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EPA ENVIRONMENTAL RESEARCH BRIEF

Biodegradation of Halogenated Hydrocarbons

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Introduction and Rationale

This research brief describes investigations conducted under a cooperative agreement between the U.S. Environmental Protection Agency and the Department of Biochemistry of Michigan State University. The investigations focus on the use of the white rot fungus *Phanerochaete chrysosporium* to degrade persistent environmental pollutants. This naturally occurring fungus is able to degrade lignin via a very non-specific and non-stereoselective mechanism. Because of this non-specific mechanism and because lignin itself is a very difficult-to-degrade compound, this research investigated the lignin degrading effects of this microorganism to degrade many synthetic and environmentally persistent organic pollutants.

Microbial degradation of contaminated material using microorganisms and/or microbial enzymes in appropriate waste treatment systems is an effective and economical method for the destruction of many hazardous organic pollutants. However, some compounds are resistant to microbial degradation. Typically, polyaromatic hydrocarbons, such as benzo[a]pyrene, and halogenated aromatic and aliphatic compounds such as DDT and Lindane, respectively, are included in this group of resistant chemicals. Because of their lipophilic nature, environmentally persistent organic pollutants often accumulate in the food chain in the body fat of animals, occupying higher trophic levels at concentrations that are often toxic, mutagenic and/or carcinogenic. Still other compounds are teratogenic or otherwise interfere with reproduction. Thus, in order for microbial treatment systems to be effective in the destruction of these more persistent compounds, microorganisms must be found or developed which can degrade these chemicals.

One strategy in the search for microorganisms capable of degrading environmentally persistent synthetic compounds is to identify and study microorganisms that degrade recalcitrant naturally occurring compounds. *P. chrysosporium* was selected for study because it degrades lignin, a naturally occurring organic compound that is extremely difficult to degrade. *P. chrysosporium* degrades lignin during idophasic metabolism (induced by nutrient nitrogen, sulfur or carbohydrate starvation) by secreting a family of unique H_2O_2 requiring hemeproteins (ligninases) that are able to catalyze the oxidative depolymerization of the insoluble lignin polymer. The soluble depolymerization products are then absorbed by the cell and metabolized to Krebs cycle intermediates and, ultimately, to carbon dioxide to complete the mineralization process.

The lignin degrading system of this fungus appears to possess a number of characteristics which make it particularly suited for use in biodegradation processes. First, the lignin degrading system is able to cleave many types of carbon-carbon and carbon-oxygen bonds which comprise the lignin molecule. Furthermore, bond cleavage occurs regardless of the conformation of chiral carbons. Thus, the lignin degrading system is non-stereoselective as well as non-specific. Second, the non-specific and non-stereoselective nature of the lignin degrading system appears to be due, at least in part, to a free radical mechanism in which low molecular weight carbon-centered free radicals or other active species serve as secondary oxidants which may catalyze lignin depolymerization or oxidation of other compounds at sites that are remote from the active site of the enzyme. Such a free

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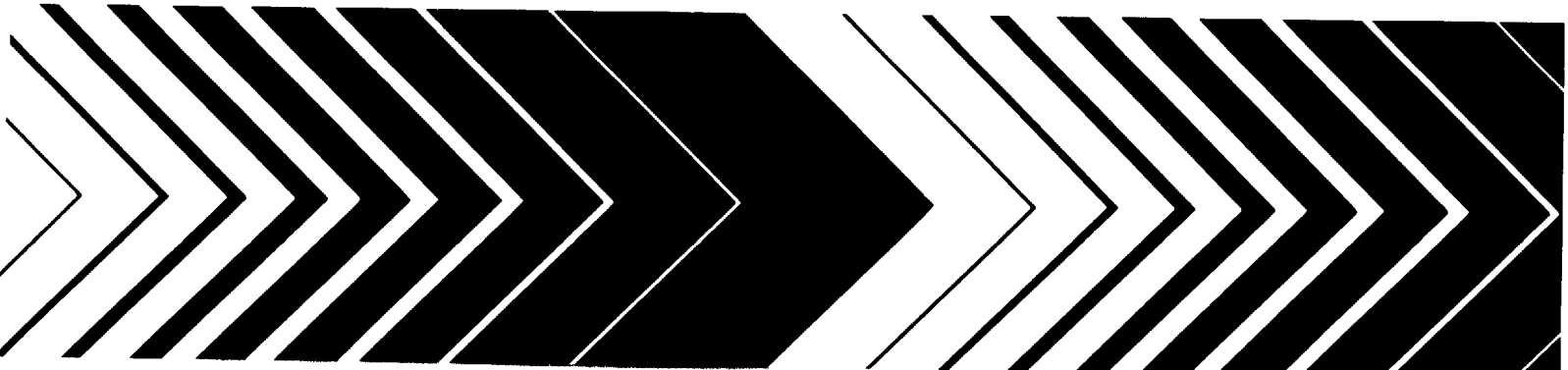

radical mechanism would have a profound effect on the biodegradation of organic pollutants if operative in the degradation of these compounds. Typically, degradative enzymes must possess very high affinites (low Km) in order for biodegradation to continue until the chemical is essentially gone. A free radical mechanism would, theoretically, allow complete conversion of a substrate to an oxidized product by a single enzyme. This is not possible with enzymes exhibiting normal Michaelis-Menton kinetics. Third, lignin is a large water-insoluble polymer. Thus, by definition, enzymes capable of catalyzing oxidative depolymerization of lignin must be able to attach extracellular, insoluble substrates. This is important because many environmental pollutants are poorly soluble in water and are usually quite tenaciously bound to organic substances in soil, making their absorption by organisms quite difficult. This limitation may be circumvented in this case because the enzymes and their activator (H_2O_2) are secreted. Fourth, the lignin degrading system is induced under nutrient (nitrogen, carbohydrate or sulfur) deficient conditions. Unlike many other biodegradative systems, substrate (i.e., lignin) is not required to be present to induce the biosynthesis of enzymes required for its biodegradation. By analogy, synthesis of lignin degrading enzymes which also attack xenobiotics would not require prior exposure of the microorganism to the xenobiotic in question, nor would enzyme synthesis be expected to be repressed when levels of the xenobiotic reached low concentrations. Fifth, many microorganisms possess the ability to catalyze only partial degradation of environmentally persistent compounds. Only the exceptional microorganism has the ability to catalyze the initial oxidation of an environmentally persistent compound as well as all of the steps in its degratory pathway to carbon dioxide, the ultimate microbial degradation product in aerobic systems. Thus, the study of *P. chrysosporium*, a microorganism which possessed a non-specific degratory system able to degrade lignin to carbon dioxide, was of great interest.

Procedures and Experimental Approach

A major objective of this study was to determine if nutrient nitrogen deficient cultures of *P. chrysosporium* could degrade a wide variety of structurally different compounds. Although degradation may be measured in many ways, mineralization of ^{14}C-labeled organic pollutants was selected as the technique-of-choice to screen compounds for their ability to be degraded by this fungus. This technique was chosen because mineralization not only demonstrates that the compound is degraded, but that degradation proceeds to $^{14}CO_2$. Mineralization also implies that intermediate degradation products are also degraded. A minor drawback to the use of this technique is the fact that mineralization is a minimal measure of biodegradation, representing only that amount of the compound that is completely degraded to carbon dioxide. In fact, degradation, as measured by disappearance of chemical would be expected to be greater than the amount mineralized until substantial amounts of all intermediates are also degraded. For use in more detailed biodegradation studies of this fungus, three model compounds were selected for specific reasons: (1) Because it is well studied and acknowledged as a persistent environmental pollutant, DDT was used to study pollutant disappearance and metabolite formation. DDT was also used in studies designed to optimize degradation; (2) Pentachlorophenol (PCP) is toxic to many fungi, including *P. chrysosporium*. Therefore, this compound was selected for use in toxicity studies and in biodegradation studies in which PCP was supplied at various concentrations; (3) The water soluble triphenylmethane dye, crystal violet (hexamethylpararosaniline), was selected for use in biodegradation studies utilizing purified enzymes.

Results

Mineralization Studies

To date, twenty-one ^{14}C-labeled compounds have been assayed for their ability to be degraded to $^{14}CO_2$ by *P. chrysosporium* (Table 1). These studies demonstrated that a wide range of structurally diverse compounds were mineralized by this fungus. These studies also show that certain structural features of these compounds affect their biodegradability. Thus, the following generalizations can be made: (1) Chlorination inhibits, but does not prevent mineralization. This phenomenon was seen for the benzoic acid/p-chlorobenzoic acid and biphenyl/polychlorinated biphenyl (Aroclors 1242 and 1254) pairs in which the chlorinated compound was always mineralized more slowly than the unchlorinated analog; (2) The presence of a substituent other than chlorine appears to be necessary for the mineralization of chlorinated aromatic compounds. Hexachlorobenzene (HCB), a perchlorinated aromatic compound, did not appear to be mineralized by this fungus. However, pentachlorophenol, a compound differing from HCB only by the presence of a hydroxyl group was relatively quickly mineralized. Similarly, the fact that PCBs were mineralized demonstrated that hydrogen atom substituents are sufficient to allow the initial oxidation of the organic pollutant in question; (3) Chlorinated aliphatic compounds were also mineralized by this fungus. Lindane and Chlordane were mineralized at rates comparable to those observed for the chlorinated aromatic compounds that were mineralized. Mirex, however, a perchlorinated compound, was poorly mineralized. Similarly, Atrazine, a widely used herbicide was also resistant to mineralization.

Involvement of the Lignin Degrading System

Three experiments have confirmed that the lignin degrading system of this fungus is responsible, at least in part, for the biodegradation of organic pollutants. First, mineralization experiments demonstrated that the temporal onset, time course, and disappearance of ^{14}C-PCP and ^{14}C-DDT mineralization was similar to those observed for ^{14}C-lignin mineralization, suggesting that all three mineralizations were carried out by the same system. Second, mineralization of ^{14}C-DDT and ^{14}C-PCP was promoted in nutrient nitrogen deficient cultures whereas it was suppressed in nutrient nitrogen sufficient cultures. This is the same pattern of promotion and suppression of mineralization as was seen for ^{14}C-lignin. Third, direct evidence for the involvement of the lignin degrading system was provided by studies in which it was shown that the H_2O_2requiring ligninases, isolated

Table 1. Twenty-one ^{14}C-labeled Compounds Degradable to $^{14}C_2$ by *P. chrysosporium*

Compound	Percent Mineralized in 30 Days
Hexachlorobenzene	<1*
Atrazine	<1
3,4,3',4'-Tetrachlorobiphenyl	2*
Naphthalene	2
Mirex	3*
DDE	4*
2,3,7,8-TCDD	4*
2-Methylnaphthalene	7
p-Chlorobenzoic Acid	8
DDT	4-8
Chlordane	10
Phenanthrene	12
Benzo(a)Pyrene	13
Aroclor 1254	14
Lindane	15
Aroclor 1242	17
Benzoic Acid	24
Biphenyl	32
Pentachlorophenol	41
p-Cresol	42
Methoxychlor	49

*60 Days

from the fungus, were also capable of oxidizing crystal violet.

Model Compound Studies

DDT Degradation

More detailed biodegradation studies were performed using DDT as a model compound in order to thoroughly document the extensive biodegradation of an acknowledged environmentally hazardous and persistent organic pollutant. Substrate disappearance studies demonstrated that approximately 50% of the DDT initially present was metabolized during the first 30 days of incubation in nutrient nitrogen starved cultures of *P. chrysosporium*. Disappearance appeared to be linear for the first 18 days of incubation after which the rate of degradation gradually declined for the rest of the thirty-day incubation period. Glucose, which was used as a growth substrate, was depleted after thirty days of incubation. Supplemental (56 mM) glucose added after 31 and 61 days of incubation resulted in substantial increased metabolism of DDT. After 90 days of incubation and two additions of glucose, less than 1% of the DDT originally present (1.7 ppm) was still detectable.

Biodegradation in some experiments was also documented by mass balance analysis which demonstrated the presence of polar metabolites. For example, in one experiment, cultures of *P. chrysosporium* which had been incubated with ^{14}C-DDT for 12 days, were extracted with hexane, followed by acidification to pH 2.0 with HCl, and extraction with methylene chloride. In these studies, 71% of the recovered radioactivity was shown to be present in the hexane fraction, while 14% and 7% were present in the acidic methylene chloride extract and the aqueous fractions, respectively, demonstrating the formation of polar and water soluble metabolites. In this study, 8% of the ^{14}C-DDT was mineralized and less than 0.1% was incorporated into insoluble portions of the mycelium after 12 days of incubation. The total mass recovery was 92%.

Metabolite formation was documented in hexane extracts of cultures obtained after various incubation times. These studies demonstrated that DDD was the predominant and, indeed, the only metabolite formed during the first three days of incubation. Between day 3 and day 6, the concentration of DDD began to decline and continued to decline for the duration of the 30-day incubation period. After day 3, the DDT metabolites dicofol (2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol), DBP (4,4'-dichlorobenzophenone), FW-152 (2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol) and two unidentified metabolites, were observed in hexane extracts of cultures incubated with DDT. In addition, two unidentified metabolites were present in acidified methylene chloride extracts. It is important to note that neither DDE nor DDD accumulated in nutrient nitrogen deficient cultures of *P. chrysosporium*. Although DDD was identified as an intermediate, it too was metabolized.

PCP Degradation and Toxicity Studies

In order to be of use in waste treatment systems, a microorganism must be able to survive in the presence of the organopollutants it is degrading. The problem is compounded by the fact that many organic pollutants have enjoyed widespread use precisely because of their fungicidal or bacteriocidal ability. Pentachlorophenol was selected for study because of its acknowledged fungicidal ability. Mineralization studies performed at low (33 ppb) concentrations showed that PCP was relatively quickly degraded by *P. chrysosporium*. Studies using high concentrations of PCP were hampered by the lethality of PCP at concentrations higher than four ppm in cultures initiated with spores. This toxicity problem was overcome by allowing the fungus to grow in culture for six days during which time a mycelial mat was formed. At this time concentrations of PCP up to at least 100 ppm were not lethal and were degraded. Although PCP at these higher concentrations suppressed respiration, as measured by $^{14}CO_2$ evolution from ^{14}C-glucose, they did not stop PCP degradation from occurring. It is noted that the water solubility of PCP at acid pH is well below 100 ppm. Thus, this concentration must be regarded as the nominal concentration in the incubation rather than the amount of PCP in true solution. Similar toxicity problems were overcome in studies using crystal violet but DDT showed no apparent toxicity at a nominal concentration of 330 ppm.

Crystal Violet Oxidation Studies

During the course of this study, it became apparent that the H_2O_2 requiring lignin degrading enzymes (collectively known as ligninases) played a role in organic pollutant degradation. Some of these enzymes were purified and their ability to oxidize crystal violet was determined. Unlike most of the compounds examined in this study, crystal violet is very water soluble. Additionally, its enzymatic biodegradation can be easily assayed spectrophotometrically.

Our studies with one of the purified ligninase enzymes indicated that crystal violet was oxidized to form a red compound that comigrated with trimethylpararosaniline during thin layer chromatography. This red compound was in turn, oxided to an unidentified colorless product. These studies also confirmed the fact that the initial oxidation of organic pollutants was accomplished by extracellular ligninases. Furthermore, we demonstrated that oxidation proceeded until the substrate (i.e. crystal violet) was no longer detectable.

Effect of Growth Substrate on Mineralization of Xenobiotics

Mineralization of lignin and of the xenobiotics examined in this study required the presence of a growth substrate. Typically, glucose (56 mM) was used as the growth substrate of choice. Studies in which the glucose concentration was varied between 23 mM and 112 mM showed that, in general, the rate and extent of mineralization increased with increasing glucose concentration. An exception to this generalization, however, was noted when the fungus was grown on 23 mM glucose. At this concentration, the greatest initial rate of mineralization was observed. However, the rate of mineralization quickly declined as the concentration of glucose became limiting.

In addition to the fact that biodegradation of organic pollutants requires the presence of a growth substrate, there are a number of other factors concerning growth substrates which are likely to affect biodegradation. For example, it was shown that growth substrates differed in their ability to support or enhance mineralization. In these studies glucose, fructose, mannose, manitol and glycerol all supported growth and mineralized 80.7, 157.7, 125.7, 48.3 and 47.5 pmoles, respectively of the 1.25 nanomoles of ^{14}C-DDT originally present during 30 days of incubation. Polyethylene glycol (PEG-4000), glycine and benzoic acid did not support growth of the fungus or mineralization of ^{14}C-DDT. Carbohydrate polymers may also serve as growth substrates. The extent of mineralization, after 30 days of incubation, was increased approximately twofold, relative to glucose, when an equivalent amount of cellulose was used as a growth substrate. Cellulose, an insoluble polymer, is a growth substrate for this fungus in nature. Furthermore, when grown on cellulose, it is possible that the fungus is better able to regulate the availability and utilization of glucose for more efficient growth and mineralization of organic pollutants.

Hydrogen peroxide is a required cofactor/activator of ligninases. When glucose serves as growth substrate, the glucose oxidase system is a major source of hydrogen peroxide. However, it is known that wood rotting fungi possess a number of carbohydrate oxidases and other enzymes that are able to produce H_2O_2. Thus, the relative ability of a growth substrate to support mineralization of organic pollutants may be dependent upon the ability of the microorganisms to generate H_2O_2 from the growth substrate. This, of course, assumes that H_2O_2 availability is rate limiting. The precise role of H_2O in degradation and the optimal conditions for growth and degradation are topics of continuing research.

Biodegradation of Organic Pollutants on Solid Matrices

Preliminary studies in which ^{14}C-labeled substrates were adsorbed onto selected soils and sediments were performed in order to determine if this microorganism could be used in the decontamination of such materials. Initial studies showed that PCP mineralization was not inhibited when PCP was adsorbed onto washed sea sand. However, when adsorbed onto top soil or peat, substantial inhibition of mineralization was observed. In other studies, mineralization of Aroclor 1242, Aroclor 1254, benzo(a)pyrene, and Lindane was found to occur only very slowly when the compounds were adsorbed onto washed sea sand. However, in other studies, substantial (40%) amounts of ^{14}C-naphthalene was mineralized following its addition to coal tar contaminated soils. These studies indicate that the solid matrix to which an organic pollutant is adsorbed will influence mineralization of the compound. Some solid matrices may cause an inhibition of mineralization while others may even accelerate mineralization. In any case the properties and effects of the solid matrix to which an organic pollutant is adsorbed must be considered in any future decontamination studies using this fungus.

Conclusions

- Based on a mineralization assay, it was shown that *P. chrysosporium* is able to degrade a broad spectrum of structurally diverse, environmentally persistent organic pollutants.

- Studies using ^{14}C-DDT as a model compound demonstrated that this compound was extensively degraded by the fungus. Degradation was studied by DDT disappearance, metabolite formation and disappearance, mass balance analysis, and ^{14}C-DDT mineralization studies.

- The lignin degrading system appears to be responsible, at least in part, for the non-specific biodegradative ability of the fungus. Indirect evidence for this comes from studies which showed that, like lignin mineralization, mineralization of organic pollutants was promoted in nutrient nitrogen deficient cultures whereas mineralization was suppressed in nutrient nitrogen sufficient cultures. Studies of crystal violet oxidation by purified ligninases provided direct evidence that the lignin degrading system is also able to degrade some organic pollutants.

- Toxicity studies showed that the model compounds, PCP and crystal violet, prevented growth when the fungus was grown from spores at concentrations of four and five ppm, respectively. However, toxicity could be circumvented by allowing the fungal cultures to grow for six days before addition of the compounds to be degraded.

Recommendations

Because of its ability to degrade a wide variety of typically difficult-to-degrade organic pollutants, it may be possible to use *P. chrysosporium* and/or its extracellular lignase(s), in selected waste treatment systems. However, a number of concerns require additional research before this technology can be applied in practical waste treatment systems.

To date, most biodegradative applications using this fungus have been hampered by the fact that the extracellular ligninases are secreted in very small amounts. Thus, a straight forward approach to increasing the biodegradative potential of this fungus would be to increase the production of the extracellular ligninases. Three approaches are recommended. First, strain development studies should be pursued to select strains that are hyper producers of ligninases. An especially attractive approach would entail development of strains in which ligninase production was "uncoupled" from the requirement for nutrient starvation. Second, culture conditions should be optimized for ligninase production since some success in this area has already been achieved. Third, gene cloning techniques should be used to create microorganisms capable of synthesizing and secreting large amounts of these enzymes.

Research focusing on the use of *P. chrysosporium* in the biodegradation of contaminated solid matrices (soils and sediments, for example) should be pursued. Preliminary studies show that organic pollutants adsorbed to some solid matrices are mineralized by this fungus but at rates that appear to be lower than those observed in aqueous culture. Also, some sediments appear to prevent mineralization altogether. In contrast, substantial mineralization (40%) of ^{14}C-napthalene was observed in coal tar contaminated soils. Thus, a major goal of future research should focus on what factors promote and what factors inhibit biodegradation in different solid matrices.

Another area deserving of special attention centers on the availability of H_2O_2. Since many transition metal complexes possess catalase type activity, H_2O_2 may be limiting in systems heavily contaminated with transition metals. The use of transition metal chelators to inhibit catalase activity may be effective and their use in such systems should be explored. Secondly, it is important to consider the substrates for H_2O_2 production. The substrate should neither repress ligninase synthesis nor produce excess H_2O_2 which would inactivate the ligninases. An ideal substrate might therefore be a complex carbohydrate that would supply a constant but appropriate concentration of substrate for the oxidase.

Organic pollutants which are adsorbed on solid matrices may, in some cases, be refractory to biodegradation because they are inaccessible to enzymatic attack or otherwise have a low bioavailability. Thus, future work should include studies aimed at increasing the bioavailabilty of these compounds. The use of selected detergents as solubilizing agents is one recommended approach.

Most research in the field of microbial degradation of organic pollutants has been performed at the level of the cell. Except for a few notable exceptions, little is known about the basic biochemistry and enzymology of these microbial processes. During the past four years much has been learned concerning the overall biodegradative abilities of *P. chrysosporium*. Similarly, much has been learned concerning the enzyme systems involved in biodegradation. For exmaple, it is now known that the ligninases are responsible for the initial oxidation of, at least, some organic pollutants. However, many of the biochemical and enzymological questions concerning biodegradation remain to be answered. This area of basic research should be pursued in order to provide a firm understanding of these processes.

Publications

The following publications describe research supported by Cooperative Agreement CR811464 between the United States Environmental Protection Agency and Michigan State University.

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