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Bioremediation capabilities of white rot fungi

Abstract

Throughout the past century, industrial, military, and farming activities have released many organopollutants into the environment. Some of these (polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCBs), trinitrotoluene (TNT), and DDT) are persistent in the environment and have potential toxic effects. Removing these organopollutants from the soil in an ecologically responsible, safe, and cost-effective way is a top concern for land management agencies. Bioremediation using various microbial organisms is one way to do this. Through intensive study of lignolytic fungi, it has been determined that these organisms produce extracellular enzymes with very low substrate specificity. This makes them suitable for degradation of many different compounds, notably organopollutants with structural similarities to lignin (PAH, PCBs, TNT, DDT). The three main lignin-modifying enzymes (LMEs) are lignin peroxidase, manganese-dependent peroxidase, and laccase. White-rot fungi contains all three enzymes and is therefore able to breakdown and mineralize several environmental pollutants into non-toxic forms. This takes place through the generation of radical species that cause the complete biodegradation of lignin polymers. This process and others like it have been extensively studied in the laboratory, showing great potential for the bioremediation capabilities of white-rot fungi. However, more research needs to be done to determine the applicability and practicality of utilizing this organism in contaminated field sites. This paper discusses the role of the lignolytic enzymes, primarily laccase, in the growing field of bioremediation.

Introduction

Background

One of the major environmental problems facing the world today is the contamination of soil, water, and air by toxic chemicals. Eighty billion pounds of hazardous organopollutants are produced annually in the United States and only 10% of these are disposed of safely (Reddy and Mathew, 2001). Certain hazardous compounds, such as polycyclic aromatic hydrocarbons (PAH), pentachlorophenols (PCP), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT),

benzene, toluene, ethylbenzene, and xylene (BTEX), and trinitrotoluene (TNT) are persistent in the environment and are known to have carcinogenic and/or mutanogenic effects. It has cost approximately $1 trillion to decontaminate toxic waste sites in the United States using traditional waste disposal methods such as incineration and landfilling (Reddy and Mathew, 2001). Due to the magnitude of this problem and the lack of a reasonable solution, a rapid, cost-effective, ecologically responsible method of cleanup is greatly needed. One growing mechanism of decontamination that may fit these requirements is bioremediation. Utilizing microorganisms to degrade toxic organopollutants is an efficient, economical approach that has been successful in laboratory studies. The next step is to apply these techniques in situ under field conditions on a large scale. This paper will review the research thus far on the potential use of microorganisms (especially white-rot fungi) in degrading some of the top pollutants throughout the world and the mechanisms involved in this process.

Characterizing organopollutants

There are several classes of chemicals that have been targeted by the USEPA as priority pollutants due to their toxic effects on the environment and human health. Six of these include PAH, PCP, PCB, DDT, BTEX, and TNT. PAHs are recalcitrant environmental contaminants that are generated from the burning of fossil fuels, coal mining, oil drilling, and wood burning (Lau et al, 2003; Verdin et al, 2004). They are not water soluble, favoring sorption to soil organic matter. Because of this they have a tendency to bioaccumulate in natural systems. They consist of 2 or more fused aromatic rings in linear, angular, or clustered arrangements. Clustered and angularly arranged ring

structures are more stable than linear arrangements, making them less biodegradable (Reddy and Mathew, 2001). Compounds with more than 3 rings are typically extremely toxic to microorganisms and, therefore, very difficult to break down into mineralizable substrates (Lau et al, 2003). PCPs have been used as wide spectrum pesticides and wood preservatives throughout the world. They are currently banned in most countries, however soil contamination continues to be a problem. PCP is toxic to most organisms at concentrations of 50ppm but some contaminated sites have concentrations greater than 1600ppm, making them very difficult to biodegrade (Aust et al, 2004). PCPs are also hydrophobic with low water solubility, increasing their persistence in the environment. DDT is an organochloride insecticide that was banned in the United States over 30 years ago. This chemical is persistent in the environment, however, biomagnifiying through the food chain. Toxic effects and population declines at higher trophic levels due to DDT have been recorded, with some studies finding stable residues in air, water, soil, sediment, fish and birds more than 10 years after it was banned (Breivik et al, 2004). PCBs were used extensively until 1993 in dielectric and hydraulic fluids, flame retardants, plasticizers, solvent extenders, textiles and printing (Reddy and Mathew, 2001). Estimates of total production of this volatile, bioaccumulative toxin range from 1.3-2 million tons worldwide (Breivik et al, 2004). BTEX compounds are components of gasoline and aviation fuels that are carcinogenic and neurotoxic to most organisms (Levin et al, 2003). They enter the environment primarily through leaking into soil, sediment, or water from underground storage tanks and pipelines. Lastly, TNT contamination is a major problem at many military complexes, with over 900,000 kg produced annually in the United States alone. It is toxic to most organisms at 50ppm but some sites have

concentrations of 4,000-12,000ppm (Boopathy, 2000). Currently, incineration is the most effective and common remediation practice, but this is extremely costly, in terms of dollars and energy used. All of these chemical compounds pose a significant threat to the health and vitality of the earth system and a sustainable method of detoxification is key.

Bioremediation

Bioremediation is defined as the application of biological processes to the treatment of pollution. Most research within the field of bioremediation has focused on bacteria, with fungal bioremediation (mycoremediation) attracting interest just within the past two decades. The toxicity of many of the above-named pollutants limits natural attenuation by bacteria, but white rot fungi can withstand toxic levels of most organopollutants (Aust et al, 2004). White rot fungi is a physiological grouping of fungi that can degrade lignin (and lignin-like substances). Four main genera of white rot fungi have shown potential for bioremediation: Phanerochaete, Trametes, Bjerkandera, and Pleurotus (Hestbjerg et al, 2003). These fungi cannot use lignin as a source of energy, however, and instead require substrates such as cellulose or other carbon sources. Thus, carbon sources such as corncobs, straw, and sawdust can be easily used to enhance degradation rates by these organisms at polluted sites. Also, The branching, filamentous mode of fungal growth allows for more efficient colonization and exploration of contaminated soil. The main mechanism of biodegradation employed by this group of fungi, however, is the lignin degradation system of enzymes. These extracellular lignin-modifying enzymes (LMEs) have very low substrate specificity so they are able to mineralize a wide range of highly recalcitrant organopollutants that are structurally

similar to lignin (Cajthaml et al, 2002; Mansur et al, 2003; Pointing, 2003, Veignie, 2004). The fact that these fungal enzymes work extracellularly allows them to access many of the non-polar, non-soluble toxic compounds that intracellular processes (such as cytochrome P450) cannot (Reddy and Mathew, 2001; Levin et al, 2003). The three main LMEs are lignin peroxidase, Mn-dependent peroxidase, and laccase. All three of these enzyme groups are stimulated by nutrient limitation (Mansur et al, 2003; Aust et al, 2004). They are most effective at degrading lignin and lignin-like substances when certain nutrient levels, primarily nitrogen, are low. Conversely, activities of these enzymes are completely suppressed in media containing high levels of nitrogen (Reddy and Mathew, 2001). This characteristic is advantageous for the fungi inhabiting highly contaminated sites with very low productivity due to toxic levels of organopollutants.

Lignin peroxidase is a glycosylated heme protein that catalyzes hydrogen peroxide-dependent oxidation of lignin-related aromatic compounds. They have a higher redox potential than most peroxidases and so are able to oxidize a wide range of chemicals, including some non-phenolic aromatic compounds (Reddy and Mathew, 2001). Mn-dependent peroxidase also requires hydrogen peroxide to oxidize Mn^{2+} to Mn^{3+}. The Mn^{3+} state of the enzyme then mediates the oxidation of phenolic substrates (Mester and Tien, 2000). Laccase, a multicopper oxidase enzyme, is the primary enzyme involved in the degradation process. It was first described in 1883, making it one of the oldest enzymes ever described (Mayer and Staples, 2002). It uses dioxygen as an oxidant, reducing it to water and it has the ability to catalyze the oxidation of a wide-range of dihydroxy and diamino aromatic compounds (Mester and Tien, 2000; Reddy and Mathew, 2001; Saito et al, 2003; Aust et al, 2004). It is most stable at a pH of 5-6 and

temperature of 45°C. (Lau et al, 2003; Mansur et al, 2003; Saito et al, 2003; Baldrian, 2004). However, this enzyme is still active at pH levels as low as 4 and as high as 7 (Mansur et al, 2003). This is beneficial in contaminated field sites with very low pH levels.

The mechanism of biodegradation depends in part, on the compound being degraded, but there are some consistent steps in the process regardless of the substrate. The ligninolytic enzymes in white rot fungi catalyze the degradation of pollutants by using a non-specific free radical mechanism (Pointing, 2001; Law et al, 2003). When an electron is added or removed from the ground state of a chemical it becomes highly reactive, allowing it to give or take electrons from other chemicals. This provides the basis for the non-specificity of the enzymes and the ability of the enzymes to degrade xenobiotics, chemicals that have never been encountered in nature (Reddy and Mathew, 2001; Pointing, 2001). The main reactions that are catalyzed by the ligninolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening. Many of these reactions result in oxygen activation, creating radicals that perpetuate oxidation of the organopollutants (Reddy and Mathew, 2001). Once the peroxidases have opened the aromatic ring structures by way of introducing oxygen, other more common species of fungi and bacteria can mineralize the products intracellularly into products such as CO_2 and other benign compounds.

Discussion

Laboratory Experiments

There have been many experiments performed in the last few years to test the degradation capabilities of white rot fungi (Pointing, 2001; Levin et al, 2003; Mansur et al, 2003; Baldrain, 2004; Verdin et al, 2004). They are almost exclusively conducted in the laboratory, providing information about the structure, function and potential uses of ligninolytic enzymes in bioremedation. Overall, they have succeeded in showing that white rot fungi, and specifically, the ligninolytic enzyme system, can efficiently remove most organopollutants from the soil in laboratory conditions. Cajthaml et al (2002) looked at the ability of purified enzymes to degrade several PAHs (anthracene, phenanthrene, pyrene, fluoranthene). They found in their in vitro studies that the ligninolytic fungi extensively degrade these compounds through both intra- and extracellular processes. After 50 days of incubation of a culture solution there was less than 1% of anthracene remaining, less than 5% phenanthrene remaining, 32% fluoranthene remaining and 7% pyrene remaining. No toxic intermediates accumulated during the incubation and the polarity and solubility of the products increased, something that would increase the likelihood of intracellular metabolism by native microorganisms but could cause problems for ground water contamination if metabolizing organisms are not present.

Levin et al (2003) tested the biodegradation of two PAHs, nitrobenzene and anthracene, by a white rot basidiomycete, *Trametes trogii*. They found that the fungus was tolerant to extremely high levels of these compounds (250-500ppm) and it was able to metabolize 90-97% of highly concentrated anthracene and nitrobenzene, respectively,

during the primary phase of fungal growth (trophophasic phase) and 100% of the compounds during the secondary phase of growth (idiophasic phase). They found high activities of laccase and Mn-dependent peroxidase during both the trophophasic and idiophasic growth stages in the fungal cultures, suggesting that the enzymes are responsible for PAH degradation. They suggested that complete degradation of PAHs by most white rot fungi likely involve cytochrome P450 enzymes as well as ligninolytic enzymes. The presence of cytochrome P450 and ligninolytic systems in most white rot fungi supports this hypothesis.

Another study aimed at testing removal capabilities by white rot fungi was conducted with TNT-contaminated soil from military complexes (Boopathy, 2000). This study analyzed two different technologies used to clean up contaminated soils: a soil slurry reactor and *in situ* bioremedation. The TNT concentrations in their soil samples were 4000-12,000mg/kg, much higher than the detection limit (0.5mg/kg). The soil slurry reactor involved mixing the contaminated soil with molasses (carbon source), water, and oxygen and maintaining a constant temperature (22°C) for two weeks. The *in situ* bioremediation technique involved placing the soil in glass columns and infusing them with different nutrient solutions, depending on their treatment (molasses, succinate, yeast extract, water control). Both techniques removed the TNT from the soil, but at different rates. The soil slurry reactor removed all of the TNT (to below detection limits) within three months while the bioremediation method took nearly 12 months to decrease TNT levels to detection limits. Despite the time advantage of the soil slurry reactor, it is a not a wholly attractive technique because it is relatively expensive. On the other hand, the *in situ* soil column is much less expensive and easier to implement.

One bioremedation technique that has gained recognition within the past few years is the application of spent-mushroom compost (SMC) to contaminated soil. This method utilizes the high levels of laccase and Mn-dependent peroxidase and high carbon contents in SMC to breakdown complex organopollutants (Lau et al, 2003; Law et al, 2003; Hestbjerg et al, 2003). There are 5kg of SMC generated for every 1kg of edible mushrooms produced, which equaled 40 Megatons of SMC in 1999 (Law et al, 2003). Putting this compost to use is a great recycling tool and potentially a sustainable approach to bioremediation. Law et al (2003) used SMC from *Pleurotus pulmonarius* to treat a PCP-contaminated water system. After 2 days of incubating contaminated water samples with various concentrations of SMC, they found that 5% SMC removed 89% of low concentration PCP samples. This was due primarily to biodegradation by ligninolytic enzymes (70%) and biosorption by the SMC (19%). Another study found that 5% SMC can completely remove certain PAH compounds from contaminated soil samples after 2 days (Lau et al, 2003). These laboratory studies provide evidence that SMC has great potential in bioremediating heavily contaminated sites.

Field Applications

A major concern associated with the budding field of bioremediation is the applicability of the laboratory research to actual large-scale contaminated field sites. The majority of the research on fungal performance has been conducted on autoclaved soil or on synthetic media. While the results overwhelmingly show white rot fungi to be efficient and successful at degrading highly toxic, complex organopollutants in these conditions, the results may not be as significant when all the natural environmental

variables are taken into consideration (native soil fauna, temperature, moisture, pH) (Reddy and Mathew, 2001; Hestbjerg et al, 2003). Very few studies actually test biodegradative capabilities under field conditions. One problem with field application deals with the strict growth conditions required for most white rot fungi. For example, *Phanerochaete chrysosporium*, a major bioremediator, has very high temperature requirements (30-37°C) for growth and ligninolytic enzyme production (Hestbjerg et al, 2003). Many white rot fungi have low competitive capabilities in the environment, as well. However, certain genera may prove useful for field application. Species of *Pleurotus* (major genera used for edible mushroom production and, hence, SMC) have much lower temperature requirements for growth and enzyme production and are less affected by native soil organisms than most other fungal species (Hestbjerg et al, 2003). Another major difficulty encountered when performing field studies are the complications surrounding chemical transformations by other soil microorganisms. Determining the chemical transformations from the fungi of interest has proven to be extremely tricky in field conditions. Radiolabelling pollutants to determine their fates is one potential solution, but it is confounded by high rates of sorption to organic matter and may not accurately represent field conditions since it is adding 'fresh' pollutant to the soil community (Reddy and Mathew, 2001).

Results from field studies, however basic, are extremely valuable for directing future research and for demonstrating complications that arise when bioremediation is applied at a large scale. The base of knowledge on bioremediation capabilities of white rot fungi is growing rapidly from laboratory studies so the next step is to utilize this pool of information in an exploratory way in the field. Considering the serious consequences

on human and ecosystem health that some of the above-mentioned contaminants create, the sooner we find a set of preliminary sustainable solutions, the better. White rot fungi may play a large role in this search, providing an environmentally-friendly, economical approach that we are really just beginning to understand.

References:

1. Aust, S. D. P. R. Swaner J. D. Stahl. 2003. Detoxification and metabolism of chemicals by white-rot fungi. *Pesticide decontamination and detoxification.* J. J. P. C. Zhu S. D. Aust A. T. Lemley Gan , 3-14. Washington, D.C.: Oxford University Press.

2. Baldrian, P. 2004. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. *Applied Microbiology Biotechnology* 63: 560-563.

3. Boopathy, R. 2000. Bioremediation of explosives contaminated soil. *International Biodeterioration & Biodegradation* 46: 29-36.

4. Breivik, K. R. Alcock Y. Li R. E. Bailey H. Fiedler J. M. Pacyna. 2004. Primary sources of selected POPs: regional and global scale emission inventories. *Environmental Pollution* 128: 3-16.

5. Cajthaml, T. M. Möder P. Kacer V. Šašek P. Popp. 2002. Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. *Journal of Chromatography A* 974: 213-22.

6. Hestbjerg, H. P. A Willumsen M. Christensen O. Andersen C. S. Jacobsen. 2003. Bioaugmentation of tar-contaminated soils under field conditions using *Pleurotus ostreatus* refuse from commercial mushroom production. *Environmental Toxicology and Chemistry* 22 , no. 4: 692-98.

7. Lau, K. L. Y. Y. Tsang S. W. Chiu. 2003. Use of spent mushroom compost to bioremediate PAH-contaminated samples. *Chemosphere* 52: 1539-46.

8. Law, W. M. W. N. Lau K. L. Lo L. M. Wai S. W. Chiu. 2003. Removal of biocide pentachlorophenol in water system by the spent mushroom compost of Pleurotus pulmonarius. *Chemosphere* 52: 1531-37.

9. Levin, L. A. Viale A. Forchiassin. 2003. Degradation of organic pollutants by the white rot basidiomycete *Trametes trogii*. *International Biodeterioration &*

Biodegradation 52: 1-5.

10. Mansur, M. M. E. Arias J. L. Copa-Patino M. Flärdh and A. E. González. 2003. The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities. *Mycologia* 95, no. 6: 1013-20.

11. Mayer, A. M. and R. C. Staples. 2002. Laccase: new functions for an old enzyme. *Phytochemistry* 60: 551-65.

12. Mester, T. and M. Tien. 2000. Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants . *International Biodeterioration & Biodegradation* 46: 51-59.

13. Pointing, S. B. 2001. Feasibility of bioremediation by white-rot fungi. *Applied Microbiology Biotechnology* 57: 20-33.

14. Reddy C.A. and Mathew, Z. 2001. Bioremediation potential of white rot fungi. *Fungi in bioremediation.* G. M. Gadd Cambridge, U.K.: Cambridge University Press.

15. Saito, T. P. Hong K. Kato M. Okazaki H. Inagaki S. Maeda Y. Yokogawa. 2003. Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated from soil. *Enzyme and Microbial Technology* 33: 520-526.

16. Veignie, E. C. Rafin P. Woisel F. Cazier. 2004. Preliminary evidence of the role of hydrogen peroxide in the degradation of benzo[a]pyrene by a non-white rot fungus *Fusarium solani*. *Environmental Pollution* 129: 1-4.

17. Verdin, A. A. L-H. Sahraoui R. Durand. 2004. Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. *International Biodeterioration & Biodegradation* 53: 65-70.