabolic pathway for 3-hydroxyl-4,4'-DCB was branched to produce the corresponding methoxylated product and to form 4-chlorobenzoic acid, 4-chlorobenzaldehyde, and 4-chlorobenzyl alcohol (Fig. 2.4). Also, 2-hydroxyl-4,4'-DCB was not methoxylated. These results are in agreement with the previous results reporting the formation of 4-chlorobenzoic acid and 4-chlorobenzyl alcohol from 4,4'-DCB by *P. chrysosporium* (Dietrich et al. 1995). Transformation of hydroxylated products produced from 4,4'-DCB into 4-chlorobenzoic acid, 4-chlorobenzaldehyde, and 4-chlorobenzyl alcohol by *Phanerochaete* cultures appears to be favored in low-nitrogen medium, but not in nitrogen-rich medium, in which the production of ligninolytic peroxidases is suppressed, indicating that ligninolytic enzymes could play a role in this conversion (Kamei et al. 2006a). It was hypothesized that a nitrate reductase enzyme was involved in dechlorination of hexachlorobiphenyl (HCB) by *P. chrysosporium*, but the specific enzyme involved in dechlorination of HCB remains yet to be identified (De et al. 2006).

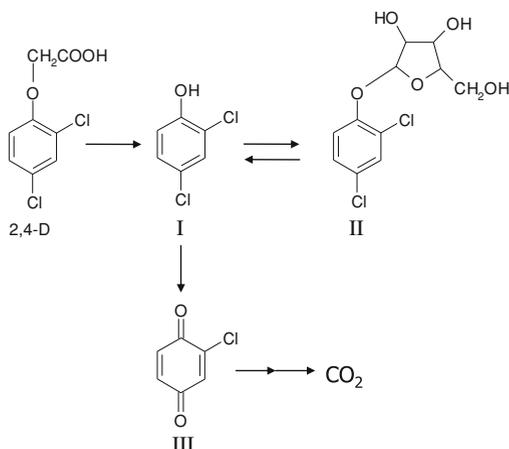
The capacity of white rot fungi to degrade hydroxylated PCBs (OH-PCBs) was also studied, since these compounds enter the environment in high concentrations

2.3.3 Phenoxyalkanoic Herbicides

This group includes 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). 2,4-D is perhaps the most commonly used broad leaf herbicide around the world; 2,4,5-T is a component of Agent Orange that was widely used as a defoliant. 2,4-D is quite susceptible to bacterial degradation and generally does not persist for long in the environment except under adverse conditions, such as low soil pH and low temperature which increase its longevity in soil. 2,4,5-T is relatively more resistant to microbial degradation and tends to persist in the environment. It has been blamed for serious illnesses in many veterans of Vietnam war, where they got exposed to Agent Orange that was used as a defoliant. 2,4-D and 2,4,5-T were also reported to be mutagenic agents. Furthermore, during the manufacture of 2,4,5-T, it gets contaminated with low levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which is very toxic to humans.

Ryan and Bumpus (1989) showed 2,4,5-T degradation by *P. chrysosporium* both in liquid culture as well as in soil. Extensive mineralization of ^{14}C -labeled 2,4-D and 2,4,5-T by *P. chrysosporium* has been demonstrated in liquid media (Pathway for 4,4'-dichlorobiphenyl Yadav and Reddy 1992, 1993). These investigators, by using a peroxidase-negative mutant of *P. chrysosporium*, demonstrated that LiP and MnP of this organism are not involved in the degradation of 2,4-D and 2,4,5-T by *P. chrysosporium* and further observed faster 2,4-D and 2,4,5-T mineralization when the wild type was grown in nutrient rich (non-ligninolytic) media. Yadav and Reddy (1993) also observed that ring-labeled 2,4-D is mineralized faster in nutrient rich (non-ligninolytic) media and that 2,4,5-T and 2,4-D were simultaneously mineralized at a higher rate when presented as a mixture. Subsequently, Reddy et al. (1997) reconfirmed that ligninolytic peroxidases were not involved in the initial cleavage reaction of 2,4-D and 2,4,5-T nor in the subsequent transformation of the side chain of 2,4,5-T and 2,4-D. However, they showed that ligninolytic peroxidases of *P. chrysosporium* and *Dichomitus squalens* were involved in the degradation of chlorinated phenolic intermediates of 2,4-D and 2,4,5-T. These results were based on the increased degradation of ring-labeled and side chain-labeled 2,4,5-T and 2,4-D by *D. squalens* on addition of Mn^{2+} (a known inducer of MnP) to the medium and on increased degradation by *P. chrysosporium* in nitrogen-limited medium (in which production of both LiP and MnP is induced). Degradation of 2,4-D by *D. squalens* appears to involve an initial ether cleavage resulting in the formation of 2,4-dichlorophenol and acetate, under conditions of nitrogen depletion when peroxidase system is induced. The chlorophenol intermediate underwent subsequent oxidative dechlorination to a benzoquinone intermediate, followed by mineralization to CO_2 (Fig. 2.5) (Reddy et al. 1997). In vitro assays with purified laccase showed no activity when 2,4-D was used, but degraded up to 60% of 2,4-dichlorophenol (Sanino et al. 1999).

Fig. 2.5 Initial steps in the proposed pathway for degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) by *Dichomitus squalens* (Reddy et al. 1997). Symbols: I: 2,4-dichlorophenol, II: 2,4-dichlorophenol xyloside, III: 2-chloro-p-benzoquinone
 Note: 2,4,5-T apparently undergoes similar transformations during its degradation



2.3.4 Triazine Herbicides

Atrazine is a commonly used triazine herbicide and is degraded by a number of white rot fungi (Masaphy et al. 1993; Mougin et al. 1994; Entry et al. 1996; Bending et al. 2002; Nwachukwu and Osuji 2007). Degradation of atrazine by *Pleurotus pulmonarius* and *P. chrysosporium* leads to the accumulation of the N-dealkylated products deethylatrazine, deisopropylatrazine, deethyl-deisopropylatrazine and hydroxyisopropylatrazine (the latter produced only by *P. pulmonarius*). Atrazine oxidation by these two appears to involve cytochrome P450 system as evidenced by inhibition of oxidation upon addition of cytochrome P450 inhibitors piperonyl butoxide and 1-aminobenzotriazole (Masaphy et al. 1996a; Mougin et al. 1997b). Transformation of atrazine did not occur in vitro using purified LiP and MnP (Hickey et al. 1994; Mougin et al. 1997b). The ability of white rot fungi to degrade atrazine when added to pasteurized and unpasteurized lignocellulosic substrates, cotton + wheat straw (CWS) was evaluated (Hickey et al. 1994; Masaphy et al. 1996b; Bastos and Magan 2009). Masaphy et al. (1996b) showed loss of nearly 70% of the total radioactivity added as ¹⁴C-ring-labeled atrazine two weeks after colonization with *Pleurotus* in pasteurized CWS, while only about 30% of atrazine loss was noted in non-inoculated CWS. No mineralization of the triazine ring was found after six weeks of incubation, but chlorinated and dechlorinated degradation products of atrazine were found. *T. versicolor* was able to grow and actively degrade atrazine in non-sterile soil with low organic matter and low water availability conditions (-0.7–2.8MPa) that limited the metabolic activity of autochthonous microbial community (Bastos and Magan 2009). These results suggested that *T. versicolor* is potentially useful for bioremediation of semi-arid soils contaminated with triazine herbicides.

P. chrysosporium was also shown to effect N-dealkylation of triazine herbicides simazine, propazine and terbuthylazine (Mougin et al. 1997b). *T. versicolor* and

P. chrysosporium degraded about 80% of simazine in a soil extract broth regardless of osmotic potential used and the range of concentrations tested (0–30 mg l⁻¹) (Fragoeiro and Magan 2005). The inoculation of the above-mentioned fungi on wood chips in soil microcosms containing simazine enhanced the degradation of the herbicide and increased the extracellular enzymes in soil (Fragoeiro and Magan 2008).

Using radiolabelled atrazine, Donnelly et al. (1993) showed that ericoid mycorrhizal fungus, *Hymenoscyphus ericae* 1318, degraded atrazine and relatively high levels of atrazine carbon was incorporated into its tissue. In general, as the nitrogen concentration increased, the extent of atrazine degradation increased.

2.3.5 Chlorinated Dioxins

Polychlorinated dibenzodioxins (PCDDs) are a group of highly toxic environmental pollutants that are confirmed human carcinogens and tend to bioaccumulate in humans and animals due to their lipophilic properties. Polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) have been shown to be degraded by several species of white rot fungi (Table 2.3). 2,7-dichlorodibenzo-*p*-dioxin (2,7-diCDD) was used as the model in most of these studies. *P. chrysosporium* degraded 50% of the added 2,7-diCDD under ligninolytic conditions while only 10% was degraded under nonligninolytic conditions, suggesting the possible involvement of LiP and MnP (Valli et al. 1992). The pathway for 2,7-diCDD degradation involved oxidative cleavage of 2,7-diCDD by LiP resulting in the production of 4-chloro-1,2-benzoquinone and 2-hydroxy-1,4-benzoquinone (Fig. 2.6), followed by cycles of oxidation involving LiP and/or MnP leading to production of hydroquinones or catechols and subsequent methylation reactions generating methoxybenzenes (Fig. 2.6). It is of interest that the white rot fungus *Panellus stypticus*, which (unlike *P. chrysosporium*) does not produce either LiP or MnP, metabolizes 2,7-diCDD and produces 4-chlorocatechol as an intermediate suggesting that its degradation system for 2,7-diCDD is different from that of *P. chrysosporium* (Sato et al. 2002). Inhibition of 2,7-diCDD degradation upon addition of the cyt. P450 inhibitor, piperonyl butoxide, suggested the possible involvement of cyt. P450 enzyme in dioxin degradation by *P. stypticus*. Mori and Kondo (2002a) reported that *Phlebia lindtneri*, *Phlebia* sp. MG-60 and an unidentified white rot fungus mineralized [¹⁴C]-2,7-diCDD to a maximum extent of 6.5%. Several of the *Phlebia* species produced one hydroxylated and two methoxylated intermediates as degradation products of 2,7-diCDD, which are different from those seen in the LiP-catalyzed reaction of *P. chrysosporium* (Mori and Kondo 2002a, b; Kamei and Kondo 2005).

Kamei et al. (2005) showed that *Phlebia* species are able to degrade higher chlorinated dioxins, such as 2,3,7-triCDD (18.4–27%), 1,2,8,9-tetraCDD (11.9–21.1%), and 1,2,6,7-tetraCDD (14.2–21.5%). Higher degradation rate of 1,2,6,7-tetraCDD compared to that of 2,3,7-triCDD by *P. lindtneri*, suggested that

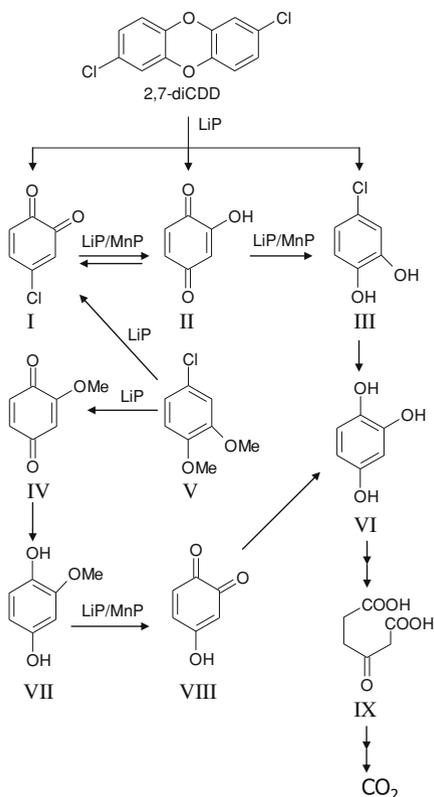


Fig. 2.6 Pathway for degradation of 2,7-dichlorodibenzo-p-dioxin (2,7-diCDD) by *P. chrysosporium* (Valli et al. 1992). Symbols: I: 4-chloro-1,2-benzoquinone, II: 2-hydroxy-1,4-benzoquinone, III: 4-chlorocatechol, IV: 2-methoxy-1,4-benzoquinone, V: 4-chloroveratrole, VI: 1,2,4-trihydroxybenzene, VII: 2-methoxyhydroquinone, VIII: 4-hydroxy-1,2-benzoquinone, IX: β -keto adipic acid; LiP: lignin peroxidase, MnP: manganese peroxidase

chlorination pattern rather than the number of chlorine substitutions affected the rate of degradation of chlorinated dioxins by this fungus (Kamei and Kondo 2005). Degradation intermediates of 2,3,7-triCDD, 1,2,8,9-tetraCDD, and 1,2,6,7-tetraCDD by *P. lindneri* included hydroxylated and methoxylated products in accordance with the pattern observed with 2,7-diCDD degradation by this organism (Fig. 2.7) (Kamei and Kondo 2005).

Phlebia brevispora, unlike several other *Phlebia* strains examined, was able to degrade 1,3,6,8-tetraCDD (28% in 28 d) and produce hydroxylated-tetraCDD, methoxy-tetraCDD, dimethoxy-tetraCDD, dimethoxy-triCDD and 3,5-dichlorocatechol as intermediates (Kamei et al. 2005). Hydroxylation of 1,3,6,8-tetraCDD was hypothesized to be the initial reaction catalyzed by cytochrome-P450 enzyme (Kamei et al. 2005). Two *Bjerkandera* strains (MS325 and MS1167), that produced

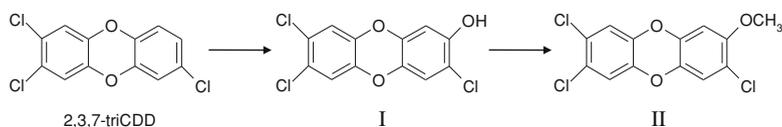


Fig. 2.7 Degradation pathway of 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-triCDD) by *Phlebia lindneri* (Kamei and Kondo, 2005). Symbols: I: monohydroxy-triCDD, II: monomethoxy-triCDD

MnP and LiP, showed 16–21% degradation of 1,3,6,8-tetraCDD in 7 d (Manji and Ishihara 2004). An additional *Bjerkandera* strain, that did not produce LiP, was unable to degrade 1,3,6,8-tetraCDD, although this does not necessarily mean that LiP is indispensable for 1,3,6,8-tetraCDD degradation in all cases.

The degradation of 2,3,7,8-tetraCDD is of high environmental interest, because it is the most hazardous dioxin. *P. chrysosporium* mineralizes 2,3,7,8-tetraCDD (Bumpus et al. 1985). Takada et al. (1996) studied degradation of 10 kinds of 2,3,7,8-substituted tetra- to octaCDDs and tetra- to octaCDFs by *Phanerochaete sordida* YK-624 and also included *P. chrysosporium* for comparison. Degradation products of 2,3,7,8-tetraCDD and octa-CDD by *P. sordida* included 4,5-dichlorocatechol and tetrachlorocatechol, respectively. Formation of these products suggested the involvement of LiP-catalyzed reaction similar to that described for 2,7-diCDD (Valli et al. 1992). However, *P. sordida* produced MnP, but no LiP and crude MnP showed degradation of the dioxins, indicating that this fungus used an alternative enzymatic system different from LiP for this purpose.

There have been a few studies to date on PCDF degradation by white rot fungi. Degradation of 2,8-diCDF by *P. lindneri* produced hydroxyl-diCDF as an intermediate, but the enzymes involved were not elucidated (Mori and Kondo 2002b). All the PCDD and PCDF congeners containing 2,3,7,8-substitutions were partially degraded by *P. sordida*, but the highest level of degradation was seen with 2,3,7,8-hexaCDD (Takada et al. 1996).

Studies on the application of white rot fungi for the cleanup of chlorinated dioxins-contaminated soils and fly ash have been sparse. Designed PCR primers for the specific amplification of *Ceriporia* sp. (MZ-340) DNA in soils have been successfully used to monitor this strain during bioremediation of contaminated fly ash *on site* (Suhara et al. 2003). The total concentration of chlorinated dioxins and furans in fly ash decreased $\geq 50\%$ by *Ceriporia* sp. in 12 weeks when compared with the control (Suhara et al. 2003). Kamei et al. (2009) studied the influence of soil properties on the growth of *P. brevispora* and its dioxin degradation ability using 1,3,6,8-tetraCDD and 2,7-diCDD as models. Slurry-state condition was found to be more suitable for fungal soil treatment than the solid state condition. When the fungus was applied to a historically contaminated paddy soil, 1,3,6,8-tetraCDD was degraded approximately 50% over 90 d of incubation. Also, the use of organic-rich soil decreased the biodegradation activity of the fungus (Kamei et al. 2009) (Table 2.3).

Table 2.3 Relevant details on the degradation of various chlorinated dioxins by white rot fungi^a

Compound	Fungus/enzyme	Comments	Reference
1-monoCDD	Cyt P450	Yeast clones expressing individual cyt P450s of the fungus show metabolism towards 1-monoCDD.	Kasai et al. (2010)
	Cyt P450	Yeast clones expressing individual cyt P450s of the fungus show metabolism towards 1-monoCDD.	Kasai et al. (2010)
2-monoCDD	LiP	LiP transformed 2-monoCDD to the corresponding cation radical as immediate product.	Hammel et al. (1986)
	<i>P. chrysosporium</i>	Identification of metabolites and elucidation of enzymatic mechanisms shown in Fig. 2.6.	Valli et al. (1992)
2,7-diCDD	<i>P. lindmeri</i>	Identification of one hydroxylated metabolite.	Mori and Kondo (2002b)
	<i>P. lindmeri</i> , <i>Phlebia</i> sp. MG-60; Unidentified -MZ-227	Mineralization of [¹⁴ C]2,7-diCDD. Nine other white rot fungi showed lower ability to mineralize.	Mori and Kondo (2002a)
	<i>P. stypticus</i>	Identification of metabolite III (Fig. 6); cyt P450 involvement in the initial oxidation of 2,7-diCDD.	Sato et al. (2002)
	<i>P. lindmeri</i>	Identification of hydroxylated, methoxylated and dimethoxylated metabolites.	Kamei and Kondo (2005)
	<i>Phlebia</i> spp.	Identification of hydroxylated and methoxylated metabolites.	Kamei et al. (2005)
2,3-diCDD	CytP450	Yeast clones expressing individual cyt P450s of the fungus show activity towards 2,3-diCDD.	Kasai et al. (2010)
2,3,7-triCDD	<i>P. lindmeri</i>	Identification of hydroxylated and methoxylated metabolites.	Kamei and Kondo (2005)
	<i>Phlebia</i> spp.	Degradation of substrate.	Kamei et al. (2005)
	<i>P. chrysosporium</i>	Low mineralization of [¹⁴ C]2,3,7,8-tetraCDD (2%).	Bumpus et al. (1985)
	<i>P. sordida</i>	Identification of 4,5-chlorocatechols as metabolite in <i>P. sordida</i> . This strain does not produce LiP.	Takada et al. (1996)

(continued)

Table 2.3 (continued)

Compound	Fungus/enzyme	Comments	Reference
2,3,7,8-tetraCDD	<i>P. chrysosporium</i> <i>P. brevispora</i>	Crude enzymes of MnP did not oxidize CDDs. Identification of monohydroxylated, monomethoxylated, dimethoxylated metabolites and 3,5-dichlorocatechol. Possible role of cyt P450	Takada et al. (1996) Kamei et al. (2005)
1,3,6,8-tetraCDD	<i>Bjerkandera</i> spp. <i>P. brevispora</i>	Degradation of substrate. Examination of the bioremediation potential in different types of soils.	Manji and Ishihara (2004) Kamei et al. (2009)
1,2,6,7-TetraCDD	<i>P. lindneri</i>	Identification of two hydroxylated and one monomethoxylated metabolites.	Kamei and Kondo (2005)
1,2,3,7,8-pentaCDD	<i>Phlebia</i> spp.	Based on degradation.	Kamei et al. (2005)
1,2,3,4,7,8-hexaCDD	<i>P. sordida</i>	Based on degradation.	Takada et al. (1996)
1,2,3,4,6,7,8-heptaCDD	<i>P. chrysosporium</i>		
1,2,3,4,6,7,8,9-octaCDD	<i>P. sordida</i>	Identification of tetrachlorocatechol as metabolite in <i>P. sordida</i> . This strain does not produce LiP.	Takada et al. (1996)
	<i>P. chrysosporium</i>	Crude MnP from this organism did not oxidize CDDs.	Takada et al. (1996)

^a *LiP* lignin peroxidase, *MnP* manganese peroxidase, *cytP450* cytochrome P450 monooxygenases. *CDD* chlorinated dibenzo-*p*-dioxins

2.3.6 Chlorobenzenes

Chlorobenzenes are used as high-boiling solvents in many industrial applications and as intermediates in the production of herbicides, pesticides, dyestuffs, and rubber. *P. chrysosporium* extensively degraded and mineralized monochlorobenzene as well as *o*-, *m*-, and *p*-dichlorobenzenes in malt extract cultures, in which LiP and MnP are not known to be produced (Yadav et al. 1995b). These results indicated that LiP and MnP were not involved in chlorobenzene degradation by *P. chrysosporium*. Additional evidence for the non-involvement of LiP and MnP was provided by using a *per* mutant that was unable to produce LiP and MnP, and the rates of degradation and mineralization of dichlorobenzene by the mutant were comparable to that of the wild type. Comparison of degradation levels with mineralization values indicated that most of the *p*-dichlorobenzene degraded was mineralized (~30%) whereas only a smaller portion of monochlorobenzene, *o*-, and *m*-dichlorobenzene was mineralized.

Degradation of 1,2,4-trichlorobenzene and 1,2,3-trichlorobenzene by *T. versicolor* in a defined medium was 91.1 and 79.6%, respectively, after 7 d of incubation (Marco-Urrea et al. 2009b). Addition of purified laccase and four different laccase mediators, such as ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) did not lead to increased 1,2,4-trichlorobenzene oxidation. However, addition of cytochrome P450 inhibitors piperonyl butoxide and 1-aminobenzotriazole strongly inhibited both dechlorination and degradation, suggesting the involvement of cytochrome P450 in 1,2,4-trichlorobenzene degradation. These findings support previous observations of the non-involvement of LiP and MnP in monochlorobenzene and dichlorobenzene degradation by *P. chrysosporium* (Yadav et al. 1995b). A degradation pathway for 1,2,4-trichlorobenzene was proposed based on the identification of the intermediates, 5-trichloromuconate, its corresponding carboxymethylene-butenolide, 2- or 5-chloro-4-oxo-2-hexendioic acid and 2- or 5-chloro-5-hydroxy-4-oxo-2-pentenoic acid on the second day of incubation, but were not present after 7 d of incubation (Fig. 2.8). This evidence, together with the near-complete dechlorination of the degraded 1,2,4- and 1,2,3-trichlorobenzene as indicated by chloride release balance, suggested possible mineralization of 1,2,4-trichlorobenzene at the end of the incubation (Marco-Urrea et al. 2009b), but this remains to be confirmed. Under the test conditions, *T. versicolor* was not able to degrade 1,3,5-trichlorobenzene, an isomer that is known to show high resistance to aerobic biotransformation. However, when extracellular hydroxyl radical production was induced via quinone redox cycling in this fungus, over 25% degradation of 1,3,5-trichlorobenzene was observed and the ratio between 1,3,5-trichlorobenzene degradation and chloride release was 1:1.9 (Marco-Urrea et al. 2009a).

Different basidiomycetes were screened for their ability to degrade and mineralize highly chlorinated hexachlorobenzene in contaminated soil (Matheus et al. 2000). Nineteen basidiomycete strains were able to colonize contaminated soils containing 5000–50000 mg of hexachlorobenzene kg⁻¹ soil, but only *Psilocybe*

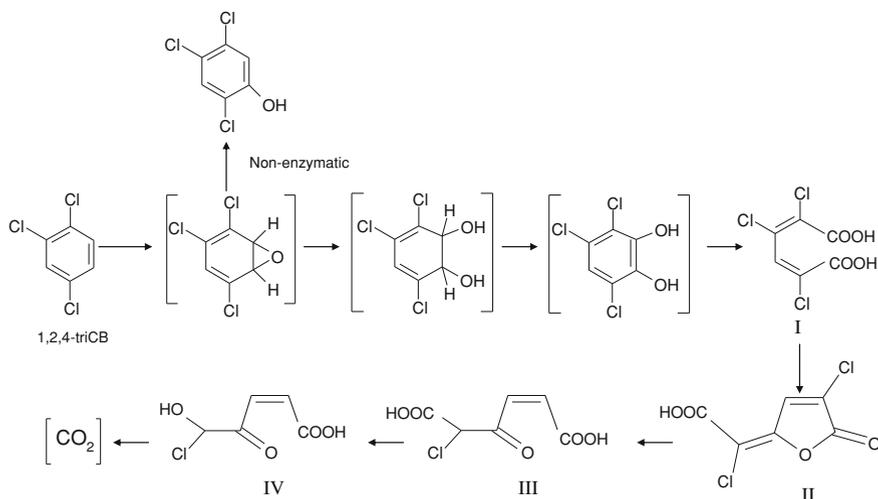


Fig. 2.8 Degradation pathway of 1,2,4-trichlorobenzene (triCB) by *T. versicolor* (Marco-Urrea et al. 2009b). Symbols: I: 2,3,5-trichloromuconate, II: (chloro-) carboxymethylenebutenolide, III: 2- or 5-chloro-4-oxo-2-hexendioic acid, IV: 2- or 5-chloro-5-hydroxy-4-oxo-2-pentenoic acid. Chemicals set into square brackets were not identified in that work

castanella showed a consistent decrease of hexachlorobenzene concentration. The amount of ¹⁴CO₂ from [¹⁴C] hexachlorobenzene, however, showed very low levels of mineralization (slightly >1%) in soils inoculated with this fungus.

2.3.7 Chlorinated Insecticides

Lindane (γ isomer of hexachlorocyclohexane) was a widely used pesticide in the past and an estimated 600,000 tons of lindane were produced globally between 1950 and 2000. There is a global ban on the use of lindane now because of its environmental persistence as a pollutant. *P. chrysosporium*, cultured under ligninolytic conditions, was reported to partially mineralize lindane in liquid cultures and in a corn-cob-amended soils inoculated with *P. chrysosporium* (Bumpus et al. 1985; Kennedy et al. 1990), but lindane degradation was not observed in vitro using purified LiP and MnP from *P. chrysosporium* (Mougin et al. 1996). However, in the presence of the cytochrome P450 inhibitor, 1-aminobenzotriazole, lindane degradation was reduced drastically suggesting the involvement of P450 in lindane degradation by *P. chrysosporium*. When α -, β -, γ -, and δ -hexachlorocyclohexane were tested for their degradation by white rot fungi, γ - and δ -isomers were degraded between 15–71% by six strains, but were found inhibitory to the other white rot fungi tested (Quintero et al. 2008). In non-sterile

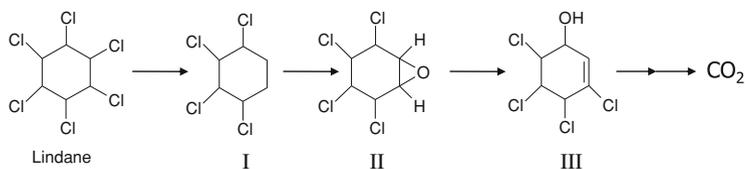


Fig. 2.9 Proposed degradation pathway for lindane by white rot fungi (Mougin et al. 1996; Singh and Kuhad 1999, 2000). Symbols: I: tetrachlorocyclohexane, II: tetrachlorocyclohexane epoxide, III: tetrachlorocyclohexenol

soil, all the hexachlorocyclohexane isomers were degraded between 8.2 and 17.5% by *B. adusta* immobilized on corncobs or woodchips. Mineralization of lindane by *P. chrysosporium*, *T. hirsutus* and *P. sordida*, occurred through the production of the intermediates tetrachlorocyclohexene, tetrachlorocyclohexene epoxide, and tetrachlorocyclohexenol, whereas only tetrachlorocyclohexenol was produced by *Cyatus bullery* (Fig. 2.9) (Mougin et al. 1996; Singh and Kuhad 1999, 2000). The effect of bioaugmentation on lindane transformation in sterile and non-sterile soils inoculated with *P. chrysosporium* has also been evaluated. In sterile soils, fungal biomass increased rapidly after inoculation with *P. chrysosporium* in the form of spores, but lindane mineralization was not seen (Mougin et al. 1997a). In contrast to this, fungus pre-grown on corncobs and added to sterile soils showed 22.8% mineralization after 60 d incubation (Kennedy et al. 1990). Conversely, either limited fungal growth or antagonist effect of autochthonous microflora on lindane degradation by *P. chrysosporium* and *B. adusta*, inoculated into non-sterile soils, was observed (Mougin et al. 1997a; Quintero et al. 2008).

DDT (1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane), the first of the chlorinated organic insecticides, was used quite heavily after World War II. High levels of DDT found in agricultural soils are of deep concern, because they present serious threats to food security and human health. The white rot fungi *P. chrysosporium*, *P. ostreatus*, *T. versicolor* and *Phellinus weirii* have been shown to mineralize DDT (Bumpus et al. 1985; Fernando et al. 1987; Purnomo et al. 2010). When glucose was added as the carbon source to nitrogen-deficient cultures of *P. chrysosporium*, 13.2% of DDT was mineralized in the first 21 d and ceased after this point. When either cellulose or starch was used as the growth substrate, substantial rates of mineralization were maintained through 90 d and over 30% mineralization of DDT was observed. *P. chrysosporium* transformed DDT to DDD during the first 3 d of incubation. Upon continued incubation, the amount of DDD decreased and oxidized degradation products 2,2,2-trichloro-1, 1-bis(4-chlorophenyl) ethanol (dicofol), 2,2-dichloro-1,1-bis(4-chlorophenyl) ethanol (FW-52), and 4,4'-dichlorobenzophenone (DBP) appeared and the fungus started to mineralize DDT. Mineralization of DDT by white rot fungi was reported to occur in nitrogen-deficient media, in which lignin-degrading enzymes such as LiP and MnP are produced, but not in nitrogen-rich media, in which the production of lignin-degrading enzymes is suppressed (Bumpus and Aust 1987). Later investigators,

however, disproved these conclusions by demonstrating DDT disappearance (not mineralization) under nitrogen-rich non-ligninolytic conditions and by demonstrating lack of transformation of DDT when exposed to a concentrated culture broth from ligninolytic cultures containing LiP (Köhler et al. 1988). Purnomo et al. (2008) proposed that cytochrome P450 is involved in DDT conversion to DDD by white rot fungi, but no experimental evidence was provided. On the basis of the reduction potential, dechlorination of dicofol to FW-52 was suggested to be produced via a free radical mechanism by LiP incubated together with oxalate and veratryl alcohol (Khindaria et al. 1995). More recently, a preliminary report appeared on DDT disappearance under different pH and oxygen concentrations in soils containing laccase, but further research is needed to demonstrate the involvement of laccase and other LME from white rot fungi in the mineralization of DDT (Zhao and Yi 2010).

It was of great interest that brown rot fungi *Gloeophyllum trabeum*, *Fomitopsis pinicola* and *Daedalea dickinsii* also showed a high ability to degrade DDT (Purnomo et al. 2008).

Aldrin, a heavily used organochlorine pesticide in the past, has been globally banned since the 1970s, because of its environmental persistence in agricultural soils and is a serious threat to human health and food security. Aldrin is converted by common soil microorganisms to dieldrin, which is the active form of the insecticide. *P. chrysosporium* was also able to carry out the epoxidation of aldrin to dieldrin together with the formation of an unidentified polar intermediate (Kennedy et al. 1990). *P. brevispora* transformed dieldrin to 9-hydroxydieldrin (Kamei et al. 2010); however, dieldrin mineralization by white rot fungi seems to be negligible (Kennedy et al. 1990).

2.3.8 Chlorinated Pharmaceutical and Personal Care Products

An expanding array of substances called 'Endocrine-disrupting compounds' (EDC) include chemicals of natural and anthropogenic origin. EDCs are widely distributed in the environment. They constitute a potential health risk to humans and aquatic life. Only a couple of EDCs are covered here. Triclosan is one such synthetic antimicrobial compound that is present in a wide range of health care products, such as tooth-paste, deodorant sticks and soaps. It has been detected in various environmental matrices due to its low degradation by conventional wastewater treatment processes. The fact, that triclosan shows remarkable toxicity towards bacteria, makes white rot fungi interesting candidates to test for biodegradation of this compound. Triclosan was degraded by seven strains of white rot fungi including *P. chrysosporium* ME 446, *P. magnoliae*, *T. versicolor*, *I. lacteus*, *P. ostreatus*, *P. cinnabarinus*, and *D. squalens*, while *B. adusta* was the only organism included in the screening that did not degrade triclosan (Cajthaml et al. 2009). *I. lacteus* and *P. ostreatus* were found to be the most efficient triclosan degraders with a degradation efficiency exceeding 90 and 80%, respectively.

In another study, triclosan was shown to inhibit the growth of *T. versicolor* over the first 3 d of incubation, but the ability of the fungus to transform triclosan to glucoside and xyloside conjugated forms and small amounts of 2,4-dichlorophenol lowered the toxicity due to triclosan and allowed the subsequent normal growth of the fungus (Hundt et al. 2000). The white rot fungus *P. cinnabarinus* also converted triclosan to the glucoside conjugate and additionally produced 2,4,4'-trichloro-2'-methoxydiphenyl (Hundt et al. 2000).

Several studies have focused on the applicability of purified laccases to oxidize and detoxify triclosan. The enzymatic transformation of triclosan by laccase was found to be optimal at pH 5 and 50°C and was negatively affected by the presence of sulfite, sulfide, cyanide, chloride, Fe(III) and Cu (II) ions that may be present in a wastewater matrix (Kim and Nicell 2006; Cabana et al. 2007). The use of polyethylene glycol (PEG) to prevent the inactivation of laccase resulted in an enhancement of triclosan degradation, but influenced negatively the toxicity of the treated solution (Kim and Nicell 2006). Immobilized laccase showed higher ability to degrade triclosan and had greater stability than free laccases (Cabana et al. 2009a, b). Degradation rates of triclosan by laccase were substantially improved through the use of laccase mediators (Murugesan et al. 2010). For example, ~90% triclosan degradation was observed in the presence of mediators, such as 1-hydroxybenzotriazole (HBT) and syringaldehyde (SYD). Other investigators did not see enhanced degradation of triclosan in the presence of mediators (Kim and Nicell 2006; Cabana et al. 2007). Two different mechanisms of triclosan degradation catalyzed by laccase were proposed. In the absence of redox mediators, laccase transformed triclosan to dimers, trimers and tetramers formed by oxidative coupling of the phenoxy radicals of triclosan (Cabana et al. 2007; Murugesan et al. 2010). Although the basic structure of triclosan was unaffected, laccase-mediated detoxification of triclosan was demonstrated using bacterial growth inhibition tests (Murugesan et al. 2010). In the presence of redox mediators HBT and SYD, the ether bond linkage of triclosan was cleaved producing 2,4-dichlorophenol, which was further dechlorinated through oligomerization reaction (in the case of HBT) or oxidative dechlorination to 2-chlorohydroquinone (in the case of SYD) (Murugesan et al. 2010). Recently, direct dechlorination of triclosan resulting in the formation of dechlorinated oligomers was shown in the presence of laccases conjugated with chitosan (Cabana et al. 2010). MnP seems to be a more promising choice than laccase for triclosan degradation, evidenced from the higher degradation rates obtained with MnP than laccase only or laccase + HOBT (Inoue et al. 2010). However, the transformation products were not described. Algal and bacterial growth inhibition tests showed that triclosan treated with MnP results in greatly reduced toxicity in the effluent.

Clofibric acid (CA) is the pharmacologically active derivative of clofibrate and several other fibrates that are used as blood lipid regulators in human medicine. Limited removal of CA, that was observed in municipal wastewater treatment plants, deserves more attention because of the risk of CA passing later barriers in partly closed water cycles. Of the four white rot fungi screened for their ability to degrade CA, only *T. versicolor* was able to degrade nearly 90% of CA (8 mg l⁻¹)

in defined liquid medium after 7 d (Marco-Urrea et al. 2009c); but three other fungi, *P. chrysosporium*, *G. lucidum* and *I. lacteus*, were unable to degrade CA under similar conditions. CA was not oxidized by the purified MnP or laccase even in the presence of redox mediators. However, CA degradation by *T. versicolor* cultures was inhibited by more than half in the presence of cytochrome P450 inhibitors piperonyl butoxide or 1-aminobenzotriazole, suggesting that P450 plays a role in CA degradation by this organism (Marco-Urrea et al. 2009c). Degradation of CA in cultures of *T. versicolor* was also attempted by inducing hydroxyl radicals, through the quinone redox cycling mechanism (Marco-Urrea et al. 2010). Based on time-course experiments, using 10 mg l^{-1} of CA, these cultures showed >80% degradations after 6 h of incubation. An intermediate showing hydroxylation of the benzene ring of CA was detected early in the incubation, but it was not detectable in the cultures after 24 h (Marco-Urrea et al. 2010).

2.4 Conclusions

White rot fungi have become increasingly attractive as candidates for designing effective bioremediation strategies, because of the broad substrate specificity of the ligninolytic enzymes which enable these fungi to degrade or mineralize quite a broad spectrum of chloro-organics (and other environmental pollutants). Even though a large majority of the bioremediation studies to date were done with *P. chrysosporium* as the model, a greater number of other genera of white rot fungi have also been studied in recent years and this may help determine the right organism with the right characteristics for carrying out a desired bioremediation application. Basic studies designed to obtain a better understanding of the mechanisms of actions as well as the basic protein and gene structures of the major extracellular ligninolytic enzymes (LiP, MnP, VP, and laccase), that catalyze degradation of chloro-organics through free radical mediated reactions, have been described. Recent studies indicate that intracellular enzymes as exemplified by cytochrome P450 monooxygenases are widespread in white rot fungi and appear to be key players involved in the degradation of a number of the chloro-organic pollutants.

Although most studies on lab or field scale bioremediation were done using bacteria, white rot fungi are beginning to gain prominence in this area, because they offer a number of advantages over bacteria for biodegradation of chloro-organic environmental pollutants. For example, reductive dechlorination of toxic PCE and TCE by bacteria usually leads to accumulation of toxic *cis*-dichloroethylene and vinyl chloride in the environment, whereas white rot fungi not only can degrade PCE and TCE to less toxic intermediates, but also have the ability to mineralize TCE. Also, compared to most degradative enzymes of bacteria, the ligninolytic enzymes of white rot fungi are non-specific and allow these organisms to degrade several classes of organo-pollutants individually or in mixtures.

Most published studies on biodegradation of chloro-organo-pollutants have been carried out in defined or complex laboratory media or in small samples of autoclaved soils and the effect of temperature, pH, moisture, nutrient (mainly C, N and S) and oxygen concentration, and small molecular weight growth/enzyme inducers on the quantitative and qualitative production of degradative enzymes, and biochemical pathways for the degradation of various pollutants have been studied. However, less number of pilot scale or field scale studies have been done to date. Because of the great versatility of white rot fungi in degrading a broad array of environmental pollutants, it is important to study bioremediation applications on a larger scale using large bioreactors and contaminated soils, sediments, and effluent streams, and other pollutant sites. Some of these studies have been reviewed by Baldrian (2008) and he described some of the limitations for the practical applicability of white rot fungi for field scale bioremediations. The specific physico-chemical properties of some chloro-organics can also restrict the application of white rot fungi. For instance, some chloro-organics, such as PCE and TCE are highly volatile, have low solubility, and are denser than water and therefore, they accumulate in the form of pools in groundwater when released into the environment. In these environments, anoxic conditions are predominant and the white rot fungi which are aerobic would require oxygen and this may add to the costs. Successful use of immobilized enzymes of white rot fungi, such as LiP, MnP, and laccases for bioremediation would obviate some of the difficulties associated with the use of whole mycelia cells. In turn, this points to the importance of detailed characterization of the ligninolytic enzymes/isoenzymes produced by different strains or species of white rot fungi that offer the most promise for different bioremediation applications, because fungal strains vary a great deal in their repertoire of enzymes/isoenzymes. Recently, application of laccases immobilized on solid supports has been explored for the treatment of contaminated effluents in adapted bioreactors. In general, immobilization of laccase on solid supports allows enhanced enzyme stability and continuous utilization of the biocatalyst for bioremediation applications. For successful bioremediation, white rot fungi should be able to survive and successfully compete with autochthonous soil flora for carbon, nitrogen, and other nutrient sources. Even though, woody materials are their natural substrates, recent studies indicate that several species of white rot fungi can survive in soil and their soil colonizing ability is comparable to those of soil-inhabiting basidiomycetes utilizing the available lignocellulosic and other substrates in soil. Also, some of the white rot fungi appear to compete well with the complex soil microbial flora.

White rot fungi are extraordinary in their versatility to degrade a large variety of complex and recalcitrant environmental pollutants that contaminate soil and groundwater ecosystems, constituting a potential danger to human and animal health. It is fairly well proven now that white rot (as well as some non-white rot fungi) have the ecological and biochemical capability to degrade important categories of toxic chloro-organic pollutants. There has been much progress in screening and identifying strains/species of white rot fungi isolated from different ecosystems that are increasingly more efficient than previously described

organisms in degrading selected chloro-organic pollutants generated by different industrial processes. There has been also much advancement in our understanding of the basic physiology, biochemistry, and molecular biology of enzymes and enzyme systems involved in the degradation of chloro-organic pollutants by white rot fungi. Current genomics and proteomics tools offer significant opportunities to determine the role and interactions of known genes and for identifying new genes involved in degradation of chloro-organics by white rot fungi. This information would also be of great interest to monitor and better understand the behaviour of white rot fungi in soils and to use them more efficiently as the agents of bioremediation in contaminated sites. More research is needed to obtain better understanding of the ecology, enzymology, genomics, and proteomics of some of the more important species of white rot fungi that appear more desirable from a bioremediation perspective. Despite of great promise of white rot fungi as bioremediation agents, a number of challenges remain to be surmounted in translating the basic knowledge on these organisms into cost-effective practical bioremediation applications.

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