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MINI-REVIEW

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Feasibility of bioremediation by white-rot fungi

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Abstract The ligninolytic enzymes of white-rot fungi have a broad substrate specificity and have been implicated in the transformation and mineralization of organopollutants with structural similarities to lignin. This review presents evidence for the involvement of these enzymes in white-rot fungal degradation of munitions waste, pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, bleach plant effluent, synthetic dyes, synthetic polymers, and wood preservatives. Factors relating to the feasibility of using white-rot fungi in bioremediation treatments for organopollutants are discussed.

Introduction

White-rot fungi

The white-rot fungi are a physiological rather than taxonomic grouping, comprising those fungi that are capable of extensively degrading lignin (a heterogenous polyphenolic polymer) within lignocellulosic substrates. The name white-rot derives from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance to the substrate. Most known white-rot fungi are basidiomycetes, although a few ascomycete genera within the *Xylariaceae* are also capable of white-rot decay (Eaton and Hale 1993).

The ability to catabolize cellulose and hemicellulose, the polysaccharides forming the other main components of lignocellulose, is fairly common as a primary metabolic process among fungi and other organisms, and occurs under a range of environmental conditions. As a result, it is not regarded as a rate-limiting step in carbon flux. Lignin, however, is extremely recalcitrant and is mineralized in an obligately aerobic oxidative process, carried out appreciably only by the white-rot fungi. This recalcitrance, possession of ligninolytic ability among relatively few taxa, and annual lignin production estimated at 20.3×10^{12} kg annually (re-calculated from Bassham 1975) contribute to lignin degradation being regarded as the rate-limiting step to carbon turnover in lignocellulose-dominated environments. Interestingly, the oxidation of lignin yields no net energy gain, and so lignin is not a substrate in primary metabolism. Rather, lignin is degraded during secondary metabolism in order to access wood polysaccharides locked in lignin-carbohydrate complexes, so providing an energy source to which other organisms do not have access (Jeffries 1990).

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The ligninolytic system of white-rot fungi

White-rot fungi variously secrete one or more of three extracellular enzymes that are essential for lignin degradation, and which combine with other processes to effect lignin mineralization. They are often referred to as lignin-modifying enzymes or LMEs. The three enzymes comprise: two glycosylated heme-containing peroxidases, lignin peroxidase (LiP, E.C. 1.11.1.14) and Mn dependant peroxidase (MnP, E.C. 1.11.1.13) (Orth and Tien 1995), and a copper-containing phenoloxidase, laccase (Lac, E.C. 1.10.3.2) (Thurston 1994). Some authors also report novel Mn-independent MnP activity in some white-rot fungi (e.g. Eggert et al. 1996). In the presence of (endogenously generated) H_2O_2, LiP catalyzes oxidation of an endogenously generated low-molecular-mass redox mediator veratryl alcohol (Reddy and D'Souza 1994), which in turn carries out an one-electron oxidation of non-phenolic aromatic nuclei in lignin to generate aryl cation radicals. These then degrade non-enzymatically to aromatic and aliphatic products, which are mineralized intracellularly. The radicals generated can carry out a variety of reactions, including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization/polymerization, and demethylation.

MnP catalyzes an H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}(as Mn^{3+}-oxalate or some other dicarboxylate) and this oxidizes phenolic components of lignin (Wariishi et al. 1992). Lac also generates radicals from a low-molecular-mass redox mediator, but in an H_2O_2-independent reaction. The mediator compound has been identified as 3-hydroxyanthranilate in the laccase-producing white-rot fungus *Pycnoporus cinnabarinus* (Bourbonnais et al. 1997), although several artificial mediator compounds such as 1-hydroxybenztriazole (HBT) and 2,2′-azino-bis-(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) are also capable of acting as Lac mediators (e.g. Pointing et al. 2000a).

Other enzymes are associated with LMEs in lignin breakdown, but are unable to degrade lignin alone. Glyoxal oxidase (E.C. 1.2.3.5) and superoxide dismutase (E.C. 1.15.1.1) produce the H_2O_2 required by LiP and MnP (Leonowicz et al. 1999). Other enzymes are involved in feedback mechanisms and serve to link lignocellulose degradation pathways. These comprise glucose oxidase (E.C. 1.1.3.4), aryl alcohol oxidase (E.C. 1.1.3.7), cellobiose: quinone oxidoreductase (E.C. 1.1.5.1), and cellobiose dehydrogenase (E.C. 1.1.99.18) (Leonowicz et al. 1999). A model for the interaction of these enzymes in the degradation of lignin by white-rot fungi has been proposed (Leonowicz et al. 1999).

The physiology of LiP, MnP, and Lac has been extensively studied using submerged liquid cultures and is comprehensively reviewed elsewhere (Buswell and Odier 1987; Boominathan and Reddy 1992; Wariishi et al. 1992; Reddy and D'Souza 1994; Hattaka 1994; Thurston 1994; Orth and Tien 1995; Leonowicz et al. 1999). LME production occurs during secondary metabolism and is induced by limited nutrient levels, particularly nitrogen. Some taxa have, however, been demonstrated to produce LiP, MnP, and Lac under conditions of nitrogen sufficiency (e.g. Buswell et al. 1984). Production of LiP and MnP is generally optimal at high oxygen tensions, but is repressed by agitation of fungi grown in submerged liquid culture (conversely, Lac production is generally enhanced by agitation). Genes encoding LiP, MnP (e.g. Gold and Alic 1993; Reddy and D'Souza 1994;), and Lac (e.g. Mansur et al. 1997) have been characterized, and current evidence suggests that all three enzymes are encoded by gene families that allow complex regulation, and production of multiple isoforms. Recent research has shown that nutrient nitrogen levels, mediator compounds, and required-metal (i.e. Mn^{2+} for MnP, Cu^{2+} for Lac) concentrations affect transcription levels of LiP (Li et al. 1994), MnP (Ruiz-Duenas et al. 1999), and Lac (Collins and Dobson 1997; Palmieri et al. 2000).

Specificity of white-rot fungal LMEs

The enzymes LiP, MnP, and Lac involved in lignin degradation are highly non-specific with regard to their substrate range; this is unsurprising considering their mode of action via the generation of radicals. A fortuitous result of this is that ligninolytic enzymes of white-rot fungi have been shown as capable of in vitro transformation or mineralization of a wide range of highly recalcitrant organopollutants with structural similarities to lignin (Fig. 1). This is particularly remarkable since many are xenobiotics that have never before been encountered in nature, and in many cases (e.g. PAH, with more than four benzene rings) white-rot fungi are the only organisms capable of their breakdown.

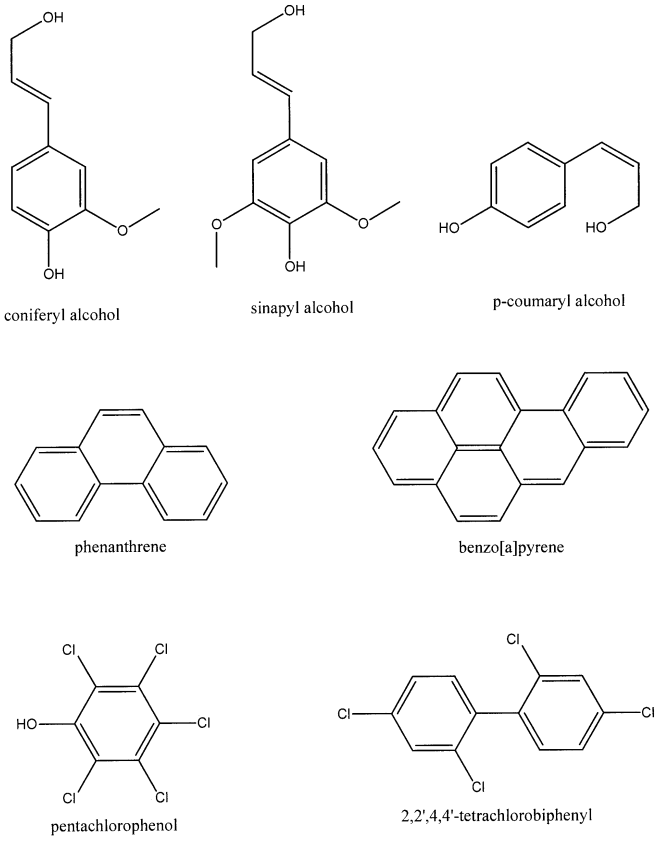

Fig. 1 Lignin monomers, polycyclic aromatic hydrocarbons (PAH), and halogenated compounds mineralized by the ligninolytic enzyme system of white-rot fungi

Evidence for LME-mediated organopollutant degradation by white-rot fungi

White-rot fungi have been demonstrated as capable of transforming and/or mineralizing a wide range of organopollutants, and in most cases LMEs have been implicated (Reddy 1995). The diversity of known substrate compounds is reviewed below; these are grouped mainly into end-use functional categories rather than chemical class, to aid the reader in locating information on biodegradability by white-rot fungi of compounds used in a given industry or process.

Munitions waste

Safe disposal of munitions waste is a constant problem for the military. Not only does the detonation risk of explosives render them hazardous, but the constituent compounds are also toxic and persistent in the environment. These are released during explosive manufacture (e.g. "red water" from TNT production) and also from underground disposal sites, where they come into soil and groundwater contact. In addition, non-explosive chemical warfare agents also present obvious problems of environmental toxicity.

TNT (2,4,6-trinitrotoluene)

World production of TNT has been estimated at 900,000 kg annually (Harter 1985). It is well known that under anaerobic conditions certain bacteria carry out reductive transformation of TNT, although mineralization does not occur (Bennett 1994). In addition a photocatalytic TiO_2-assisted reduction of TNT has been demonstrated, but without significant mineralization (Hess et al. 1998). Recent research, however, has shown that white-rot fungi are capable of oxidative TNT attack, offering potential for mineralization of explosives. There are no reports of white-rot fungi being isolated from TNT contaminated soils, although members of the genera *Alternaria*, *Aspergillus*, *Penicillium*, and *Trichoderma* have been isolated from composted TNT, with limited TNT biotransformation ability (Bennett et al. 1995). This may reflect their tolerance to the compound rather than an ability to mineralize it, since non-TNT-mineralizing strains of *Trichoderma viridae* and *Cladosporium resinae* have been shown to grow in the presence of up to 200 ppm TNT in an agar-based growth medium (Bayman and Radkar 1997). To date, fungal mineralization of TNT has been demonstrated only for certain white-rot basidiomycetes.

Transformations of TNT result in formation of the dinitrotoluenes (DNTs) 2-amino-4,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, and 4-amino-2,6-dinitrotoluene. These compounds are generally not degraded further, or are dimerized to even more persistent azo- and azoxy dimers (Bumpus and Tatarko 1994). Several white-rot fungi are able to transform TNT to DNTs (e.g. Donnelly et al. 1997), as are many bacteria and mitosporic fungi. Significantly, only white-rot fungi have been shown as capable of DNT degradation, and mineralization to CO_2. *Phanerochaete chrysosporium* has been the organism of choice in such studies (Bumpus and Tatarko 1994; Hawari et al. 1999; Jackson et al. 1999; Hodgson et al. 2000). That this degradation is carried out by ligninolytic enzymes has been conclusively demonstrated by mineralization studies using purified MnP (Scheibner and Hofrichter 1998; Van-Aken et al. 1999). Addition of the surfactant Tween-80 to ligninolytic cultures of *P. chrysosporium* enhanced TNT mineralization two-fold (from 13.9% to 29.3%, over 24 days), although this was not due to enhanced LME production (Hodgson et al. 2000). Such mineralization has been shown to reduce mutagenicity of aqueous TNT wastes by up to 94%, as measured using the *Salmonella*/microsome bioassay (Donnelly et al. 1997).

Most mineralization studies have employed relatively low TNT concentrations; however, one study has shown that *P. chrysosporium* can germinate from spores, grow vegetatively and transform TNT at 20 ppm (Spiker et al. 1992). Furthermore, when TNT was added to established liquid-grown cultures, the fungus was able to transform TNT at levels of up to 100 ppm, although further germination and vegetative growth were inhibited. This may not reflect tolerance to TNT in soils, indeed Fernando et al. (1990) demonstrated TNT mineralization by *P. chrysosporium* in soils when present at levels of up to 10 000 ppm.

Other munitions

Nitroglycerin (glycerol trinitrate) mineralization has been demonstrated by *P. chrysosporium* in mixed culture with bacteria; however, anaerobic mineralization of this compound by bacteria occurs at a faster rate (Bhaumik et al. 1997). Transformation of the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) has also been demonstrated for *P. chrysosporium*, although without complete mineralization (Bayman et al. 1995). The mass-produced chemical warfare agent Yperite (bis(2-chloroethyl) sulphide) has been shown to be completely mineralized by the white-rot fungus *Trametes (Coriolus) versicolor*, although the role of the ligninolytic enzyme system has yet to be confirmed (Itoh et al. 1997).

Pesticides

Organochlorine insecticides such as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), lindane, and the aldrins (aldrin, dieldrin, endosulfan) have been manufactured and applied in vast quantities since the 1940s. Organochlorine herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2-methyl-4,6-dichlorophenoxyacetic acid (MCPA) have also been widely used, with the concomitant generation of dioxins. These compounds found infamy as components of "Agent Orange," used extensively as a defoliant during the Vietnam War. Although the use of organochlorines has largely been discontinued in developed countries, their use continues in some developing nations (Alloway and Ayres 1993). Organochlorines are persistent in the environment, and as a result of biomagnification through the food chain, several organochlorine-linked toxic effects/population declines at higher trophic levels have been recorded (Alloway and Ayres 1993). Other classes of pesticide, including organophosphorous, methylcarbamate, and synthetic pyrethroid insecticides that were developed to replace organochlo-

rines also present problems of toxicity and persistence. Similarly, newer herbicides such as chlorophenoxyalkanoates, triazines, and other hydrocarbons (i.e. PCBs) have also been found to create toxicity and persistence problems in the environment. The reader's attention is drawn to the similarity in chemical structure between certain pesticides and other classes of compound that are degraded by white-rot fungi (i.e. PAH, PCBs, chlorinated synthetic polymers, wood preservatives). The biodegradation of these compounds is further discussed in later sections of this review.

Organochlorines

Although DDT is highly persistent, it is degraded slowly in the environment. Certain bacteria are capable of DDT mineralization, with the proposed pathway involving reductive de-chlorination, followed by further dechlorination, oxidation, and decarboxylation prior to ring cleavage (Bumpus and Aust 1987). White-rot fungi have also been shown as capable of mineralizing DDT. Three strains of *P. chrysosporium* plus *Pleurotus ostreatus*, *Phellinus weirii*, and *Polyporus versicolor* were able to mineralize 5.3–13.5% of added ^{14}C-radiolabeled DDT, dicofol, and methoxychlor over 30 days under ligninolytic growth conditions (Bumpus and Aust 1987). The role of LMEs was not established, and further work showed extracellular LMEs of *P. chrysosporium* were incapable of DDT degradation (Kohler et al. 1988). Biodegradation of DDT can result in toxic and persistent metabolites. One study has shown that ^{14}C-radiolabeled 1,1-dichloro-2,2-bis(4-chlorophenyl)ethene (DDE), an extremely toxic and persistent DDT-breakdown product, is mineralized to $^{14}CO_2$ by *P. chrysosporium* (Bumpus et al. 1993). The mineralization of the dioxin 2,7-dichlorodibenzo-*p*-dioxin by *P. chrysosporium* has been demonstrated (Valli et al. 1992). In this study purified LiP and MnP were capable of mineralization in a multi-step pathway involving sequential oxidation, reduction, and methylation reactions to remove the two Cl atoms and carry out ring cleavage. *P. chrysosporium* has also been shown to mineralize 9.4–23.4% of ^{14}C-radiolabeled aldrin, dieldrin, heptachlor, chlordane, lindane, and mirex over 30 days (Kennedy et al. 1990). Of these, only lindane and chlordane underwent extensive biodegradation. No involvement of LMEs has yet been demonstrated in organochlorine pesticide mineralization. A model for white-rot fungal pesticide degradation has recently been proposed, in which distinct hydrolytic and oxidative (including cytochrome P-450 mediated) pathways are involved, on the basis of metabolite analysis of *P. chrysosporium* degradation of endosulfan (Kullman and Matsumura 1996). One study has also studied bio-sorption of lindane to non-viable biomass of *Rhizopus oryzae* (not a white-rot fungus) (Young and Banks 1998). Adsorption of the negatively charged lindane to negatively charged mycelium was facilitated by hydrogen ions acting as bridging ligands.

Biodegradation of the organochlorine herbicide 2,4,5-T and 2,4-D by *P. chrysosporium* has been demonstrated (Ryan and Bumpus 1989; Yadav and Reddy 1993a), although the role of ligninolytic enzymes in this process was not confirmed. Studies on the metabolism of 2,4-D and MCPA by *Aspergillus niger* (not a white-rot fungus) revealed that ester hydrolysis and hydroxylation reactions are involved in catabolism of these herbicides (Faulkner and Woodcock 1964).

Other pesticides

The organophosphate insecticides are not generally persistent, and *P. chrysosporium* has been demonstrated to mineralize 12.2–27.5% of ^{14}C-radiolabeled chloropyrifos, fonofos, and terbufos during an 18-day incubation (Bumpus et al. 1993). No confirmation that this was due to the action of LMEs was obtained, and hydrolytic cleavage of the organophosphates fenitrothion and fenitrooxon by the non-ligninolytic fungus *Trichoderma viridae* (Baarschers and Heitland 1986) suggested that the process is not mediated by LMEs. The chlorinated triazine herbicide 2-chloro-4-ethylamine-6-isopropylamino-1,3,4-triazine (atrazine) is recalcitrant in the environment, although the white-rot fungi *P. chrysosporium* (Mougin et al. 1994) and *Pleurotus pulmonarius* (Masaphy et al. 1993) have both been demonstrated to transform atrazine, yielding hydroxylated and *N*-dealkylated metabolites.

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are produced by chlorination of biphenyl, and many different congeners are produced which vary in their degree of substitution. They have a variety of industrial uses including dielectric fluids, flame retardants, heat-transfer fluids, hydraulic fluids, organic diluents, plasticizers and solvent extenders. They have been used extensively and are now ubiquitous in the environment, where they present problems of toxicity and persistence (Alloway and Ayres 1993). Aerobic bacteria are known to attack less-chlorinated (1–6 Cl substitutions) congeners via a dioxygenase, although more highly chlorinated congeners are not transformed (Abramowicz 1990). Some reductive dechlorination of these compounds has, however, been recorded for anaerobic sediment bacteria (Abramowicz 1990).

Numerous studies have shown that white-rot fungi including *Coriolopsis polyzona*, *P. chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor* are all capable of significant PCB (with 1–6 Cl substitutions) removal (as measured by substrate disappearance) in vivo (Zeddel et al. 1993; Yadav et al. 1995; Novotny et al. 1997). There appears to be little evidence for congener specificity, with removal rates in most studies not correlated to the degree of chlorine substitution. In some studies, high

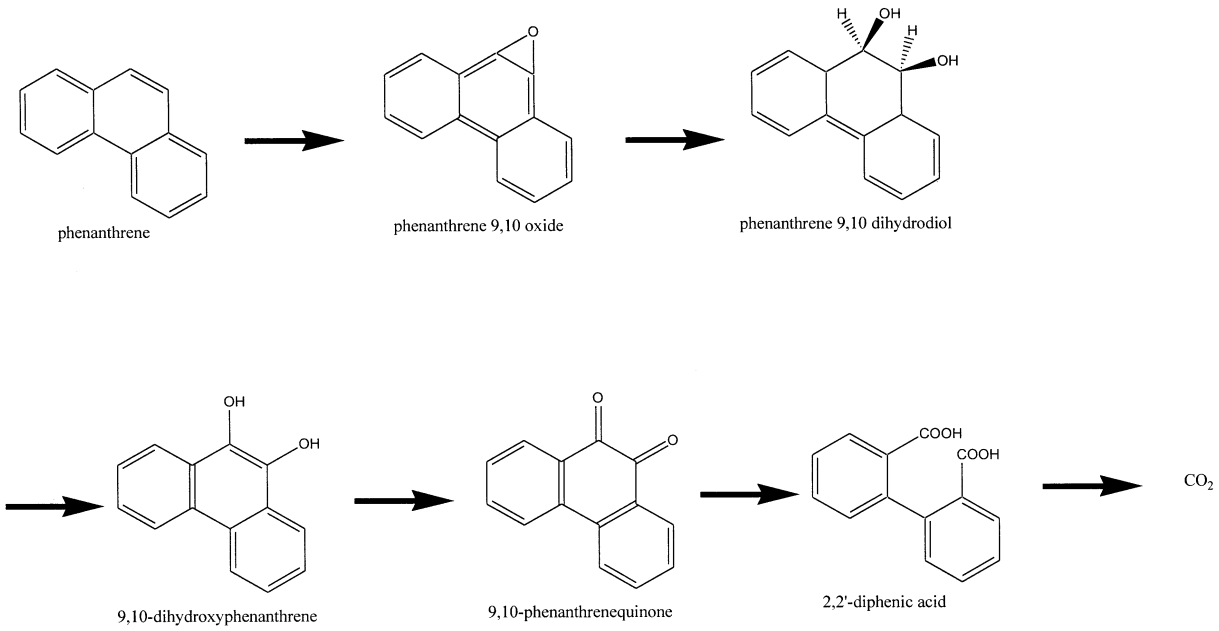

Fig. 2 Proposed degradation pathway for phenanthrene by white-rot fungi (after Bezalel et al. 1996). The formation of dihydrodiol, quinone, and diphenic acid metabolites has been shown to vary among species

levels of PCB removal were correlated to high LME production by fungi (e.g. Novotny et al. 1997), although in other studies PCB removal was independent of any LME production or activity in the culture medium (e.g. Kremar and Ulrich 1998). Studies using ^{14}C-radiolabeled PCBs show that *Coriolopsis polyzona* (Vyas et al. 1994), *P. chrysosporium* (Dietrich et al. 1995), and *Trametes versicolor* (Beaudette et al. 2000) are capable of mineralizing PCBs, but the exact role of LMEs in this process is not clear. The dehalogenation of PCBs by a commercial preparation of horseradish peroxidase and purified Lac of *T. versicolor* has been demonstrated (Dec and Bollag 1995), and this is probably due to LME-mediated free radical production. Furthermore, rates of peroxidase- and Lac-mediated (from *T. versicolor*) PCB attack were stimulated in the presence of a 2,6-dimethoxyphenol co-substrate (Roper et al. 1995). The ability to transform PCBs has also been recorded for some non-ligninolytic taxa including ectomycorrhizal and mitosporic fungi (Dmochewitz and Ballschmitter 1988; Donnelly and Fletcher 1995).

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are benzene homologues formed from the fusion of four or more benzene rings. For the purpose of evaluating white-rot fungal mineralization, the definition of PAH here is extended to also include the three-ringed homologues anthracene and phenanthrene. A considerable number of individual PAH are found, and these arise from natural oil deposits and vegetation decomposition, in addition to considerable anthropogenic production from the use of fossil fuels in heating and power production, wood burning, vehicular transport, runoff from bitumen roads, waste incineration, and industrial processes (Alloway and Ayres 1993). These compounds present huge problems of toxicity and persistence in the environment. Significantly, the white-rot fungi are the only organisms capable of significant PAH mineralization, although it is worth mentioning that some mitosporic and ectomycorrhizal fungi have also been shown to oxidize PAH, although to a lesser extent than white-rot fungi (Gramss et al. 1998). Despite apparent non-LME mediated PAH mineralization by some white-rot fungi grown under non-ligninolytic conditions (e.g. by *P. chrysosporium*, Dhawale et al. 1992), there is substantial and conclusive evidence that ligninolytic enzymes are involved in PAH mineralization by white-rot fungi (Fig. 2). A (cell-associated) cytochrome P-450 monooxygenase mediated PAH transformation reaction is also thought to occur in certain white-rot fungi (Sutherland et al. 1995; Bezalel et al. 1997).

Several studies have shown that diverse white-rot fungi are capable of PAH mineralization, and that rates of mineralization correlate with the production of LMEs (e.g. Field et al. 1992; Sack et al. 1997). Several taxa, including *P. chrysosporium* (e.g. Bumpus 1989), *Pleurotus* sp. (e.g. Bezalel et al. 1996a), and *Trametes versicolor* (e.g. Morgan et al. 1991) are noteworthy for their significant PAH-mineralizing capabilities. Experiments with purified cell-free enzyme extracts have confirmed the role of LMEs in PAH attack. Extracellular preparations of LiP from *P. chrysosporium* were among the first to be shown as capable of PAH oxidation (Haemmerli et al. 1986; Hammel et al. 1986; Bumpus 1989). More recently, the purified LiP of *Nematoloma forwardii* has been shown to oxidize anthracene and pyrene in the presence

of the mediator veratryl alcohol (Guenther et al. 1998). LiP-mediated PAH metabolism is thought to occur via an one-electron oxidation to yield quinone products. The theoretical upper limit for LiP substrates is an ionization potential (IP) of around ~7.55, however, white-rot fungal cultures typically transform PAH with IP far higher than this. Moreover, LiP is often not expressed in (known LiP-producing) white-rot fungal cultures incubated with PAH. Since MnP and Lac appear to be the predominant LMEs produced during PAH metabolism, most research has focused on these enzymes. The purified MnP (in a MnP-lipid peroxidation system) of *P. chrysosporium* has been shown to oxidize twelve 3–6 ring PAH (Bogan and Lamar 1995; Bogan et al. 1996a, b, c). The metabolism of PAH was closely correlated with IP of the substrate, even for those above the threshold IP for either Mn^{3+}- or LiP- mediated reactions. Further evidence of lipid-peroxidation-coupled MnP-mediated PAH oxidation was observed for *Phanerochaete laevis*, which produced predominantly polar products, with no significant quinone accumulation (Bogan and Lamar 1996). By contrast, MnP of *N. forwardii* (in a lipid-peroxidation-coupled reaction) oxidized anthracene and pyrene to produce quinone products (Guenther et al. 1998). These were not further metabolized, but some transformation of hydroxylated products occurred. PAH transformation by MnP has also been shown to be limited by Mn^{2+} availability (Bogan and Lamar 1996). Purified Lac from *Trametes versicolor* has been shown to oxidize a range of 3–5 ring PAH in the presence of the chemical mediators HBT and ABTS (Collins et al. 1996; Johannes et al. 1996; Majcherczyk et al. 1998). A similar PAH substrate range was found for purified Lac of *Coriolopsis gallica* (Pickard et al. 1999). Significant oxidation of PAH with IP values below ~7.45 eV (e.g. anthracene, pyrene, benzo[a]anthracene, benzo[a]pyrene) occurred with Lac of *Trametes versicolor*. PAH of higher IP also underwent oxidation (e.g. acenapthene, acenapthylene, flourene), although with no obvious correlation between oxidation and IP value (Majcherczyk et al. 1998). Results appear to vary between investigators and fungi. For example, Collins et al. (1996) found that *Trametes versicolor* Lac was unable to oxidize phenanthrene and attributed this to the high IP (IP 7.91) of the substrate, conversely the Lac of *Coriolopsis gallica* was capable of oxidizing phenanthrene (Pickard et al. 1999). In all cases quinone degradation products were formed.

Bleach-plant effluent

The production of high-quality paper requires a chlorine-mediated bleaching process to remove color associated with the 5–10% residual lignin in pulp. As a result, large aqueous volumes of toxic, low molecular mass, halogenated lignin degradation products are released into the environment from bleach-plants. These include chlorolignins, chloro-phenols, chloro-guaiacols, chloro-catechols, and chloro-aliphatics (Neilson et al. 1991). Such effluents typically contribute 50–60% of the biochemical oxygen demand (BOD) from a pulp mill's total waste (Virkola and Honkanen 1985). Anaerobic (reductive) dehalogenation of such compounds by bacteria occurs (Rintala and Puhakka 1994); however, the knowledge that white-rot fungi can mineralize halogenated organic compounds offers exciting prospects for oxidative bioremediation treatments.

Oxidative demethylation and dechlorination of bleach-plant effluent, with associated decolorization, has been demonstrated for *P. chrysosporium* (Michel et al. 1991; Jaspers et al. 1994). These studies showed that MnP was responsible for most decolorizing activity, with minor involvement of LiP. High decolorization efficiencies (up to 85% chloro-guaiacol degradation) by Lac of *Trametes versicolor* have also been demonstrated (Archibald et al. 1990; Limura et al. 1996).

Synthetic dyes

Synthetic dyes are chemically diverse, with those commonly used in industry divided into those of azo, triphenylmethane or heterocyclic/polymeric structure (Gregory 1993). They are used extensively in the biomedical, foodstuff, plastic, and textile industries, where it is estimated 10–14% of dye is lost in effluent during the dyeing process (Vaidya and Datye 1982). Synthetic dyes share a common feature in that they are not readily biodegradable; when discharged to the environment they are therefore persistent and many are also toxic (e.g. Michaels and Lewis 1985). Early studies showed that polymeric dyes were decolorized by ligninolytic cultures of *P. chrysosporium*, and that inhibitors of lignin degradation also inhibited dye decolorization (Glenn and Gold 1983). Subsequent work has revealed the ability to decolorize a wide array of azo, triphenylmethane and heterocyclic dyes among white-rot fungi (Pasti-Grigsby et al. 1992; Pointing et al. 2000b). Although most studies have used the "standard" test organism *P. chrysosporium*, dye-decolorizing ability among diverse taxa (e.g. 16 different fungal strains; Rodriguez et al. 1999) and white-rot fungi with very different LME-producing characteristics has also been recorded (e.g. *Pycnoporus sanguineus* decolorized azo and triphenylmethane dyes while producing Lac as the sole LME, Pointing and Vrijmoed 2000). The role of sorption in dye decolorization (by removal of dye from solution, without degradation, due to binding/uptake by mycelia) appears to be minimal. Non-ligninolytic cultures of *P. chrysosporium* were found to have 11–49% of total azo and heterocyclic dye bound to the mycelium (Cripps et al. 1990). Conversely, sorption accounted for less than 3% of azo and triphenylmethane dye removal by ligninolytic (dye-decolorizing) cultures of *P. sanguineus* (Pointing and Vrijmoed 2000).

The involvement of LMEs in the dye decolorization process has been confirmed in several independent studies using purified cell-free enzymes. LiP of *P. chrysospo-*

rium has been shown to decolorize azo, triphenylmethane, and heterocyclic dyes in the presence of veratryl alcohol and H_2O_2 (Cripps et al. 1990; Ollikka et al. 1993). It is interesting that the ability of enzyme preparations to decolorize identical dyes varied between the two studies. The latter demonstrated different specificities between lignin peroxidase isoforms, and this may help to explain some of the variability in results obtained between laboratories (i.e. that differential expression of lignin peroxidase isoforms by different taxa or culture conditions may result in variable dye-decolorizing ability). The MnPs of *Bjerkandera adusta* and *Pleurotus eryngii* have also been shown to catalyze dye decolorization (Heinfling et al. 1998). The enzymes from both fungi were unusual in that they did so in Mn^{2+}-independent reactions (i.e. the Mn^{3+}-lactate complex was not the oxidative agent created by the enzyme). Two Lac isoenzymes purified from *Trametes hispida* were able to catalyze decolorization of several synthetic dyes (Rodriguez et al. 1999).

White-rot fungi have been shown as superior dye decolorizers, particularly in comparison to prokaryotes which are generally poor or non-decolorizers (e.g. Yatome et al. 1981). Even the lignin-transforming actinomycete *Streptomyces chromofuscus* is a weak decolorizer compared to *P. chrysosporium* (Paszczynski et al. 1992). The question as to whether fungal dye decolorization represents mineralization has been addressed by relatively few studies. Mineralization rates of 23.1–48.1% for a wide range of ^{14}C-ring-labeled azo dyes after a 12-day incubation with *P. chrysosporium* have been recorded (Spadaro et al. 1992). Those dyes with hydroxyl, amino, acetamido, or nitro substitutions to aromatic rings were mineralized more extensively than those with unsubstituted rings. Another study confirmed the ability of ligninolytic cultures of *P. chrysosporium* to mineralize ^{14}C-radiolabeled azo dyes, yet found no correlation between aromatic ring substitution pattern and mineralization rates (Paszczynski et al. 1992). Toxic intermediates probably do not accumulate during dye decolorization and mineralization, although experimental evidence is lacking.

Synthetic polymers

Synthetic polymers (“plastics”) are used widely in modern society. Their contribution to landfill and high visibility as discarded waste due to their recalcitrance make studies on plastic biodegradability of particular environmental relevance. Biodegradation of several synthetic polymers with diverse chemical composition has been recorded, although few of these have been attributed to LMEs of white-rot fungi. Polyurethane, which is formed from condensation of polyisocyanate and polyol, is used as a base material in many industries. Polyurethanes are known to be biodegradable, and bacterial degradation, via the action of polyurethane esterases and depolymerases, of polyurethane, polyester polyurethanes, and polyether polyurethanes has been reported, (Nakajima et al. 1999). Fungal degradation of polyurethane has also been reported; a species of *Curvularia senegalensis* (not a white-rot fungus) was shown to degrade the polymer via secretion of an extracellular esterase (Crabbe et al. 1994). The ability of white-rot fungi to degrade polyurethanes has not been tested. Polyethylenes are widely used in the manufacture of plastic products. Three ligninolytic bacteria (*Streptomyces* spp.) and *P. chrysosporium* were tested for their ability to degrade polyethylenes in pure culture, but only the bacteria were capable of significant degradation (Lee et al. 1991). Seven white-rot fungal species were evaluated for their ability to depolymerize polyvinylchloride (PVC), a widely used synthetic textile, under ligninolytic culture conditions (Kirbas et al. 1999). Significant depolymerization (as measured by loss in C-H bonds using FTIR) was achieved by *P. chrysosporium*, *Pleurotus sajor caju*, and *Polyporus versicolor*, with lower levels of depolymerization observed for four other *Pleurotus* species. Although the experiment was carried out in LME-inducing culture medium, no attempt was made to quantify enzyme production or demonstrate substrate degradation by purified enzymes. The role of LMEs in this process cannot therefore be confirmed at this stage. Polyvinyl alcohol (PVA) is a polymer with commercial applications in adhesive manufacture and paper/textile coatings, due especially to its exceptional cellulose-bonding ability. A hydrolase-mediated degradation of PVA has been reported for the bacterium *Pseudomonas vesicularis*, with purification of a 2,4-pentanedione hydrolase (Kawagoshi and Fujita 1998). Oxidative PVA degradation was recorded for the white-rot fungus *Pycnoporus cinnabarinus* in liquid culture (Larking et al. 1999). Degradation rates correlated with production of Lac; no peroxidases were secreted by the fungus. Other white-rot fungi may have potential to degrade this polymer since it has been shown that peroxidase-type degradation of PVA using Fenton’s reagent is possible (Larking et al. 1999). Nylon is a linear amide-linked polymer widely used in the textile industry. Significant degradation of a nylon-66 membrane was observed for *P. chrysosporium* and *Trametes versicolor* under ligninolytic culture conditions (Deguchi et al. 1997). A nylon-degrading enzyme was later purified and characterized from an un-named white-rot fungus (IZU-154) (Deguchi et al. 1998). The characteristics of the purified protein were identical to those of MnP, although the method of catalysis was shown to involve peroxidase-oxidase reactions (as seen for horseradish peroxidase), rather than the peroxidase-Mn^{3+} type of reaction more typical of MnPs. Electron micrographs revealed that nylon degradation involved an initial stripping off of the surface, followed by regular horizontal grooves of erosion on polymer fibers.

Wood preservatives

The organic wood preservatives creosote and pentachlorophenol (PCP) have been widely used due to their high

efficacy, although their use now has largely been discontinued as a result of environmental concerns. Creosote is a coal-tar distillation product, comprising a highly heterogenous PAH mixture (which includes 16 of the United States EPA priority-listed pollutants). Although worldwide use is declining, consumption of creosote in the USA alone was estimated at 336×10^6 l in 1993 (Micklewright 1994). PCP is a benzene ring with five Cl substitutions, and is listed as a priority pollutant by the United States EPA. The use of PCP is now restricted in the USA, although consumption for wood treatment in 1993 was estimated at 9.4×10^6 kg (Micklewright 1994). The use of PCP has been discontinued in Finland, Japan and Sweden. Preservative-treated wood in-service is not considered a pollution threat, but contamination of soil and groundwater at manufacture and treatment plants has raised environmental concerns.

Creosote

Evidence for creosote-mineralizing capability among white-rot fungi can be inferred from PAH-mineralizing abilities (see preceding section on PAH), although it is important to consider the following. Firstly, creosote is a complex mixture which may be more toxic to fungi than single PAH or simple mixtures of the component PAH which are commonly used in laboratory studies. One study has shown that soft-rot fungi isolated from creosote-treated wood can actively grow on filter paper impregnated with 100% w/w creosote (Ribichich and Lopez 1996), suggesting fungal populations may adapt to this otherwise toxic mixture. Secondly, biodegradation of PAH as single compounds may occur at different rates and with different pathways than for complex PAH mixtures such as creosote (e.g. Kennes and Lema 1994). These, and other considerations, are discussed in the subsection of this review entitled "Bioremediation using LME-producing white-rot fungi."

Pentachlorophenol

The biodegradation of PCP has been extensively researched, and studies on the bioremediation of this compound have perhaps received more attention than that of any other organo-pollutant (e.g. the recent and comprehensive review by Litchfield and Rao 1998). The complete mineralization of PCP, involving oxidation and reductive dehlogenation steps, has been recorded for *Arthrobacter* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Rhodococcus* sp., and methanogens, with successful bioremediation technologies based on prokaryotes having been developed for PCP treatment (reviewed in Litchfield and Rao 1998).

P. chrysosporium has been demonstrated to mineralize up to 50.5% of ^{14}C-radiolabeled PCP when grown under ligninolytic culture conditions (Mileski et al. 1988), although no confirmation that LMEs were involved was obtained. Growth of seven species belonging to the white-rot fungal genus *Phanerochaete* was severely reduced in the presence of even low levels (5 ppm) of PCP (Lamar et al. 1990), although *P. chrysosporium* and *P. sordida* were able to grow, albeit at a reduced rate, in the presence of 25 ppm PCP. Both these fungi degraded PCP, which occurred in a two-stage process. Firstly, a rapid and extensive (64% and 71% respectively) depletion of PCP resulted in accumulation of the product pentachloroanisole (PCA) (quinone intermediates, expected for LME-mediated oxidation, were not assayed for in this study). This intermediate was then slowly mineralized by both fungi. No confirmation that LMEs were involved in this process was obtained, and PCP mineralization by some white-rot isolates has been found to occur under non-ligninolytic culture conditions. Further work, however, demonstrated up to 70% PCP mineralization by cell-free enzyme preparations of *P. chrysosporium.* Generation of the expected oxidation intermediate strongly suggested a role for extracellular peroxidases, although cell-associated (cytochrome P-450 mediated?) oxidation of PCP by *P. chrysosporium* also occurred (Lin et al. 1990). The role of Lac in PCP degradation is unclear. *Trametes versicolor* was able to mineralize ^{14}C-radiolabeled PCP (21% in 17 days) while secreting Lac (but not peroxidases) in a ligninolytic culture medium, however no correlation between Lac secretion and degradation was observed (Ricotta et al. 1996). Purified Lac was able to degrade PCP but produced chloranil as a metabolite, suggesting a different method of catalysis compared to the peroxidase-mediated reactions of *P. chrysosporium*.

Other compounds

Our knowledge about the range of organopollutants capable of degradation by white-rot fungal LMEs is likely to increase as research continues. The preceding sections have reviewed evidence for degradation of certain "high profile" compounds that have received considerable research attention. Other structurally similar pollutants have received less attention, but evidence suggests that they are also transformed or mineralized by the ligninolytic enzyme system of white-rot fungi. For example benzene, toluene, ethylbenzene, and xylenes (BTEX, used in gasoline and aviation fuels) are degraded by *P. chrysosporium* (Yadav and Reddy 1993b).

Bioremediation using LME-producing white-rot fungi

The use of microorganisms, either naturally occurring or introduced, to degrade pollutants is called bioremediation. The aim of bioremediation is to reduce pollutant levels to undetectable, non-toxic, or acceptable (i.e. within limits set by regulatory agencies) levels. Bioremediation technology is receiving increasing attention for the restoration of polluted sites where biodegradation

is occurring slowly or imperceptibly, and operating bioreactors that facilitate contact between pollutants and microorganisms, and encourage rapid pollutant degradation. In bioremediation, the goal is to completely mineralize organopollutants to CO_2, or in the case of metals to remove them by sorption or transformation to a less toxic form. It is not within the scope of this review to consider the theory and practice of bioremediation in detail; the reader is directed to recent reviews by Alexander (1994) and Allard and Neilson (1997) for further reading. It is, however, worth identifying the major requirements for successful bioremediation in order to better understand the recent progress in research on white-rot fungi. These are (from Alexander 1994):

a. Microorganisms must exist that have the needed catabolic activity.
b. Those organisms must have the ability to transform the compound at reasonable rates and bring the concentration to levels that meet regulatory standards.
c. They must not generate products that are toxic at the concentrations likely to be achieved during the remediation.
d. The site must not contain concentrations or combinations of chemicals that are markedly inhibitory to the biodegrading species, or means must exist to dilute or otherwise render innocuous the inhibitors.
e. The target compound(s) must be available to the microorganisms.
f. Conditions at the site or in a bioreactor must be made conducive to microbial growth or activity. For example, an adequate supply of inorganic nutrients, sufficient O_2, or some other electron acceptor, favorable moisture content, suitable temperature, and a source of C and energy for growth if the pollutant is to be cometabolized.
g. The cost of the technology must be less or, at worst, no more expensive than that of other technologies that can also destroy the chemical.

Most bioremediation currently conducted on a commercial scale utilizes prokaryotes, with comparatively few and recent attempts to use white-rot fungi. White-rot fungi, however, offer advantages over bacteria in the diversity of compounds they are able to oxidize, notably the larger PAH. The extracellular nature of white-rot fungal LMEs and low-molecular-mass mediators may also enhance bioavailability of pollutants to white-rot fungi in situations where bacteria, with their cell-associated pollutant catabolism, may not have access. The most significant difference between bacterial and white-rot fungal pollutant catabolism relates to their biochemistry. Bacteria generally utilize organopollutants as a nutritional C and/or N source, whereas substrate oxidation by LMEs of white-rot fungi yields no net energy gain. An additional C and N source is therefore required for primary metabolism by white-rot fungi. White-rot fungi are obligate aerobes, whereas bacteria can transform and mineralize certain pollutants under aerobic, microaerophillic, and anaerobic conditions.

Bioremediation technology can be separated into two approaches: in situ treatments and bioreactors. In the following sections, factors relating to the feasibility of using white-rot fungi for organopollutant removal employing these approaches are considered, with reference to published academic literature (It should be noted, however, that details of bioremediation projects are not always readily available in the public domain).

In situ bioremediation

In practice, bioremediation of organopollutants in situ generally applies to contaminated soils. Two approaches are recognized: biorestoration, in which the physicochemical nature of the soil (e.g. nutrients, aeration) is altered to encourage indigenous microorganisms to degrade the pollutant, and bioaugmentation, in which a known pollutant-degrading microorganism is introduced to the contaminated soil (with or without physico-chemical alteration). Despite their ubiquity in many soils, white-rot fungal involvement has not been documented in biorestoration research. Conversely, bioaugmentation of soils with white-rot fungi appears to show some potential.

Much of the research on organopollutant transformation in soils by white-rot fungi has focused on PAH, either singly or as mixtures in creosote or other coal/oil-derived products, since the larger PAH are not substrates for soil bacteria. It is important to bear in mind, however, that the ability of white-rot fungi to mineralize PAH under laboratory conditions does not necessarily mean they are capable of doing so in soil. For example, one study has shown that whereas *Trametes versicolor* mineralized anthracene, benz[*a*]anthracene, and dibenz[*a,h*]anthracene in soil, *P. chrysosporium* and *Pleurotus sajor-caju* (also known PAH-mineralizing fungi) carried out only a limited transformation of these PAH in soil, with significant accumulation of the dead-end metabolite 9,10-anthracenedione (Andersson and Henrysson 1996). Reports of PAH degradation by white-rot fungi in soil are mixed. For example, up to 49% of added benzo[*a*]pyrene (among the most recalcitrant PAH) was removed from soil by *Pleurotus ostreatus* after 3 months incubation (Eggen and Majcherczyk 1998). In soils spiked with a mixture of 3–7-ring PAH, degradation of 3–4-ringed PAH was not enhanced above levels observed for indigenous (bacterial) microflora upon addition of *Pleurotus* sp. Conversely the 5–7-ringed PAH, which were not attacked by indigenous soil bacteria, were removed 29–42% by *Pleurotus* sp. (Gramss et al. 1999). Another study reports significant removal of 3-ring (85–95%) and 4-ring (24–72%) PAH from creosote-contaminated soil by *Phanerochaete sordida*, although larger PAH persisted at their original concentrations (Davis et al. 1993). In another study, *Pleuotus ostreatus* removed 48% of 5-ring PAH from creosote-contaminated soil, in addition to 87–89% of 3- and 4- ring PAH (Eggen 1999). A decrease in total petroleum

hydrocarbon concentration from 32 g/kg to 7 g/kg within 12 months in soil microcosms was observed for *Coriolus* (*Trametes*) *versicolor* (Yateem et al. 1998).

Degradation of PCP within soil by white-rot fungi has also been demonstrated, although currently used commercial bioremediation treatments for PCP-contaminated soil generally employ indigenous microflora (Borazjani and Diehl 1998). *Trametes versicolor* mineralized 29% of added PCP during 42 days of growth in soil (Tuomela et al. 1999). Similar high removal levels have been reported for *Phanerochaete* spp. (Lamar and Dietrich 1990), *P. chrysosporium*, and *Lentinula edodes* (Okeke et al. 1996). In all cases, low levels of anisole transformation products were detected in soil. Other compounds such as atrazine (e.g. Hickey et al. 1994) and TNT (Fernando et al. 1990), are also transformed by white-rot fungi during growth in soil.

A common feature in soil-degradation studies using white-rot fungi has been the generally low or unpredictable level of transformation and mineralization compared to laboratory studies using submerged liquid culture (e.g. Boyle et al. 1998). Contributing factors include bioavailability of the compound and growth of the fungus. Bioavailability of organopollutants is generally low in soils (e.g. PAH; Breedveld and Karlsen 2000) due to their poor solubility in water and adsorption to soil particles, although partially oxidized PAH have been shown to have increased bioavailability and biodegradability in soil (Meulenberg et al. 1997). Increased bioavailability and degradation of PAH by white-rot fungi have been demonstrated upon addition of non-ionic surfactants to soil (Bogan et al. 1999), and this may have applications in soil treatments. Soil conditions must be favorable for growth of white-rot fungi; factors such as soil moisture content, C/N ratio, and temperature have been demonstrated to affect colonization rates of white-rot fungi in soil (Okeke et al. 1996; Eggen and Sveum 1999). Interactions with indigenous microbial populations should also be considered. Soil bacteria have been shown to inhibit the growth of *P. chrysosporium* under laboratory conditions (Radtke et al. 1994), whilst in other studies addition of white-rot fungi (*Pleurotus* sp.) to soil has resulted in inhibition of indigenous soil bacteria (Gramss et al. 1999). Co-existence of white-rot fungal inoculum and indigenous microflora is probably desirable, since they have been shown to mineralize PAH as consortia (Kotterman et al. 1998; Gramss et al. 1999). In addition to considering growth acclimation of white-rot fungi in soil, it is also important to assess expression of LMEs, since this is independent of primary metabolism. The expression of LMEs by white-rot fungi during PAH degradation within soil has been demonstrated using RT-PCR (Bogan et al. 1996a, b), although this may not occur in all cases due to the high variability of soils and fungal physiology.

Further considerations are necessary when considering full-scale in situ bioremediation treatments using white-rot fungi. A suitable carrier for introducing inoculum to the soil must be found. Lignocellulosic materials such as corn cobs (Rodriguez et al. 1998) or alfalfa straw (Boyle 1995) can be directly inoculated with white-rot fungi, or spent mushroom compost (containing fungal mycelium) can be used (Eggen 1999). Other studies suggest modifying such carriers by coating with alginate-immobilized spore suspensions (Lestan and Lamar 1996). A reliable method for detecting colonization by the test fungus and efficacy of the bioremediation must also be available. One study has shown that detection of *P. chrysosporium* in soil with a mixed microbial population is possible using PCR and restriction fragment analysis (Johnston and Aust 1994), and molecular techniques undoubtedly have potential for detection of introduced bioremediation organisms. In assessing the efficacy of bioremediation in soil, it is important to monitor reductions in toxicity in addition to actual organopollutant depletion. For example, the Tradescantia-micronuclease test has been successfully used to demonstrate reduced genotoxicity in creosote-contaminated soils treated with white-rot fungi (Baud-Grasset et al. 1993). It is also worth considering that many polluted soils may contain a highly heterogenous mixture of pollutants, and this may affect white-rot fungal colonization and organopollutant removal. Nevertheless synthetic dye (Pointing et al. 2000b) and PAH (Baldrain et al. 2000) degradation by white-rot fungi have been shown to proceed in the presence of "polluting" levels of trace metals.

Bioreactors

Bioreactors are used extensively for waste treatment prior to discharge and in bioremediation, with aerobic and anaerobic treatments for the processing of solid (i.e. compost), slurry, and liquid wastes established. Most of these utilize indigenous microorganisms (e.g. PCP, Borazjani and Diehl 1998; TNT, Boopathy 2000), although some of the organopollutants treated are also known substrates for white-rot fungi. Comparatively little attention has been given to the use of white-rot fungi in bioreactors, although their good growth in soil and lignocellulosic material suggests they have potential in composting of solid waste. One application may lie in the decolorization of synthetic dye-laden wastewaters, since decolorizing efficiency in bioreactors with white-rot fungi immobilized on a solid phase frequently approaches 100% (e.g. *P. chrysosporium*, Das et al. 1995; *Pycnoporus cinnabarinus*, Schleiphake and Lonergan 1996; *Trametes versicolor*, Leidig et al. 1999).

Concluding remarks

Evidence for the ability of white-rot fungi to transform or mineralize a range of organopollutants has been reviewed here. Although in several cases the role of LMEs in this process has been confirmed, there are still unanswered questions about the catabolism of these com-

pounds, particularly the precise role and regulation of LMEs. Bioremediation using microorganisms is already an established technology, although almost all currently employed treatments use prokaryotes. Treatments employing white-rot fungi offer the possibility to expand the substrate range of existing treatments via biodegradation of pollutants that cannot be removed by prokaryotes (or by chemical means). White-rot fungal bioremediation treatments may be particularly appropriate for in situ remediation of soils, where recalcitrant compounds (e.g. the larger PAH) and bioavailability are problematic. A further application may lie in the operation of bioreactors for certain compounds (e.g. synthetic dyes) in liquid waste, where near-100% degradation efficiencies have been achieved using white-rot fungi.

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