IRG/WP 94-50021

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Section 5 Environmental Aspects

Remediation of Pentachlorophenol- and Creosote-Contaminated Soils Using Wood-Degrading Fungi

Richard T Lamar and T. Kent Kirk

Institute for Microbial and Biochemical Technology
Forest Products Laboratory
One Gifford Pinchot Drive, Madison, WI 53705, USA.

IRG Secretariat
Box 5607
S-114 86 Stockholm
Sweden

Remediation of Pentachlorophenol- and Creosote-Contaminated Soils Using Wood-Degrading Fungi.

R. T. Lamar and T. K. Kirk
Institute for Microbial and Biochemical Technology

Abstract

Microbiological treatment of hazardous wastes has generally been associated with the use of bacteria. During the past decade a significant body of evidence has accumulated that demonstrates that fungi, in particular white-rot fungi, have the ability to degrade a wide range of hazardous organic compounds (xenobiotics) and thus might also be useful for treatment of materials contaminated with these compounds. Our work has focused on the development of a soil remediation technology that is based on the xenobiotic-degrading abilities of these fungi. This work has demonstrated that the technology is useful for remediation of pentachlorophenol-contaminated soils and may also be useful for creosote-contaminated soils. In this presentation the fungi and their xenobiotic-degrading abilities will be described and a summary of applications of this technology to remediation of PCP and creosote-contaminated sites and a discussion of technological developments necessary for commercialization of the technology will be given.

Introduction

Until very recently, the literature concerning biotreatment of hazardous organic compounds (xenobiotics) dealt almost exclusively with bacteria. It is now becoming apparent that fungi, especially wood-degrading fungi, also play an important role in xenobiotic degradtion, and that they have potential for remediating contaminated soils and waters. The higher basidiomycetous fungi probably play the major role in recycling the carbon in lignocellulosics, the most abundant renewable organic materials on earth. Thousands of species of these fungi degrade the complex structural component lignin, thereby gaining access to the cellulose and hemicelluloses, which with lignin, make up lignocellulosics. During recent years, it has become clear that the lignin-degrading enzyme system of these fungi is quite nonspecific and can degrade a wide variety of man-made as well as natural non-lignin compounds. This paper--and our research--concerns the deliberate harnessing of the xenobiotic-degrading abilities of lignin-degrading fungi for remediating soils contaminated with xenobiotics. This work has demonstrated that fungal bioaugmentation, where the fungi are added to the soil via a solid substrate inoculum, is useful for remediation of pentachlorophenol (PCP)-contaminated soils and may also be useful for creosote-contaminated soils. In this paper we describe: the fungi and their xenobiotic-degrading abilities; our experiences on the application of fungal bioaugmentation to remediation of PCP- and creosote-contaminated soils and; aspects of fungal bioaugmentation that will require further developments before commercialization of the technology, will be described.

Lignin-degrading fungi

Lignin-degrading fungi are ubiquitous in nature. The most familiar are those that produce mushrooms, brackets (conks) and other sporophores on decaying trees, wood, forest litter, and other lignocellulosics. These organisms cause the white-rot type of wood decay, and are closely related to the litter-decomposing fungi. The most vigorous lignin-degraders are white-rot wood decay fungi, which are mainly basidiomycetes belonging to the orders Agasicales, Aphyllophorales,

and Tremellales. A few ascomycetes belonging to the order Sphaeriales also cause white-rot wood decay. It is the lignin-degrading fungi that have been most intensively studied for bioremediation, and it is their lignin-degrading system that seems to be important in such applications. The most studied fungus both for lignin and xenobiotic degradation is *Phanerochaete chrysosporium.* The ligninolytic system of this organism is thought to be composed of lignin peroxidase (LiP), Mn-dependent peroxidase (MnP) and glyoxal oxidase. The latter oxidizes the fungal metabolites glyoxal and methyl glyoxal with reduction of O_2 to H_2O_2, which activates the peroxidases. Lignin peroxidase oxidizes nonphenolic aromatic nuclei in lignin by one electron to generate aryl cation radicals, which degrade nonenzymatically via many reactions. Most of those reactions result in polymer cleavages, generating both aromatic and aliphatic products which are taken up by the hyphae and mineralized. Manganese peroxidase in the presence of H_2O_2, oxidizes Mn^{2+} to Mn^{3+}, which in turn can oxidize phenolic units in lignin. The aromatic metabolite veratryl alcohol seems to play multiple roles, including stimulation of the production of the enzymes and electron transfer reactions during substrate degradation. For recent reviews of lignin degradation by *P. chrysosporium,* see Kirk (1988) and Eriksson et al. (1990). Both the extracellular and intracellular components of this system apparently play roles in xenobiotic degradation.

Xenobiotics degraded by lignin-degrading fungi

Interest in using lignin-degrading fungi for degrading xenobiotics originated from research on their use to decolorize kraft pulp bleach plant effluents. These effluents contain both polymeric, chlorinated, heavily oxidized fragments of lignin, which are responsible for the highly colored nature of the effluents, and a complex mixture of chlorinated phenols and other low molecular weight components. Several lignin-degrading fungi, including *P. chrysosporium,* were found to decolorize the effluent (Eaton et al. 1982), and *P. chrysosporium* was shown to remove low molecular weight chloro-organics (Huynh *et al.* 1985). The results of these investigations led to further studies that demonstrated that *P. chrysosporium* and other fungi are able to degrade a broad range of structurally diverse xenobiotics. The list of chemicals that are mineralized by white-rot fungi ranges from the insecticides DDT and Lindane, to wood-preserving chemicals, including PCP and polynuclear aromatic (PNA) creosote components including anthracene and phenanthrene, to polychlorinated biphenyls and chlorinated dioxins.

Many of these compounds are substrates for LiP, which presumably is involved in their initial degradation. For example, Hammel et al. (1986) reported that oxidation of anthracene by LiP leads to anthraquinone, which is further metabolized by intact cultures. Other compounds mineralized by the cultures, including DDT and phenanthrene, are not substrates for LiP. However, subsequent transformation products for phenanthrene have are substrates (Tatarko and Bumpus 1993). Pentachlorophenol is oxidized by LiP with formation of tetrachloro-*p*-benzoquinone (Hammel and Tardone 1988), but whether this compound is an intermediate in its mineralization by *P. chrysosporium* is not yet known. Compounds that are mineralized but are not substrates for LiP or MnP presumably are degraded by an alternate extracellular or intracellular enzyme system, perhaps the same system that further degrades lignin decomposition products. Essentially nothing is known about the intracellular system, making its description an important subject for further research.

Studies of PCP degradation in soil by lignin-degrading fungi

Bioaugmentation of PCP-contaminated soils with selected lignin-degrading fungi has been shown to cause a rapid and extensive decrease in PCP concentrations in a variety of soils under ideal laboratory conditions (Lamar et al. 1990a, Lamar et al. 1990b). For example, PCP decreases in 3 soils, that were spiked with 50 mg PCP kg^{-1} soil and inoculated with the lignin-

degrading fungus *P. chrysosporium*, averaged 98% after 8 wks (Lamar et al. 1990a). Although this fungus has been shown to mineralize PCP in aqueous culture (Lamar et al. 1990b, Mileski et al. 1988), decreases of PCP in the three soils were primarily due to conversion to non-volatile products. The nature of these products, whether they were soil-bound or extractable was greatly influenced by soil type. The extractable and primary transformation product in PCP-spiked Marshan soil cultures of both *P. chrysosporium* and another lignin-degrading fungus, *P. sordida* was pentachloroanisole (PCA) (Lamar et al. 1990b). In *P. chrysosporium* cultures, 46% of the overall 96% PCP decrease was due to conversion to and accumulation of PCA. However, both fungi were able to degrade some of the PCA. For example, after peak PCA accumulation (1 wk) where 64% of the PCP was methylated, there was an 18% decrease in the anisole concentration during the following 7 wk in *P. chrysosporium* cultures. Decreases of PCA in *P. chrysosporium* and *P. sordida* soil cultures was not suprising because the anisole was mineralized in liquid cultures of both organisms to similar extents as was PCP (Lamar et al. 1990b).

In Batavia and Zurich soil microcosms inoculated with *P. chrysosporium* conversion to nonextractable products was the predominant fate of PCP (Lamar et al. 1990a). The nature of the nonextractable compounds has not been elucidated. However, chlorophenols including PCP have been coupled to humic materials via copolymerization reactions catalyzed by fungal phenoloxidases such as laccases and peroxidases in in vitro reactions (Dec and Bollag 1990, Bollag et al. 1988, Sakar et al. 1988) and in soil microcosms (Cheng et al. 1983). The resulting xenobiotic-humic acid hybrid polymers are non-toxic (Lyr 1962, Bollag et al. 1988) and relatively resistant to microbial degradation in soils (Dec and Bollag 1988). Direct and indirect evidence that copolymerization of xenobiotic and humic materials occurs in soils as a result of the activity of fungal phenol-oxidizing enzymes includes the immobilization of 2,4-dichlorophenol by a laccase from *Geotrichum candidum* in soil columns (Shannon and Bartha 1988) and the humification of benzo(a)pyrene by *P. chrysosporium* in soil microcosms (Qiu and MacFarland 1991), respectively.

We have reported significant but less extensive PCP decreases (i.e. 88% to 91%) in two small-scale field studies (e.g. total weight of soil for all treatments $\leq$ 24 tonnes). In these studies the ability of lignin-degrading fungi to decrease concentrations of PCP as the sole soil contaminant (Lamar and Dietrich 1990) and in mixtures with creosote (Lamar et al. 1993, Davis et al. 1993) was evaluated. In the first study, inoculation of a sandy gravel soil pH 9.6 contaminated with a commerical PCP-containing wood preservative (250-400 mg PCP kg^{-1} soil) with either *P. sordida* or *P. chrysosporium* resulted in an overall decrease of 88% to 91% of the PCP in the soil in 6.5 weeks (Lamar and Dietrich 1990). The second study was a field treatability study that was conducted at the former Brookhaven Wood Preserving pole-treatment facility in Brookhaven, MS. The objectives of the treatability study were to evaluate the ability of several lignin-degrading fungi to decrease the concentrations of PCP and PNA components of creosote in a clay soil pH 3.8 contaminated with K001 sludge and to select the best fungal treatment for use in a large-scale demonstration study that was conducted at the same site. K001 sludge is described as bottom sediment sludge from the treatment of waste waters from wood preserving processes that use creosote ad/or PCP (CFR 1990). Intial soil concentrations of PCP and total PNA analytes averaged 700 mg kg^{-1} and 2000 mg kg^{-1}, respectively. The best fungal treatment was inoculation of the soil with *P. sordida.* This resulted in an 89% removal of PCP after 8 wks (Lamar et al. 1993).

In the first field study, a small percentage (8 to 13%) of the KIP decrease caused by *P. chrysosporium* or *P. sordida* was due to methylation and accumulation of PCA (Lamar and Dietrich 1990). In this case, decreases in PCA concentrations after peak accumulations of the anisole, which occured 3 wks after inoculation, were insignificant. However, the physiological activity of the fungi during the period after peak PCA accumulation may have been severly restricted by low soil temperatures that averaged 10°C. Decreases in the PCP concentrations during ths period were small. relative to those that occurred during the first 3 wks. However, a slower rate of PCP decrease

during the last 3 wks of the study may have been caused by a limited residual supply of readily available PCP, and/or by restricted physiological activity caused by low soil temperatures. In the treatability study, approximately 11% (80 mg kg^{-1}) of the PCP was transformed to PCA after 4 wks (Lamar et al. 1993). However, after 8 wks only 3% of the overall PCP decrease was caused by formation of PCA. Thus, *P. sordida* caused a substantial decrease in the PCA concentration. This decrease occurred concurrently with significant PCP decreases. The results of these field studies (Lamar and Dietrich 1990, Lamar et al. 1993) and the soil microcosm study (Lamar et al. 1990a) indicated that both *P. chrysosporium* and *P. sordida* can degrade PCA once it is produced.
In the treatability study there was also a 70% removal of PNAs in soil inoculated with *P. sordida* after 8 wks (Davis et al. 1993). Toxicity analyses using seed germination and root elongation tests demonstrated significant detoxification of a creosote-contaminated soil after treatment with *P. chrysosporium* (Baud-Grasset et al. 1993). Extent of detoxification was highly correlated to decreases in the total concentration of 3- and 4–ring PNAs. The ability of *P. chrysosporium, P. sordida* and other fungi (Loske et al. 1990) to cause substantial decreases in PNA concentrations suggests that, with improvements in the extent of PNA removal, they might also be useful for remediation of creosote-contaminated soils

The feasibility of large-scale fungal bioaugmentation was evaluated by assessing the ability of the lignin-degrading fungus *P. sordida* to decrease the soil concentrations of pentachlorophenol (PCP) and 13 priority pollutant PNA creosote components in a field-demonstration study. This study was also performed at the Brookhaven, MS site. Inoculation of the contaminated soil with the fungus (fungal treatment) at a rate of 10% (dry wt inoculum dry wt $soil^{-1}$) was compared to amending the soil (amended control) with sterile inoculum substrate also at a 10% rate (dry wt substrate dry wt soil-1) and no treatment (nonamended control), for their effects on PCP and PNA concentrations over a 20 week period. The fungal treatment and controls were chosen on the basis of results obtained from the above-mentioned field treatability study that was conducted during the previous year (Lamar et al. 1993, Davis et al. 1993). The initial soil PCP and total measured PNA concentrations for the three treatments averaged 1058 mg kg^{-1} and 1210 mg kg^{-1}, respectively. The concentrations of PCP and PNAs varied greatly throughout the contaminated soil volume. Thus initial concentrations also varied between the treatability and demonstration studies. Despite very low initial levels of fungal biomass, determined from ergosterol levels in the inoculum and inoculated soil, there was a 64% PCP decrease to a residual concentration of 362 mg kg^{-1} in the fungal treated soil after 20 weeks of treatment. This residual concentration was statistically less than the 715 mg kg^{-1} (30% decrease) and the 985 mg kg^{-1} (19% decrease) PCP concentrations observed in the amended control and the non-amended control soils, respectively. There were significant decreases in the concentrations of 3- and 4-ring PNAs in all treatments. With the exception of acenapthene, decreases in the concentrations of 3-ring PNAs were significantly greater in the nonamended control soil than in either the amended control or fungal treated soils. Conversely, decreases in the concentration of 4-ring PNAs tended to be greater in the fungal treated and amended control soils than in the nonamended control soils. The concentrations of 5- and 6-ring PNAs were not decreased significantly by any of the treatments.

Comercialization of Fungal Bioaugmentation

The results of these field investigations demonstrate that (1) lignin-degrading fungi can be successfully inoculated into and cultivated in soils contaminated with relatively high concentrations of PCP (up to 1,000 mg kg^{-1}) alone or in combination with creosote (4,000 mg kg^{1} total measured PNAs) and (2) concentrations of PCP and low-molecular-weight PNAs are greatly reduced as a result of the activity of these fungi. The abilities of these organisms to decrease the concentration of PCP in two soils with very diverse chemical and physical

characteristics lends support for the continued development of a fungal-based treatment technology to remediate soils contaminated with organic pollutants. Several important components of the fungal technology require additional development before this technology can become commercially viable. First, fungal inocula that are easy to apply must be able to be produced economically. Next, continued investigation of contaminant fate in fungal inoculated soils and fungal strain selection is required. Finally, the extent of contaminant degradation must be improved.

The development of an inexpensive and effective fungal inoculum is crucial to successful implementation of fungal-based soil remediation. Currently, the production and delivery of the inoculum is the most expensive component (currently $1.32/kg) of the technology. In the small-scale field trials, only modest amounts of fungal inoculum ($\leq$ 500 kg per fungus) were applied. Scale-up of fungal bioaugmentation, as it is currently practiced, will require the production and delivery of much greater quantities of inoculum. For example, at an inoculation rate of 10% (dry weight basis), inoculation of 100,000 tonnes of soil would require 25,000 tonnes, wet weight (60% moisture content) of inoculum. Inoculum costs can be reduced by developing more economical production methods and by producing inocula with greater inoculum potential (i.e. weight of fungal biomass produced per weight of inoculum). The latter will allow inoculation rates to be reduced, thus lowering the costs of inoculum.

Mechanized techniques for inoculum application will also be necessary for large-scale operations. In the demonstration study, use of a large screw auger grain mixer to mix the inoculum into the contaminated soil resulted in severe dimunition of inoculum potential. Therefore, in addition to low costs and ease of application, inocula that maintain inoculum potential through the mixing process need to be developed. Wood chips and a nutrient-fortified mixture of sawdust and grains have served well as substrates for fungal inocula in these field studies. These materials provide a niche in which the lignin-degrading fungi can outcompete indigenous microbes and survive the toxic environment of the contaminated soil. For large-scale applications, the delivery and application of the necessary quantities of high-quality inocula made with these materials would be difficult to economize in their present form. Novel production and inoculum formulations involving lignocellulosic or other substrates are expected to result in economical inocula that more effectively take advantage of the niche (i.e. the substrate(s)) and growth habits of these organisms.

The effectiveness of remediation treatments is currently assessed by measuring, through direct chemical analysis, the extent of contaminant(s) removal and/or by determining if contaminants are transformed to innocuous products. Very little is known about the fate of contaminants in soil due to the activity of lignin-degrading fungi. We have found that *P. chrysospurium* removes PCP *per se* from soils primarily by converting it to soil-hound products (PCP-humic material copolymers) (Lamar et al. 1990a). Similarly, *P. chrysosporium* enhanced the polymerization of the creosote component benzo[a]pyrene into soil humic materials (Qiu & McFarland 1991). For certain contaminants the fungal technology works, apparently by transforming pollutants by decreasing their bioavailability through stabilizing them by polymerization with soil organic matter. This type of transformation may not be the fate for all the hazardous compounds that these organisms are able to degrade. The nature and stability of the such pollutant-humic copolymers or soil-bound residues is not known. Work with chemically synthesized humic acid-xenobiotic copolymers suggests that xenobiotics bound to humic materials through enzymatic polymerization reactions are relatively stable (Bollag & Liu 1985, Haider & Martin 1989). The nature, stability, and toxicity of the soil-bound transformation products, under a variety of conditions, require continued evaluation.

More than 1,500 species of lignin-degrading fungi have been estimated to exist in North America alone (Gilbertson 1980). Most of the research on the pollutant-degrading abilities of these organisms has been focused on relatively few species and on *P. chrysosporium* in particular. Fungal strains with superior biotransformation abilities are expected to be identified through simple screening procedures.

Although the most effective fungal treatments in the Oshkosh and Brookhaven studies decreased PCP concentrations by approximately 90%, a significant amount of PCP remained in the soil at the end of each study. Insufficient removal of contaminants is a common drawback of bioremediation treatments in general. In both field studies, the ultimate extent of PCP removal probably was restricted by the extremely low soil temperatures encountered toward the end of the investigations because of the negative effect of these temperatures on fungal growth and activity, and therefore the length of the treatment period. However, the residual PCP simply may have been unavailable to the fungi. In addition to providing optimum environmental conditions for fungal growth and activity, techniques to enhance the availability of contaminants to fungal or other microbial degradation systems may facilitate more complete contaminant removal.

In summary, the development of a technology that employs lignin-degrading fungi to remediate contaminated soils is promising. Actual implementation of the technology on a commercial scale will require further developments in inoculum production and in techniques to improve the effectiveness of the treatment.

Literature Cited

Bollag J-M, Shuttleworth K L, Anderson D H (1988) Laccase-mediated detoxification of phenolic compounds. *Appl. Env. Microbiol.* 54:3086-3091.

Cheng HH, Haider K, Harper SS (1983) Catechol and chlorocatechols in soil: degradation and extractability. *Soil Biol. Biochem.* 15:311-317.

Code of Federal Regulations. (1990) Volume 40, Part 261. *Identification and Listing of Hazardous Waste*. Revised July 1, 1990. Office of the Federal Register, National Archives and Records Administration.

Davis MW, Glaser JA, Evans JW and Lamar RT. (1993) Field evaluation of *Phanerochaete sordida* to treat creosote-contaminated soil. *Env. Sci. Technol.* 27:2572-2576.

Dec J, Bollag J-M (1990) Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions. *Arch. Env. Contam. Toxicol.* 19:543-550.

Dec, J and Bollag, J -M (1988) Microbial release and degradation of catechol and chlorophenols bound to synthetic humic acid. *Soil Science Society of America Journal* 52: 1366-1371.

Eaton, D C, Chang, H -M, Joyce, T W, Jeffries, TW, and Kirk, T K (1982) Method obtainsfungal reduction of the color of extraction-stage kraft bleach effluents. *Tappi* 65: 89-92.

Eriksson, K-E, Blanchette, RA and Ander, P. (1990) *Microbial and enzymatic degradation of wood and wood components*. Springer Verlag, Berlin. 407 p.

Hammel, KE and Tardon, PJ (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidase. Biochemistry 27:6563-6568.

Hammel, KE, Kalyanaramen, B, Kirk, TK (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium. J Biol. Chem.* 261:16948-16952.

Huynh, V-B, Chang, H-M, Joyce, TW, and Kirk, TK (1985) Dechlorination of chloro-organics by a white-rot fungus. *Tappi J.* 69:98-102.

Kirk, TK (1988) Lignin degradation by *Phanerochaete chrysosporium. ISI Atlas of Science:Biochemistry* 1:71-76.

Lamar RT, Dietrich DM (1990) *In situ* depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. *Appl. Env. Microbiol.* 56:3093-3100.

Lamar RT, Glaser JA, Kirk TK (1990a) Fate of pentachlorophenol (PCP) in sterile soils inoculated with the white-rot basidiomycete *Phanerochaete chrysosporium*:Mineralization, volatilization and depletion of PCP. *Soil Biol. Biochem.* 22:433-440.

Lamar RT, Larsen MJ , Kirk TK (1990b) Sensitivity to and degradation of pentachlorophenol by *Phanerochaete* spp. *Appl. Env. Microbiol.* 56:3519-3526.

Lamar RT, Evans JW, Glaser JA (1993) Solid-phase treatment of pentachlorophenol-contaminated soil using lignin-degrading fungi. *Env. Sci. Technol.* 27: 2566-2571.

Loske D, Hutterman A, Majcherczyk A, Zadrazil F, Lorsen H and Waldinger P (1990) Use of white-rot fungi for the clean-up of contaminated sites. In: *Advances in biological treatment of lignocellulosic materials*; Coughlan MP, Collaco MYA, Eds.; Elsevier Applied Science: London, England; pp 311-321.

Lyr H (1962) Detoxification of heartwood toxins and chloropheols by higher fungi. *Nature* 195:289-290.

Milesk GJ, Bumpus JA, Jurek MA & Aust SD (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium. Appl. Env. Microbiol.* 54:2885-2889.

Qui, X and McFarland, MJ (1991) Bound residue formation in PAH contaminated soil composting using Phanerochaete chrysosporium. *Hazardous Waste and Hazardous Materials* 8:115-125.

Sarkar J M, Malcolm R L, Bollag J-M (1988). Enzymatic coupling of 2,4-dichlorophenol to stream fulvic acid in the presence of oxidoreductases. *Soil Sci. Soc. Am. J.* 52:688-694.

Shannon, MJR, and Bartha, R (1988) Immobilization of leachable toxic soil pollutants by using oxidative enzymes. *Appl. Env. Microbiol.* 54:1719-1723.

Tatarko, M and Bumpus, JA (1993) Biodegradation of phenanthrene by *Phanerochaete chrysosporium*: on the role of lignion peroxidase. *Letters in Applied Microbiology* 17:20-24.

PRINTED WITH SOY INK
on recycled paper