Utilization of the *Phanerochaete chrysosporium* Ligninolytic System

and the Potential for Toxaphene Degradation.

by

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IN

ENVIRONMENTAL ENGINEERING

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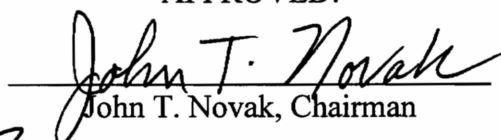

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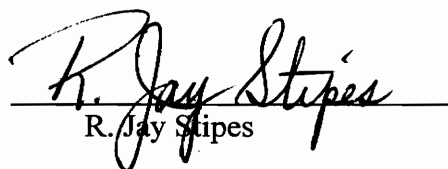

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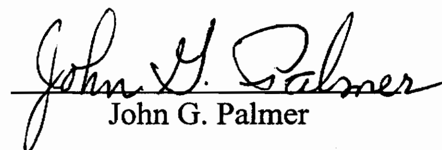

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UTILIZATION OF THE *PHANEROCHAETE CHRYSOSPORIUM* LIGNINOLYTIC SYSTEM AND POTENTIAL FOR TOXAPHENE DEGRADATION.

by

Matthew H. Stolte

John T. Novak, Chairman

Department of Environmental Engineering

(ABSTRACT)

The idiophasic production of nonspecific extracellular enzymes by the Basidiomycete fungus *Phanerochaete chrysosporium*, referred to as the ligninolytic system, has been reported by many researchers to be capable of degrading a broad spectrum of environmentally persistent compounds including some alkyl halide chemicals. Cultivation of the fungus and the ability of the ligninolytic system to degrade the alkyl halide pesticide toxaphene were explored in pure liquid cultures. The effects of culture growth temperature and toxaphene concentration were evaluated. The extent of toxaphene conversion with time was determined using three different indicators; toxaphene gas chromatographic elution patterns, the determination of free chloride concentrations by ion chromatography analyses, and the distribution of chloride in aqueous and biomass fractions via total organic halide analyses. Oxidation of the azo dye, Orange II, via spectrophotometric measurements was employed to determine the activity and reactivation of the fungal ligninolytic system.

Degradation of toxaphene in the *P. chrysosporium* cultures was limited, with a

maximum of 7% of the original mass of chloride originating from the toxaphene being released over a three week period. The main removal mechanism of the toxaphene from solution was observed to be adsorption to the mycelium biomass. Ligninolytic activity was equally active at temperatures less than optimum for maximum growth of the fungus which implies that the ligninolytic system can be effective with reduced temperature requirements. Elevated pH conditions did not effect ligninolytic activity indicating that high more neutral pHs, which are characteristic of soil properties, do not inhibit ligninolytic activity. Reactivation of the ligninolytic system was accomplished in a nonimmersed liquid culture system. The immobilized configuration appeared to be an effective system for cultivating the ligninolytic system on a continuous basis.

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1. INTRODUCTION

Alkyl halide pesticides are ubiquitous in our environment. Because they are resistant to biological and abiotic degradation, they have been widely used to prevent parasitic destruction of cash crops. However, due to their persistence, bioaccumulation, and high toxicity to animal populations, many of these pesticides have been banned by the Environmental Protection Agency (EPA) and are listed on the national priority pollutant list (Rochkind et al. 1986). As of July 1, 1992, with the enactment of Code of Federal Regulations (CFR) 268, land disposal restrictions have been set for many of these compounds whereby incineration and bioremediation are the only acceptable treatment technologies specified by the EPA. Incineration is costly and may also cause secondary pollution problems. Bioremediation is an attractive alternative due to the potentially reduced costs associated with on-site biotreatment of contaminated soils.

Toxaphene is a camphene derived chlorinated insecticide and was the most widely used alkyl halide insecticide throughout the United States during the late 1970s and early 1980s and has become one of the pesticides restricted by the EPA. Due to its recalcitrant properties it may persist for years, resulting in high concentrations in runoff, bioaccumulation, and potential exposure to humans from areas with latent toxaphene contamination. The Tennessee Valley Authority (TVA) is currently interested in finding alternatives for decontaminating crop duster landing strips in Mississippi and Pennsylvania that have been abandoned for more than ten years and still contain levels of toxaphene as

high as 200 mg/kg (Young 1992). Due to its potential health risk, toxaphene is limited to a level of 5μg/L on the national drinking standards list. Organisms have the capability to concentrate toxaphene from air, water, and food sources to levels that are many times above ambient concentrations (Rohman 1984). The 1993 EPA Great Lakes Initiative has specifically targeted toxaphene and classified it as a bioaccumulation chemical of concern (Smith 1993).

The published literature contains little information about systems to degrade toxaphene by nonthermal means. The Dames and Moore environmental consulting firm has devised an experimental approach that employs heat, ultraviolet rays from the sun, and metal catalysts to volatilize and dehalogenate toxaphene from soils (Nicholas 1993). An anaerobic landfarming technique has been researched by the University of California (Mirasatari et al. 1987) to degrade toxaphene bound to sediment in a pesticide waste disposal ditch. A limited number of researchers have reported that toxaphene can be biologically reduced by the fungus *Phanerochaete chrysosporium*. Katayama et al. (1991) reported the use of a dual system of ultraviolet (UV) light and a mutant UV resistant strain of *P. chrysosporium* (BU-1) to degrade toxaphene in petri dishes. Research has been conducted in an attempt to elucidate biological systems that can degrade recalcitrant alkyl halide compounds and it has been reported by several researchers that *P. chrysosporium* is capable of oxidizing a broad spectrum of environmentally persistent compounds including some alkyl halide chemicals (Barr et al. 1994).

Phanerochaete chrysosporium has been researched extensively over the last twenty

years due to its ability to mineralize lignin and is a Basidiomycete fungus that causes a white rot during decay (Garraway et al. 1984). Upon the depletion of carbon, nitrogen, and sulfur nutrients the fungus employs nonclassical cometabolic mechanisms to produce a variety of idiophasic nonspecific extracellular enzymes. The condition under which these enzymes, most commonly known as lignin peroxidases (LiP) and manganese dependent peroxidases (MnP), are produced is referred to as ligninolytic activation. The ligninolytic system has several advantages over conventional bacterial systems for degrading pollutants. These are; the system is extracellular, it is nonspecific, activation of the system is independent of contaminant concentration, and the fungus has the capability to utilize substrate that is not readily obtained by bacteria or microorganisms present in most biological systems (Barr et al. 1994). In order to utilize the degradative capabilities of the fungal system it is important to have an understanding of how to activate and monitor the system for ligninolytic conditions.

The purpose of this study was to evaluate the feasibility of *P. chrysosporium* to degrade toxaphene and to determine culture conditions that might enhance the activity and reactivation of the ligninolytic system. The ability of *P. chrysosporium* to degrade toxaphene was evaluated in liquid medium cultures under static ambient conditions. Temperature requirements were explored at 20 °C, 30 °C, and 40 °C. The fungus was incubated in cultures containing toxaphene concentrations at 3, 20, and 200 mg/L to evaluate toxic effects by the pesticide on the fungus. The extent of toxaphene conversion over time was evaluated using three different indicators; GC elution patterns, free chloride

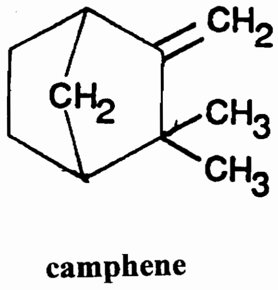
concentrations by ion chromatography analyses, and the distribution of chloride in the aqueous and biomass fractions via total organic halide analyses. A spectrophotometric technique which measured the ligninolyitc oxidation of an azo dye, Orange II, was employed to research the activity and reactivation of the fugal ligninolytic system in liquid cultures. Culture conditions of temperature, pH, and additives (nitrogen, hydrogen peroxide, and veratryl alcohol) in liquid growth medium were varied to determine the effects on ligninolytic activity. A nonimmersed liquid culture system which consisted of the fungus immobilized on porous pieces of polyurethane foam was constructed to determine the feasibility in reactivating the ligninolytic system. Overall the main objective of the project was to obtain an understanding of how to manipulate the fungal system, monitor enzyme activity, and determine the feasibility by which the ligninolytic system can be utilized for the aerobic biodegradation of the alkyl halide pesticide toxaphene.

2. LITERATURE REVIEW

A. Description of Toxaphene

Toxaphene (see Figure 1) is an alkyl halide insecticide produced by chlorinating camphene. It is 70% chlorine by weight and is comprised of a complex mixture of more than 170 chlorinated congeners with an overall average elemental composition of $C_{10}H_{10}Cl_6$ (Hawley 1987). In 1976 more than 1900 tons of toxaphene were applied in the continental United States making it the most widely used insecticide. It was used most extensively on soybean, cotton, and peanut plants. In addition, toxaphene has also been used against insect pests associated with livestock, sunflowers, ornamental plants, corn, wheat, rice, barley, and many vegetable crops. Toxaphene has also been used for the eradication of unwanted rough fish from lakes to support sport fishing (Rohman 1983). In October 1982 the EPA publicly announced its decision to terminate the registration of toxaphene for most uses. Land disposal restrictions were placed on toxaphene with the enactment of CFR 268, on July 1, 1992.

Due to the heavy use of toxaphene throughout the United States prior to 1982, residual concentrations within soils can be found. Toxaphene has been detected in air samples taken in the northern United States and in the Canadian-northwest territories. In the southeastern United States, rainwater containing atmospheric toxaphene has been documented as contributing significantly to the contamination of a South Carolina estuary (Rohman 1983). The Tennessee Valley Authority (TVA) in Muscle Shoals, Alabama is



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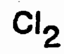
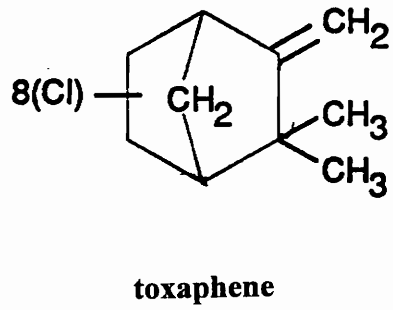




toxaphene

Figure 1. Toxaphene congeners produced by chlorinating camphene.

interested in finding alternatives for decontaminating farm land in Mississippi and Pennsylvania that contain high levels of toxaphene. Abandoned crop duster landing strips in Mississippi, which have been out of service for more than ten years, are contaminated with toxaphene at concentrations greater than 200 mg/kg. The TVA is dealing with similar sites throughout the southeastern United States (Young 1992).

B. Approaches Used to Degrade Toxaphene

Review of the published literature for this research project yielded only a small amount of information on nonthermal systems to remove toxaphene from soils. A proprietary Hydrolytic Terrestrial Dissipation (HTD) system has been designed by the Dames and Moore environmental consulting firm (Tallahassee, Florida) to remove toxaphene from contaminated soils. The HTD system uses heat and ultraviolet rays from the sun, along with metal catalysts, to volatilize and dehalogenate toxaphene from soils (Nicholas 1993).

Mirasatari et al. (1987) performed studies to degrade toxaphene using insitu anaerobic landfarming techniques. Sediment in a toxaphene contaminated pesticide waste disposal ditch was amended with 10% manure and flooded with water to attain anaerobic conditions. Toxaphene residues were reduced from 63 to 23 mg/kg over a 4 month period and degradation activity was still evident 8 months after the initial treatment.

The literature review for this project has identified two authors who reported that toxaphene can be degraded by *P. chrysosporium*. Barr et al. (1994) has complied an extensive summary on the current understanding of the mechanisms by which *P.*

chrysosporium degrades complex pollutants and in the summary have reported that the nonspecific enzyme system of the fungus is capable of degrading toxaphene to carbon dioxide. However, specific research results outlining the extent to which toxaphene is mineralized by the fungus was not included in their summary.

Katayama et al. (1991) reported the use of a dual system of ultraviolet (UV) light and a mutant UV resistant strain of *P. chrysosporium* (BU-1) to degrade toxaphene in petri dishes. The disappearance of toxaphene was monitored by extracting the contaminant with an organic solvent from sacrificial experimental cultures and quantifying the remaining toxaphene by gas chromatography. A 1 mg/L concentration of toxaphene was reported to be degraded 21 ± 1% over a 7 day period by exposing petri dishes inoculated with the mutant BU-1 strain to a 254 nm UV source. Control cultures that were inoculated with the BU-1 strain and not irradiated with the UV source resulted in an 8 ± 6% decrease in toxaphene over the same time period. Although these results indicated limited toxaphene degradation, Katayama et al. (1991) were successful in degrading other chlorinated pesticides, such as, DDT, dieldrin, heptachlor, and 3,4,3',4'-tetrachlorobiphenyl (TCB) to nondetectable concentrations in 21 days using the dual treatment (see Figure 2). Therefore, the *P. chrysosporium* system has been applied in liquid cultures to initiate the biological breakdown of toxaphene and to mineralize similar complex halogenated pesticides.

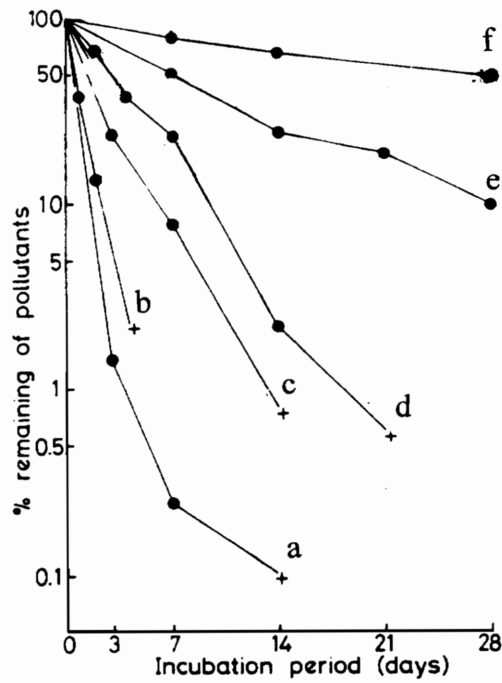


Figure 2: Research results from Katayama et al. (1991) showing the degradation of 1 mg/L of (a) DDT, (b) TCDD, (c) heptachlor, (d) TCB, (e) dieldrin, and (f) toxaphene in petri dish cultures of *P. chrysosporium* with UV irradiation. Note, points a, b, c, and d are below detectable levels.

C. The *P. chrysosporium* Lignin Degrading System.

Phanerochaete chrysosporium is a Basidiomycete fungus and causes a white rot during wood decay (Garraway et al. 1984). Over the last twenty years much attention has been drawn to *P. chrysosporium* by the forest products industry due to its ability to mineralize lignin. The fungus has several characteristics that have made it beneficial for research which include rapid growth, relatively high temperature growth optimum, and the ability to degrade a variety of complex organic pollutants (Kirk 1987).

Fungi are heterotrophic eukaryotic organisms that require organic compounds as a source of carbon and energy. The primary energy source for heterotrophs is chemical energy supplied by the oxidation of complex organic compounds such as polysacharides, into simpler constituents (Rochkind et al. 1986). Organic nutrients are often available to fungi as large, insoluble macromolecular complexes, which must first be degraded to smaller substituents and then internalized and used as a source of energy (Bumpus et al. 1985). The most abundant natural organic material is cellulose, a complex organic high polymer carbohydrate compound that is the fundamental constituent of all vegetable tissues (wood, grass, cotton, etc.) (Hawley's 1987). Therefore, a wide variety of naturally occurring microorganisms, including fungi, degrade cellulose to sustain metabolic activities. Cellulose is bound up in the tissues of plants and is coated with a natural plastic type material known as lignin.

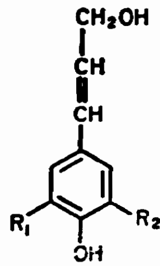
Lignin makes up 20-30% of most wood material and 5-15% of most agricultural crop residues (Garraway et al. 1984). The main structure of natural lignin consists of complex

high molecular weight phenylpropane (C_6-C_3) branched polymers linked through seven major types of alkyl (C-C) and ether (C-O-C) linkages. Three substituted phenyl alcohols have been recognized as the basic polymeric structure of lignin: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The ratio of these acids is dependent on the plant species (see Figure 3). The complex chemical structure of the phenyl alcohol lignin polymer makes it resistant to attack by most organisms. Fungi are the only microorganisms that have been clearly shown to be capable of degrading lignin (Griffin 1981).

D. Growth Requirements and Ligninolytic Activity of *P. chrysosporium*

The Forest Products Laboratory operated by the Forest Service under the auspices of the United States Department of Agriculture, in Madison, Wisconsin has been one of the pioneering research laboratories in determining optimum growth conditions for the fungus and elucidating mechanisms by which the oxidizing system breaks down lignin. Kirk et al. (1976) reported that lignin was degraded to CO_2 by *P. chrysosporium* and that a growth substrate such as cellulose or glucose was required, indicating that lignin was oxidized by the fungus as a secondary source of energy. Results in this research revealed a linear relationship between the amount of carbohydrates supplied and the amount of lignin degraded to CO_2 by the fungus.

Keyser et al. (1978) researched the relationship between *P. chrysosporium* growth, nutrient nitrogen assimilation, and ligninolytic activity in stationary liquid medium batch



I	$R_1 = R_2 = H$	= *p*—Coumaryl alcohol
II	$R_1 = H, R_2 = OCH_3$	= Coniferyl alcohol
III	$R_1 = R_2 = OCH_3$	= Sinapyl alcohol

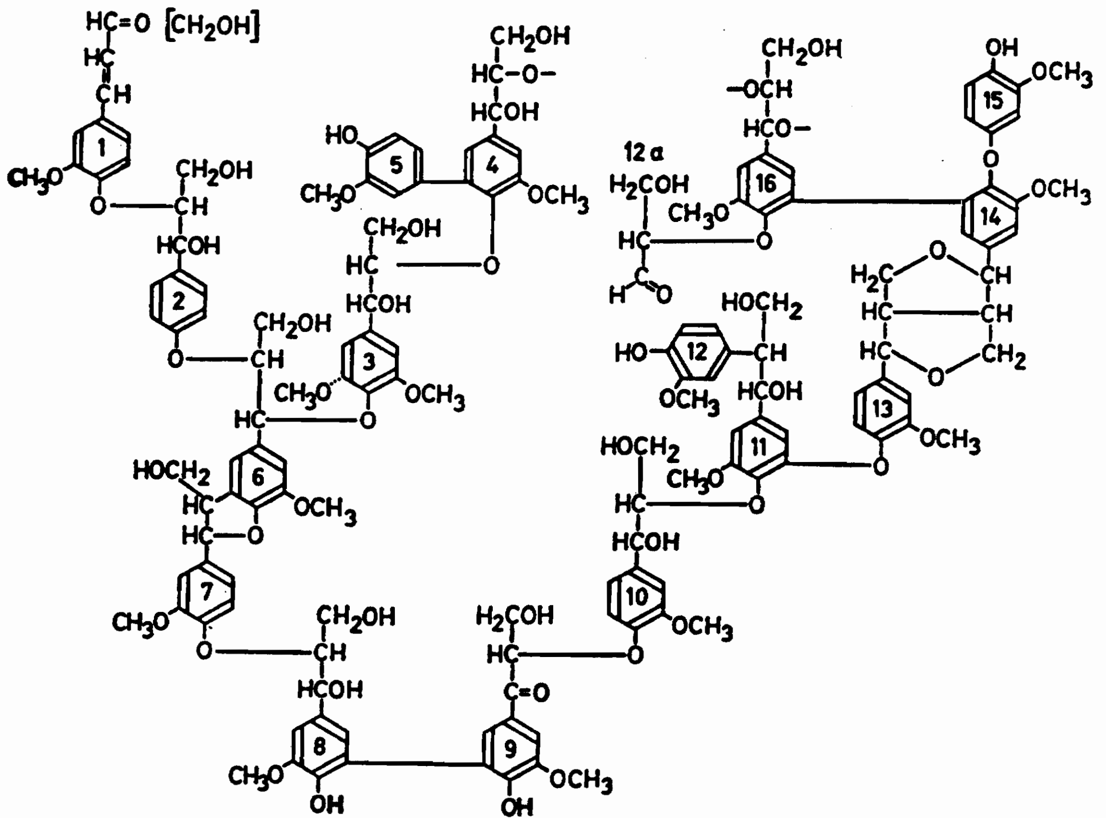


Figure 3: The structure of lignin. The three basic subunits of lignin (top) and a schematic formula for conifer lignin (Garraway et al. 1984).

cultures. The studies revealed that metabolism of lignin was maximal at low concentrations (approximately 2 mM) of nutrient nitrogen and confirmed that the presence or absence of lignin in cultures had no influence on the level or time at which ligninolytic activity occurred. Keyser et al. (1978) outlined the sequence of events that formed the ligninolytic system of the fungus: 0 to 1 day, germination, linear growth, and depletion of nutrient nitrogen; 1 to 2 days, cessation of linear growth, and nitrogen starvation; 3 to 4 days, appearance of ligninolytic activity; 5 to 10 days maximum ligninolytic activity maintained; and 10 to 15 days, sporulation of cultures. The research concluded that the ligninolytic system must be highly nonspecific due to its ability to degrade the structurally diverse lignin subunits.

Kirk et al. (1978) studied the influence of oxygen, pH, nitrogen, inorganic nutrients, and active mixing of liquid cultures on *P. chrysosporium* growth and degradation of synthetic lignin in shallow batch cultures. Results from the research determined that the ligninolytic system was a highly aerobic process. Elevated concentrations of O_2 in the headspace of non-agitated cultures increased ligninolytic activity. Degradation of a synthetic lignin polymer was nonexistent with 5% O_2 in N_2, and a 2- to 3-fold enhancement was observed over ambient air conditions (21% O_2) in cultures flushed with 100% O_2. Agitation of static liquid cultures resulted in the production of mycelial pellets which greatly suppressed ligninolytic activity. The pH of the medium was found to be critical for lignin degradation with optimum rates observed between pH 4.0 to 4.5 and substantial suppression of ligninolytic activity occurring below pH 3.5 and above pH 5.5. The optimum pH for

growth was reported to be at a pH between 5.0 to 5.5. The source of nutrient nitrogen (NO_3^-, NH_4^+, or amino acids) was reported as having little influence on lignin decomposition. However the concentration of nitrogen was critical, and ligninolytic activity was suppressed in cultures containing nonlimiting nitrogen concentrations. Thiamine was the only vitamin required for growth and activation of ligninolytic activity. Optimum growth temperatures for the fungus were determined to be between 39-40 °C. Some results of research by Kirk et al. (1978) are shown in Figure 4. These data show the relationship between mycelial growth, carbohydrate depletion, and ligninolytic activity in nitrogen rich and nitrogen limited cultures. Maximal rates of synthetic lignin degradation, measured by $^{14}CO_2$ production, were observed between day 3 and day 18 of incubation.

Nutritional regulation of the ligninolytic system with regards to concentrations of nitrogen, carbohydrate, sulfur, phosphorous, and inorganic nutrients was researched by Jefferies et al. (1981). Results from the research showed that ligninolytic activity was activated not only by the consumption of limited nitrogen but also activated by the depletion of carbohydrates in nitrogen rich cultures. The overall productivity of the ligninolytic system in the nitrogen rich cultures was dependent upon the initial amount of carbohydrate supplied. Carbohydrate limited cultures led to the appearance of ligninolytic activity earlier than nitrogen limited cultures. Cultures grown with limiting carbohydrate (4.4 mM cellobiose) and limiting nitrogen (2.6 mM) resulted in slower initiation of ligninolytic activity than in cultures supplied with excess nitrogen and limited carbohydrates. Addition of carbohydrates to experimental growth solutions during lignin metabolism increased ligninolytic activity

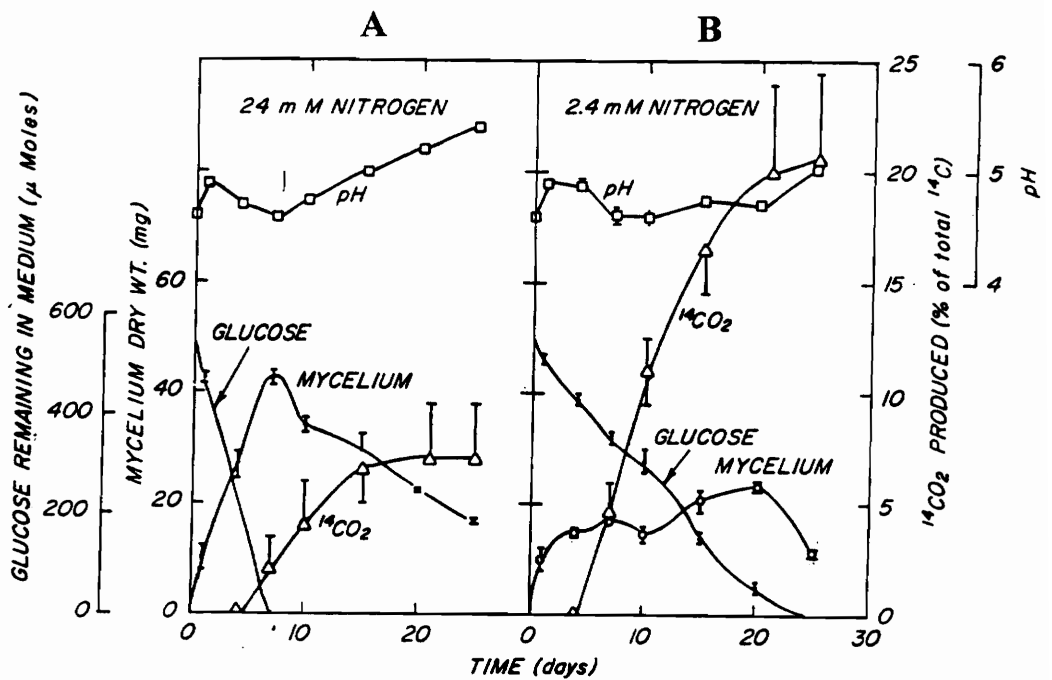


Figure 4: Ligninolytic activity in nitrogen rich (A) and nitrogen limited (B) cultures of *P. chrysosporium* with synthetic lignin, from Kirk et al. (1978). Note that carbohydrate (glucose) concentrations were depleted more quickly in nitrogen rich cultures, but ligninolytic activity was lower ($^{14}CO_2$ concentrations).

when nitrogen was limited and suppressed ligninolytic activity when nitrogen was in excess. Jefferies et al. (1981) calculated the ratio of carbohydrate, nitrogen, and sulfur concentrations which led to simultaneous depletion of nutrients under optimum conditions to be 2.2 mg of carbohydrate, 54 μg of nitrogen, and 0.5 μg of sulfur for each 1 mg (dry weight) of mycelium.

Jefferies et al. (1981) summarized the sequence of events that indicated ligninolytic activity in nitrogen limited cultures. After nitrogen was depleted from solution, a 1- to 2-day lag period occurred. Substantial changes in intracellular amino acids and protein concentrations were observed during this lag period and the system reached an intermediate metabolic equilibrium. Ligninolytic activity was observed as the physiological equilibrium was reached. Mycelial dry weight decreased as carbohydrates were depleted from solution and ligninolytic activity was initiated. This research also noted that the wood-destroying *P. chrysosporium* are well adapted to nitrogen-deficient environments and are able to recycle and conserve this nutrient, which is usually present at low concentrations in wood substrates.

The extent to which sulfur affected ligninolytic activity was not clearly defined. Jefferies et al. (1981) concluded that sulfur concentrations may not directly affect the activity of the enzymes but may magnify the extent to which elevated nitrogen concentrations inhibit ligninolytic activity.

Results from research performed by Reid et al. (1979) and Jefferies et al. (1981) indicated that ligninolytic activity was not affected by limited or excessive concentrations

of phosphorous.

The affects of inorganic nutrients Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and MoO_4^{2-} on ligninolytic activity were also researched by Jefferies et al. (1981). It was concluded that the only metal that significantly affected ligninolytic activity was Mn^{2+} which was inhibitory at elevated concentrations. Increased concentrations of Ca^{2+} and Mg^{2+} countered the inhibitory effects of Mn^{2+}; therefore the balance of these trace metals was shown to be important for ligninolytic activation.

Research by Faison et al. (1984) reported an increase in ligninolytic activity in cultures amended with the secondary metabolite, 3,4-dimethoxybenzyl (veratryl) alcohol and H_2O_2. Incubation of cultures with 0.2 mm veratryl alcohol caused a twofold increase in ligninolytic activity: however the rate at which lignin was oxidized to CO_2 was equivalent to controls which contained no veratryl alcohol. Hydrogen peroxide added at a concentration of 0.03 mM did not significantly increase ligninolytic activity over control cultures. Kirk et al. (1985) reported a five-fold increase in ligninolytic activity with nitrogen limited medium amended with veratryl alcohol (0.4 mM) and excess trace metals.

E. Characterization of the Secondary Metabolic Process of *P. chrysosporium.*

Two categories of metabolism exist in microorganisms and plants; primary metabolism and secondary metabolism. Primary metabolism, such as the breakdown of cellulose by fungi, is essential for the life of the organism. It involves catabolic, amphibolic, and anabolic pathways to provide the energy and biosynthesis for cellular development and

functions. Biosynthesis of compounds that have no essential role in the development and primary growth of the organism (such as the oxidation of lignin by the *P. chrysosporium* ligninolytic system) is referred to as secondary metabolism. Secondary metabolites released by organisms (such as the extracellular enzymes produced by *P. chrysosporium*) are dependent upon precursors and energy acquired from primary metabolism. Secondary metabolism is often not expressed until the growth phase approaches completion, as is evident in the depletion of nitrogen in the *P. chrysosporium* ligninolytic system (Drew 1983).

In secondary metabolism there are two important phases to distinguish; primary growth phase (trophophase) and the production of secondary metabolites (idiophase). An understanding of the trophophase-idiophase relationship is important in maximizing secondary metabolite production. The distinction between phases is less defined in filamentous microorganism such as fungi. Jefferies et al. (1981) concluded that activation of the *P. chrysosporium* ligninolytic system follows a nonclassical cometabolic process differing from the traditional definition of cometabolism in the sense that lignin degradation and primary growth of the fungus do not occur simultaneously.

The main mechanism behind the ligninolytic system of the fungus has been reported to be the nonclassical idiophasic production of enzymes that break down complex organic material. There are many enzymes that are believed to be involved with the ligninolytic system, and four main enzymes have been identified in the ligninolytic process: ligninase, laccase, manganese peroxidase, and H_2O_2-producing enzymes (see Table 1). An extensive summary of the research that has resulted in the discovery and characterization of

ligninolytic enzymes has been compiled by Kirk (1987). Tien et al. (1984) identified and purified an extracellular lignin-degrading enzyme referred to as ligninase. Pure cultures of the ligninase were shown to oxidize several lignin-related compounds and required H_2O_2 for activity and therefore is referred to as lignin peroxidase (LiP). Kirk (1987) has suggested that LiPs are the main enzymes that catalyze extensive oxidation of non-phenolic and phenolic units in lignin. His research indicated that the LiP resembles horseradish peroxidase in many of its properties although it has a higher oxidation potential. Veratryl alcohol has been identified as a secondary metabolite of *P. chrysosporium* and oxidation of this substrate by LiP has been reported by Barr et al. (1994) to enhance the degradation of anisyl substrates by LiP. The research suggested that veratryl alcohol was oxidized to a cation radical which may act as a diffusible 1-electron oxidant to interact with other substrates. Other research by Tien et al. (1984) has discounted this postulate and suggested that veratryl alcohol does not act as an oxidant but only protects LiP from inactivation from anisyl substrates. The stimulatory effects of veratryl alcohol with respect to LiP activity has been documented by several researchers but the mechanisms have not been clearly elucidated.

Laccase has been identified in most white rotting fungi and has been demonstrated to catalyze the oxidation of phenolic compounds to phenoxy radicals. *P. chrysosporium* does not produce extracellular laccases, and therefore it has been demonstrated that laccase is important but not essential for ligninolytic activity (Kirk 1987).

Table 1: Enzyme activities implicated in the ligninolytic system (Kirk et al. 1987).

Enzyme Activity	Cofactors/ Substrates	Consequence of Action
Ligninase	H_2O_2	Aromatic nuclei oxidized to cation radicals
Laccase	O_2	Phenols oxidized to phenoxyl radicals
Mn peroxidase	H_2O_2, Mn^{II}	Mn^{II} oxidized to Mn^{III} (Mn^{III} oxidizes phenols to phenoxyl radicals)
H_2O_2-producing enzymes	Various organic substrates	O_2 reduced to H_2O_2

Manganese peroxidase (MnP) is an extracellular enzyme produced by *P. chrysosporium* and exists as multiple isoenzymes, the significance of which is not clearly understood. The MnP functions by oxidizing Mn^{II} to Mn^{III}, which has been isolated and demonstrated to oxidize phenols to phenoxy radicals (Kirk 1987).

Hydrogen peroxide producing enzymes have been isolated from *P. chrysosporium* cultures and are believed to supply H_2O_2 required for ligninase and Mn peroxidase activation. Several different intracellular and extracellular oxidases have been discovered that supply H_2O_2 to the ligninolytic system (Kirk 1987).

Ligninase is the only enzyme shown to oxidize non-phenolic as well as phenolic units in lignin, and therefore is believed to have a greater degradative potential than Mn peroxidase or laccase. Kirk (1987) has isolated the degradation products of lignin oxidized by *P. chrysosporium* via solvent extraction, purification techniques, and ^{13}C-NMR spectroscopy. These studies have revealed that fungal attack decreases the methoxyl, phenolic, and aliphatic hydroxyl contents and increases aliphatic hydrocarbon structures. Not all breakdown of lignin material can be linked to ligninase, indicating that there are additional enzymes involved in the ligninolytic process that remain unidentified.

Kirk et al. (1987) reported that concentrated aliquots of purified enzymes isolated from ligninolytic cultures of *P. chrysosporium* did not degrade lignin to a greater extent than unpurified crude ligninase isoenzymes in whole culture experiments. Kirk postulated three reasons for this occurrence: 1) some type of enzyme component is inactivated during preparation or catalysis; 2) key enzyme components reside on the cell surfaces and are absent

in the studied cell-free preparations; 3) cofactors produced continuously by the cells are absent in the isolated preparations. These observations indicated that the ligninolytic system utilizes several intracellular and extracellular processes to implement the oxidative system and that whole cultures of the fungus appear to be more effective in overall degradative capabilities than isolated enzymes.

F. Techniques for Monitoring Ligninolytic Activity

In order to utilize the degradative capabilities of the ligninolytic system it is crucial to have an understanding of how to monitor for ligninolytic conditions. Initial ligninolytic experimental studies by Kirk et al. (1978), Keyser et al. (1978), and Jefferies et al. (1981) determined ligninolytic activity of the fungus by monitoring the production of $^{14}CO_2$ gas evolved from liquid cultures inoculated with *P. chrysosporium* amended with synthetic ^{14}C-lignin compounds.

Tien et al. (1984) utilized an alternative approach to identify ligninolytic activity of the fungus, which employed monitoring the H_2O_2-dependent conversion of veratryl alcohol to veratrylaldehyde. The ligninolytic assay utilized a standard reaction mixture (0.5 mL volume) containing 0.4 mM veratryl alcohol and 0.15 mM H_2O_2 in 100 mM of sodium tartrate (pH 3.0). Each assay tube contained approximately 300 μL of culture supernatant and the reaction was initiated by the addition of the H_2O_2 to the supernatant. The linear increase in absorbance was monitored spectrophotometrically at a wavelength of 310 nm. The efficiency of the assay was dependent upon the concentration of ligninolytic material

in solution and the purity of veratryl alcohol and H_2O_2 reagents used to initiate the oxidative reaction. This technique is the most commonly used assay to measure ligninolytic activity in the research reviewed for this project.

Glenn et al. (1983) researched the use of polymeric dyes PolyB-411, Poly R-481, and Poly Y-606 as possible alternatives to radiolabeled lignin as a substrate alternative to indicate ligninolytic activity. Like lignin degradation, the decolorization of these dyes by *P. chrysosporium* occurred during secondary metabolism, was suppressed in cultures grown in the presence of high levels of nitrogen, and was strongly dependent upon oxygen concentration in the cultures. The dyes were added at a concentration of 0.02% to liquid medium. At specified time intervals a volume of the extracellular culture medium was removed and diluted 10-fold with water. A ratio of the maximum and minimum absorbance of the dyes were determined on a spectrophotometer and were recorded for different time intervals in which the dye was incubated with the fungus. A decrease in the absorbance ratio signified a degradation of the dye and onset of ligninolytic activity. Platt et al. (1985) followed the work of Glenn et al. (1983) to demonstrate the use of Poly-B411 for the screening of fungi for ligninolytic activity and Cripps et al. (1990) reported the use of the *P. chrysosporium* ligninolytic system to oxidize three azo dyes, Topaeolin O, Congo Red, and Orange II. Orange II incubated with nitrogen-limited cultures was completely decolorized after 5 days of incubation. Examination of a number of fungi showed that the dye decolorization occurred only for fungi with a known lignin-degrading ability. The results from these studies suggest that the decoloration of polymeric and azo dyes incubated with

P. chrysosporium offer a simple approach to identify ligninolytic conditions. This method provides a simple, rapid, and quantitative spectrophotometric assay to monitor ligninolytic conditions achieved by *P. chrysosporium* cultures and reduces the use of radiolabeled substrates that may be difficult to obtain and eliminates sensitive assays that require considerable labor or expertise and money (Glenn et al. 1983).

G. Degradation of Complex Organic Compounds by *P. chrysosporium.*

As previously discussed in this literature review, *P. chrysosporium* produces several nonspecific extracellular enzymes such as LiP and MnP in response to the depletion of carbon, nitrogen, and sulfur nutrients which are capable of degrading complex organic compounds. The ligninolytic system has been employed by several researchers to degrade a wide range of complex pollutants. Some of these are; polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins (Hammel et al. 1986, and Bumpus 1989), polychlorinated biphenyls (PCB) (Eaton 1985), DDT (Bumpus et al. 1987), Pentachlorophenol (PCP) (Mileski et al. 1988), Crystal Violet (Bumpus et al. 1988), Thianthrene (Schreiner et al. 1988), TNT (Fernando et al. 1990), and Phenanthrene (Sutherland et al. 1991). An extensive list of environmental pollutants degraded by *P. chrysosporium* has been compiled (see Table 2) by Barr et al. (1994).

There are several advantages that are associated with the ligninolytic system of the *P. chrysosporium* over conventional bacterial systems for treating complex organic pollutants. Barr et al. (1994) have compiled an extensive review of the benefits and current

Table 2. Environmental pollutants degraded by the white rot fungus *Phanerochaete chrysosporium* (Barr et al. 1994).

Polycyclic aromatic compounds
- Benzo[a]pyrene
- Pyrene
- Anthracene
- Chrysene

Chlorinated aromatic compounds
- Pentachlorophenol
- 4-Chloroaniline
- Polychlorinated biphenyls
- Dioxin

Pesticides
- DDT
- Lindane
- Chlordane
- Toxaphene

Polymeric and Azo dyes
- Crystal Violet
- Azure blue
- Orange II

Munitions
- TNT (2,4,6-trinitrotoluene)
- RDX (cyclotrimethylenetrinitroamine)
- HMX (cyclotetramethylenetetranitramine)

Others
- Cyanides
- Azide
- Aminotriazole
- Carbon tetrachloride

understanding of mechanisms by which *P. chrysosporium* degrades complex pollutants. The advantages that the ligninolytic system has over conventional bacterial systems are; the system is extracellular, it is nonspecific, activation of the system is independent of contaminant concentration, and the fungi have the capability to utilize substrate that is not readily obtained by bacteria or microorganisms present in most biological systems.

Due to the extracellular release of enzymes, it has been reported by several researchers (Barr and Aust 1994) that *P. chrysosporium* can withstand higher concentrations of toxic pollutants and degrade chemicals that are not readily soluble. Most bacteria degrade pollutants using intracellular mechanisms which require uptake of the pollutant into the organism's cells. Bacteria are limited by the concentration of a pollutant that must be transported into the cell for oxidation by intracellular enzymes and must maintain a low enough concentration of the contaminant to eliminate toxic effects to the organism.

The nonspecific nature of the degradative mechanisms employed by *P. chrysosporium* has been shown to be effective on a large variety of reduced and oxidized chemicals. Pollutants such as creosotes and PCBs that have traditionally required a mixed consortium of bacteria to oxidize constituents at varying steps of the degradation process have been mineralized solely by the ligninolytic system.

Growth of the fungus is dependent upon a primary growth substrate such as cellulose or glucose and the ligninolytic system is initiated by nutrient limitation, therefore the system is not directly influenced by the toxicity or concentration of most pollutants. Conventional bacterial systems that degrade pollutants typically follow first-order kinetics. In contrast the

ligninolytic system is able to degrade low level pollutants to nondetectable or nearly nondetectable levels due to the fact that the production of the degradative enzymes is not dependent upon the presence of target pollutants. The nonspecific ligninolytic system is also uniquely differing from bacterial systems because it does not require preconditioning to a particular pollutant.

The lignin-degrading system allows the fungus to utilize carbon sources that are normally inaccessible by other microbes and therefore, the *P. chrysosporium* organism has a competitive edge to access bound substrate for energy. Inexpensive growth materials such as corn cobs or wood chips, which are not effectively used by most microorganisms as substrate, have been used to cultivate the fungi in soils.

H. Mechanisms by Which *P. chrysosporium* Degrades Pollutants

It is well documented throughout the literature that the nonspecific degradative properties of the *P. chrysosporium* ligninolytic system is due to the formation of highly reactive free radicals. The fundamental principle behind radical reactions is that bonds are broken up by the reaction of odd-electron species (McMurry 1984). Free radicals are highly reactive and initiate many chain reactions. The mechanisms by which the fungus degrades pollutants via free radicals have been elucidated by many researchers. Barr et al. (1994) have outlined five major mechanisms that have been reported as being responsible for degradation of complex organic contaminants by *P. chrysosporium*; direct oxidation, indirect oxidation, reduction reactions, plasma membrane potential reactions, and the production of highly

reactive oxygen species. A brief discussion on each of these mechanisms follows.

One mechanism that has been attributed to the ligninolytic system is the direct oxidation of pollutants. Peroxidases, including LiP and MnP, utilize hydrogen peroxide to promote a one-electron oxidation of chemicals to free radicals. Many diverse compounds have been shown to be directly oxidized by the lignin peroxidases, such as benzo(a)pyrene, dioxins, and cyanides. This is believed to occur through the oxidation of the pollutant via electron transfer from nonspecific peroxidases that do not require physical binding of the contaminant to the enzyme. The LiP intermediates have been reported to have higher reduction potentials than other peroxidases, therefore the number of chemicals oxidized has been shown to be greater than with horseradish peroxidase. Barr et al. (1994) have reported that the oxidative reactions following the direct reaction of a chemical with LiP often results in carbon-carbon bond cleavages, benzylic alcohol oxidations, demethylations, hydroxylations, and dimerizations. Some contaminants that have been reported to be degraded by this mechanism are methoxybenzenes, pyrene, polycyclic aromatic hydrocarbons, and 2,4,5-trichlorophenol.

Research has shown that the addition of a chemical that is oxidized to a free radical by LiP can result in the subsequent oxidation of chemicals that are not directly oxidized by the enzyme, a process that is referred to as indirect oxidation. The mechanisms by which indirect oxidation occurs are still being debated within the research community. The production of veratryl alcohol (3,4-dimethoxy benzyl alcohol) by *P. chrysosporium* from either lignin or glucose is believed to be a major component in the indirect oxidation of

chemicals. Veratryl alcohol has been identified as an excellent substrate for LiP and is oxidized to a cation radical, which can then oxidize other chemicals that are not directly oxidized by LiP. Another potential mechanism has been discovered by Barr et al. (1994) in which veratryl alcohol is used to produce molecular oxygen from H_2O_2. H_2O_2 is believed to be oxidized to a superoxide anion radical by a veratryl alcohol cation radical which can initiate the indirect oxidation of other chemicals. Lignin, the herbicide 3-amino 1,2,4-triazole, and various organic acids have been reported as being oxidized by the indirect LiP-dependent process (Barr et al. 1994).

Many of the pollutants that have been shown to be degraded by the ligninolytic system are highly oxidized chemicals, such as polychlorinated biphenyls, chlorinated phenols, and nitroaromatic munitions. For these highly oxidized pollutants it appears that they must first be reduced before further oxidative conversions can take place. Therefore, the fungi must have some type of mechanism to reduce these chemicals. One mechanism that has been discovered by Barr et al. (1994) involves a second veratryl cation radical mechanisms initiated by LiP. The veratryl cation has been reported by Barr et al. (1994) to oxidize chemicals, but in some cases the radical formed from this oxidation can also result in reduction reactions . It has been reported that oxalic acid is secreted by *P. chrysosporium* and serves as an electron donor for this mechanism (Barr et al. 1994). It appears that the highly oxidized chlorinated pollutants such as carbon tetrachloride, 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (DDT), 2,4,6-trinitrotoluene (TNT), and pentachlorophenol (PCP) can be metabolized by the fungus through this LiP-dependent reductive pathway. Additional

reduction mechanisms have been associated with the production of MnP from the fungi. It has been reported that MnP typically oxidizes Mn^{+2} to $MN^{+3,}$ and the Mn^{+3} species is involved in oxidative reactions. However, in the presence of hydroquinones and Mn^{+2}, MnP has been identified as catalyzing the reduction of chemicals. The Mn^{+3} species can oxidize hydroquinones to their corresponding semiquinone radicals, which can then act as reducing agents. It has been reported that *P. chrysosporium* produces a number of quinones during the degradation of lignin or when grown on glucose. Because the fungus also excretes a quinone reductase, it is believed that the MnP-dependent reductive mechanism using hydroquinones as the electron donors is a major mechanism in the degradation of oxidized environmental pollutants (Barr et al. 1994).

An alternative method of reduction used by many microbes, including filamentous fungi, involves the maintenance of a proton gradient across the plasma membrane. It has been reported that several redox active dyes are reduced by various fungi via such a membrane potential. Barr et al. (1994) have demonstrated that a plasma-membrane-dependent redox system of *P. chrysosporium* was responsible for the reduction of TNT to amino congeners. The reduction of TNT was found to be dependent upon the presence of live intact mycelia.

Oxygen markedly influences the degradation of chemicals by the ligninolytic system (Kirk 1987). Many researchers have reported increased degradation rates in cultures incubated with 100% O_2 headspace as opposed to ambient air. Therefore, a fourth important mechanism used by *P. chrysosporium* has been characterized by Barr et al. (1994). Research

has shown that veratryl alcohol produced during the ligninolytic process is oxidized to a cation radical and then reacts further with molecular oxygen to produce a superoxide ($O\cdot-$), which has been shown to be important in regulating the oxygen concentration in the environment surrounding the fungus and additional production of hydroxyl radicals (-OH) and H_2O_2. The production of hydroxyl radicals is believed to be an important component in the breakdown processes associated with the degradation of halogenated highly oxidized pollutants (Barr et al. 1994).

There are a variety of mechanisms that have been associated with the *P. chrysosporium* ligninolytic system, which make it unique from conventional bioremediation systems that employ a selective group of microbes to degrade a particular contaminant. Frequently bacterial systems require various oxidative and reductive enzymes that are induced by the particular pollutant for degradation to occur. The white rot fungus uses very nonspecific free radical mechanisms initiated by extracellular enzymes produced upon nutrient depletion and independent of contaminant activation. The degradation mechanisms that employ enzymes, such as LiP and MnP, have been shown to promote a wide variety of oxidative and reductive reactions, as well as highly reactive oxygen species that degrade a variety of pollutants (Barr et al. 1994).

I. Degradation of Alkyl Halide Pesticides by *P. chrysosporium*

Kennedy et al. (1990) performed a comparative study on six alkyl halide insecticides (lindane, chlordane, aldrin, dieldrin, heptachlor and mirex) to determine the biodegradation

of these compounds by *P. chrysosporium*. The study was performed in aqueous and soil cultures inoculated with spore suspension and corncobs respectively. Results from the study concluded that of the six compounds only lindane and chlordane underwent extensive biodegradation as evidenced by the fact that 9.4% and 23.4% of the respective compounds were degraded to $^{14}CO_2$ over a 30 day period in liquid medium and 60 days in soil-corncob cultures. The remaining compounds underwent limited transformations to intermediate metabolites but were not significantly mineralized.

Data collected in the study revealed substantial differences in the ability of *P. chrysosporium* to degrade alkyl halide insecticides similar in structure. The results showed a difficulty in the ligninolytic system to degrade uniform hexachlorocyclopentadiene rings of aldrin and dieldrin and the multicyclic structure of perchloromethenocyclobuta-pentalene (mirex) (see Figure 5). In this investigation it was shown that chlordane was mineralized to CO_2 and heptachlor was not, even though both compounds are very similar in structure. Heptachlor has a double bond between the C-2 and C-3 bonds and is not chlorinated at C-2. Chlordane has a single bond between the C-2 and C-3 carbon atoms and is chlorinated at C-2. It was concluded by Kennedy et al. (1990) that the initial oxidation of chlordane by the ligninolytic enzymes must occur at the C-2 or C-3 carbon atoms and the remaining hexachloro-cyclopentadiene portion of the molecule is subject to further attack and degradation to CO_2.

The chemical structure of toxaphene is similar to the compounds examined by

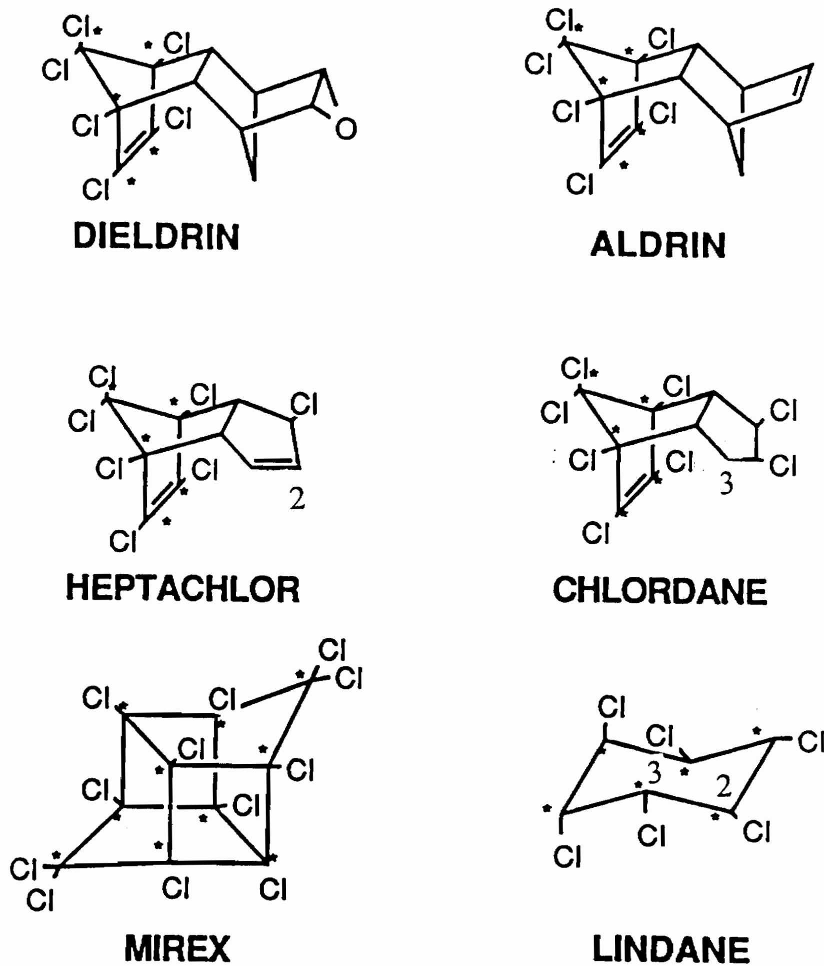


Figure 5: Structures of the alkyl halide insecticides used by Kennedy et al. (1990) in biodegradtion experiments with *P. chrysosporium*. Lindane and chlodane were mineralized by the ligninolytic system.

Kennedy et al. (1990). Toxaphene has a cyclohexane ring as its backbone structure and has no intercyclic double bonds. It would appear logical that the initial attack of toxaphene by the ligninolytic system would be on the C-2 and C-3 carbon atoms similar to what has been observed by Kennedy et al. (1990) with lindane and chlordane degradation.

J. Systems Employed for *P. chrysosporium* Ligninolytic Production

There are several systems that have been used in an attempt to apply the ligninolytic enzymes on a larger scale. A brief summary of systems that have been used to manipulate the fungus for large scale production of ligninolytic enzymes and biodegradation of complex chemicals follows. Initial research performed by Kirk et al. (1978) was unsuccessful in producing ligninolytic conditions in ordinary stirred tank reactors. Agitation of liquid medium in which *P. chrysosporium* was cultivated suppressed ligninolytic activity. It was theorized that agitation of liquid cultures caused the mycelium to form pellets which reduced the hyphae surface area to oxygen. The oxygen concentration in the pellets was reduced to such a point that the production of ligninolytic enzymes was limited to the outer surfaces of the pellets. Most of the literature has reported the use of bench scale static liquid cultures in 250 mL flasks to cultivate the fungus and produce ligninolytic enzymes.

A bench scale rotating biological contactor (RBC) disc fermenter reactor system has been used by Kirk et al. (1985) with a mutant strain of *P. chrysosporium* to scale-up the production of ligninolytic enzymes. The ligninolytic enzymes produced by the mutant strain on the RBC apparatus were reported as being homologous to the ligninolytic system

sustained by the wild fungus culture produced in static liquid cultures. The use of an RBC system to cultivate *P. chrysosporium* is not prevalent in the literature because the wild strain does not adhere well to the surface of plastic discs.

Jager et al. (1985) produced ligninolytic conditions in a stir tank fermenter by amending the medium of agitated cultures with Tween 20 and Tween 80 detergents. The ligninolytic activity was reported to be equal to or higher than activity obtained in stationary cultures or disk fermentors. These findings indicated that it was possible to scale up the production of ligninolytic enzymes in stirred tank fermertors and decrease the suppressed ligninolytic activity effects that had been associated with previous stirred culture systems for enzyme production.

A composting system amended with *P. chrysosporium* was employed by McFarland et al. (1989) to enhance the biodegradation rates of benzo(a)pyrene at 100 mg/kg concentrations. The addition of corncobs inoculated with *P. chrysosporium* increased the mineralization of benzo(a)pyrene in soil over a sixty day period from 5% to 12%. Additional experiments showed that compost systems exposed to 100% oxygen atmosphere more than doubled the mineralization rate of the contaminant, from 0.31 benzo(a)pyrene mg/kg compost material-day to 0.85 mg benzo(a)pyrene/kg compost material-day. Soil pH did not appear to be as important factor as initially believed. The fungus was observed to control the pH of its microenvironment, allowing the extracellular enzymes to metabolize contaminants in a system which had a bulk alkalinity pH. Oxygen availability appeared to be an important process variable which significantly affected the efficiency of the compost

system to enhance transformation rates of the contaminant.

Lamar et al. (1990) employed a large scale landfarming type system to research the ability of *P. chrysosporium* to bioremediate soil contaminated with pentachlorophenol (PCP) and creosote remaining from a wood preserving facility. Sterile aspen wood chips were tilled into the soil at a loading of 2.5% (w/w dry weight basis), and fungal inocula were subsequently applied to the soil with a proprietary substrate consisting of nutrient fortified grain-sawdust material at a loading of 10% (w/w dry weight basis). The soil water content was maintained at 20% and was tilled once a week to provide aeration. The initial concentration of PCP in the soil was 1000 μg/g and after 56 days of treatment the initial PCP concentration was reduced to 300 μg/g. A large portion of the original PCP was observed to be converted to undesirable intermediates (pentachloroanisole) which were not readily degraded. Factors that were observed to effect the efficiency of the system were temperature and the inability of the contaminant to contact the fungal degradative enzymes.

An immobilized system consisting of fugal mycelium grown on porous pieces of poylurethane foam saturated with liquid medium and exposed to gaseous oxygen was used by Dosoretz et al. (1993). This system was one of the few systems reported in the literature where ligninolytic conditions were achieved with the wild *P. chrysosporium* strain under nitrogen-sufficient conditions (24 mM NH_4^+). Dosoretz has attributed the effectiveness of this system to the way in which it mimics the conditions in which *P. chrysosporium* is found in nature, that is, the fungal mycelium is immobilized on porous foam medium saturated with liquid substrate and readily exposed to an aerobic environment. Dosoretz et al. (1993) were

able to achieve eight times-higher LiP activity under excess nutrient conditions compared with limiting nutrient conditions. This immobilized system appears to be highly effective in the mass production of ligninolytic enzymes.

K. Summary of the Literature Review

A review of the literature has shown that there are very few studies that have demonstrated an effective approach in mineralizing toxaphene, and no large scale biological treatment systems have been found. The highly oxidized, halogenated, and complex organic structure of toxaphene causes toxic effects on conventional bacteria that may attack it as a source for primary substrate. Katayama et al. (1991) has reported the use of a dual system of UV light and a mutant strain of the fungus *P. chrysosporium* (BU-1) to degrade toxaphene by 40% over a four week period in petri dishes. In contrast, a substantial amount of research has been performed on the idiophasic oxidative enzymes produced by *P. chrysosporium*, referred to as the ligninolytic system, and have shown its ability to degrade recalcitrant organic compounds such as lignin, polycyclic aromatic compounds, chlorinated aromatic compounds, alkyl halide pesticides, polymeric dyes, and munitions. The *P. chrysosporium* fungus is readily cultivated in liquid or solid cultures at elevated temperatures (40 °C) and on a variety of formulated media or inexpensive substrates. The degradative mechanism behind the ligninolytic system of the fungus is the production of nonspecific intracellular and extracellular enzymes, lignin peroxidases and manganese peroxidases, which are produced in nitrogen and carbohydrate limited conditions 2 to 3 days after primary

mycelia growth has terminated. Ligninolytic activity has been reported to be stimulated in cultures amended with hydrogen peroxide, the intermediated metabolite veratryl alcohol, and excess oxygen conditions. Several different systems such as RBCs, large scale fermenters, composting, landfarming, and immobilized substrate systems have been used in an attempt maximize production of the oxidative enzymes and to apply the ligninolytic system on a larger scale. An immobilized system consisting of fungal mycelium grown on porous pieces of polyurethane foam saturated with liquid medium offers a promising approach in applying the ligninolytic system on a larger scale. During the last twenty years, research has elucidated several mechanisms by which the ligninolytic system oxidizes complex organic compounds. However, the development of an effective system that employs the *P. chrysosporium* ligninolytic system to degrade alkyl halide pesticides, like toxaphene, on a large scale has not been reported.

3. METHODS, MATERIALS, and PROCEDURES

A. Experimental Approach

The objectives of this research were to evaluate the ability of *P. chrysosporium* to degrade toxaphene and to study the decoloration of an azo dye as a technique to monitor ligninolytic activity of the fungus. Discussion of the project is broken into three sections. The first section describes experimental procedures and analytical techniques employed throughout the entire project. Section two describes the series of experiments designed to evaluate the degradation of toxaphene by *P. chrysosporium*. The final section describes a technique that measured the oxidation of an azo dye added to the DMS medium to monitor fungal ligninolytic activity.

B. Analytical Techniques and Procedures

B.1 Aseptic Techniques

Aseptic techniques were used in the research project to maintain purity of the fungal cultures. All glassware, subculturing tools, growth medium, and utensils used to prepare the inoculum or to extract toxaphene were sterilized in an autoclave. The autoclave was monitored to ensure that a minimum temperature of 121 °C and 15 pounds of pressure existed for at least 15 minutes. All subplating of stock cultures, preparation of inoculum solutions, and manipulation of culture organisms were conducted in a laminar flow 0.5

micrometer air-filtered sterile hood. Addition of toxaphene into microcosms, consisting of 250 mL Erlenmeyer flasks, was performed in a sterile box constructed out of plexiglass. The sterile box was placed inside a fume hood to reduce toxaphene exposure. The box was placed over the microcosms and toxaphene stock solutions. A 70% (vol/vol) solution of ethanol was sprayed within the confines of the box to create a sterile environment and then a toxaphene stock solution was added to the microcosms. Purity of *P. chrysosporium* on stock culture plates and in microcosms was checked on a regular basis by microscopic observation.

B.2 Growth Medium for *Phanerochaete chrysosporium*

A culture of the fungus *P. chrysosporium* (ATCC# 24725BKM, F-1767) was obtained from Dr. Robert F. Hickey (Michigan Biotechnology Institute, Lansing, Michigan). Cultures were grown in 2,2-dimethyl succinate (DMS) medium following a recipe provided by Dr. Hickey. In order to eliminate extraneous sources of chloride that could potentially interfere with mass balance experiments, $CaCl_2{\cdot}2H_2O$ and NaCl were replaced with $Ca(H_2PO_4)_2$ and $NaH_2PO_4{\cdot}H_2O$. Unless otherwise stated, the *P. chrysosporium* basal medium contained the following per liter of distilled H_2O: D-glucose, 20 g; NH_4NO_3, 0.048 g; KH_2PO_4, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.5g; $Ca(H_2PO_4)_2$, 0.1 g; 2,2-dimethylsuccinic acid (DMS), 3.7 g; mineral solution, 10.0 mL; thiamine, ~100 μg. Mineral solution was prepared in 1 liter volumes of distilled H_2O containing: nitrilotriacetate, 1.5 g; $MgSO_4{\cdot}7H_2O$, 3.0 g; $MnSO_4{\cdot}H_2O$, 0.5 g; $NaH_2PO_4{\cdot}H_2O$, 1.5 g; $FeSO_4{\cdot}7H_2O$, 100 mg; $(CoNO_3)_2{\cdot}6H_2O$, 210 mg;

$Ca(H_2PO_4)_2$, 0.7 g; $ZnSO_4 \cdot 7H_2O$, 180 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; H_3BO_3, 10 mg; $NaMoO_4 \cdot 2H_2O$, 12mg; and $AlK(SO)_4 \cdot 12H_2O$, 20 mg. The growth solution was mixed and then the pH of the solution adjusted to 4.5 using a 10 N NaOH solution. Growth solutions for microcosms were prepared by pouring a volume of the DMS liquid medium into individual 250 mL Erlenmeyer flasks and capping them with a 50 mL Griffin low-form beaker. The microcosms were then processed through an autoclave to create a sterile environment for experimental runs. DMS stock-culture solid-medium plates were prepared by adding 1.5% agar to the nutrient solution, processing the solution through an autoclave, and pouring the mixture into sterile Petri dishes. The plates were allowed to cool to room temperature in the sterile hood.

Stock cultures of the fungus were stored in the dark at room temperature and maintained on DMS and 1.5% agar plates. The stock plates were subcultured under aseptic conditions once every three to six weeks by transferring an agar plug to fresh DMS plates.

B.3 Inoculum Preparation for *P. chrysosporium*

The inoculum consisted of filtered (glass wool) conidial suspensions. Conidia were taken from 3 to 6 week old stock plates, suspended in Ultapure water. The absorbance of the conidial suspension solution was measured on a spectrophotometer and diluted until a 0.5/cm reading was obtained at an Absorbance of 650 nm, (A_{650nm} = 0.5/cm is approximately 2.5 x 10^6 spores/mL), (Kirk et al., 1978). The conidial suspension was incubated at 30 °C for 24 hours and then a 1 mL aliquot was added to each experimental flask as inoculum.

B.4 Chemicals

Toxaphene, also referred to as technical grade chlorinated camphene (CAS#: 8001-35-2, melting range 65-90 °C), was obtained from AccuStandard Inc., (New Haven, Connecticut). The stock solution of toxaphene added to the experimental flasks was prepared by dissolving pure amber colored waxy toxaphene in methanol. Approximately 100 mg of toxaphene to 5 mL of methanol solvent was prepared as stock solution and diluted accordingly. A substance having a LD_{50} less than 400 mg/kg of body weight is considered to be very toxic (Hawleys 1987). Toxaphene is a highly toxic pesticide with a reported LD_{50} = 45 mg/kg. Precautions were taken throughout the research project to reduce the chance of human exposure to toxaphene. All procedures were performed in an operational fume hood with the use of gloves, goggles, and protective laboratory clothing. Waste generated from the experiments was retrieved, stored in separate containers, and properly disposed of at the conclusion of the experiment.

The azo dye Orange II, (4-[(2-hydroxy-1-naphthalenyl)azo]benzenesulfonic acid monosodium salt) was purchased from the Aldrich Chemical Co., Milwaukee, Wisconsin. Experimental flasks were prepared with 0.1% (wt/wt) Orange II dye in DMS liquid medium.

B.5 Microcosm Culture Setup and Sampling

Biodegradation of toxaphene by *P. chrysosporium* was evaluated in static DMS growth medium microcosms. Each microcosm consisted of a 250 mL Erlenmeyer flask aseptically filled with 30 mL of DMS growth medium solution and capped with a 50 mL

Griffin low-form beaker. The 50 mL flask overlapped the top of the Erlenmeyer flask by approximately one and a half inches, preventing foreign material from contaminating the cultures and allowing air to passively move into the interior of the microcosm and maintain aerobic conditions. Inoculation of the microcosms was followed by addition of toxaphene in a methanol stock solution to the DMS medium. Control microcosms were prepared and monitored parallel to live microcosms to distinguish between biological and abiotic toxaphene disappearance. The sampling scheme employed sacrificial microcosms for each data point. A sufficient number of flasks were prepared to collect a minimum of three active and two control microcosms for each data point of interest.

B.6 Extraction of Toxaphene from Microcosms

Microcosms containing 30 mL of DMS growth medium, toxaphene, and *P. chrysosporium* mycelium were removed from the incubator and extracted with neat hexane solvent for toxaphene analyses by gas chromatography (GC).

Experiments with different solvent mixtures, (neat hexane, neat methylene chloride, and a hexane:acetone (4:1) mixture), were conducted to determine which solvent would exhibit the highest extraction efficiency for toxaphene from liquid cultures. Twelve flasks were prepared, each with a 30 mL volume of liquid DMS medium and with toxaphene at a concentration of 10 mg/L. Each solvent solution was then used to extract toxaphene from four of the spiked flasks with 3 x 30 mL of the respective extraction solvents. Neat hexane exhibited an 84% extraction efficiency for the 10 mg/L toxaphene sample, which was greater

than a 75% extraction efficiency using methylene chloride and 75% for the hexane:acetone mixture (see Appendix A).

Neat hexane was chosen to be the extraction solvent for the rest of the project due to the high extraction efficiency and no requirement for a solvent exchange procedure prior to GC analyses. To assess the extraction efficiency of toxaphene a recoverability test was performed using neat hexane and four different concentrations of toxaphene in DMS growth solution. Toxaphene concentrations ranged from 200, 100, 20, and 4 mg/L. After extraction with 3 x 30 mL volumes of hexane the respective toxaphene recoveries were 79%, 82%, 85%, and 72% (see Appendix A).

A surrogate compound was chosen after experimenting with three organic compounds that were similar to toxaphene in organic structure. Alpha-BHC, endrin ketone, and mirex were obtained from the EPA Repository for Toxic and Hazardous Materials. A concentration of 0.4 μg/mL of each of the aforementioned compounds was added to hexane and analyzed by GC to compare elution times with that of toxaphene (see Appendix A). Alpha-BHC was chosen as the internal standard of choice because of its similar structure to toxaphene and its elution time, which did not coelute with toxaphene. Alpha-BHC recovery was greater than 90% when extracted in hexane, (see Appendix A).

Each toxaphene extraction and analysis procedure involved adding alpha-BHC (NSI Environmental Solutions, Research Triangle Park, NC) as a surrogate to the flask, sonicating the microcosm solutions for two minutes to break up the mycelial pellets that formed over time, and then extracting the toxaphene from the solution with 3 x 30 mL of hexane solvent

in a seperatory funnel. Following the extraction process, 50 μL of 1.8 mg/L of Restek Pesticide Surrogate Mix containing 2,4,5,6-tetrachloro-meta-xylene and decachloro-biphenyl (Restek Corp., Bellefonte, PA) was added to a 1 mL aliquot of the organic extract solution as a GC internal standard.

B.7 Gas Chromatography Analysis

Gas chromatography analyses were performed using a Hewlett Packard 5890 series II GC equipped with an electron capture detector (ECD). Refer to Appendix A for GC operating conditions. Two different chromatography columns were employed throughout the project for toxaphene analyses. Both columns were similar in performance and were used at different times depending on availability. The two columns used were a Restek RTX-5 (Restek Corp., Bellfonte, PA) and a J & W Scientific DB5MS (J&W Scientific, Folsom, CA). Prior to sample analyses, five point calibrations for toxaphene and alpha-BHC surrogate were performed. Toxaphene calibration standards were run at 0.6, 1.2, 2.5, 5.0, and 10.0 mg/L concentrations. Alpha-BHC standards ranged from 0.02, 0.03, 0.06, 0.12 and 0.25 mg/L. Calibration standard solutions were run with 0.09 mg/L of internal standard (Restek Pesticide Surrogate Mix) to monitor instrumental response variations. An R^2 value not less than 0.95 was obtained from the linear regression run on all calibration runs, (see Appendix A). Toxaphene and alpha-BHC midrange continuing calibration check standards were analyzed following fifteen consecutive microcosm samples to establish system reproducibility. Due to the multi component nature of toxaphene, which has more than 170

chlorinated isomers, chromatographic quantitation was performed by identifying nine specific peak retention times and monitoring their change in peak height (see Figure 6). The change in peak height at the specified retention times indicated a change in the overall toxaphene concentration.

B.8 Ion Chromatography Analysis

Analysis for free chloride in DMS buffer solution was performed using a Dionex Suppressed Conductivity Detection Ion Chromatography system, (Dionex Corporation, Sunnyvale, California). A 2.0/0.75 mM $Na_2CO_3/NaHCO_3$ buffer eluent solution was pumped at a rate of 1.0 mL/min via a non-gradient pump through an Anion Separator #9 (AS9-SC) column and into a Dionex conductivity detector. Bromide was added to samples prior to analysis at a concentration of 1.0 mg/L as surrogate to monitor system performance and sample preparation techniques (see Appendix B). A four point calibration was performed on the system prior to analyses to establish the working range of the detector and to quantify free chloride concentrations derived from a linear response factor. The calibration standard concentrations ranged from 0.25, 0.5, 1.0, and 4.0 mg/L for chloride, and bromide calibration standards ranged from 0.5, 1.0, 2.0, to 8.0 mg/L.

B.9 UV-Vis Spectrophotometry Analysis

A Beckman DU-640 UV-Vis spectrophotometer equipped with an automatic "sipper cell" and a 1.0 cm path length was used to monitor changes in Orange II dye absorbance.

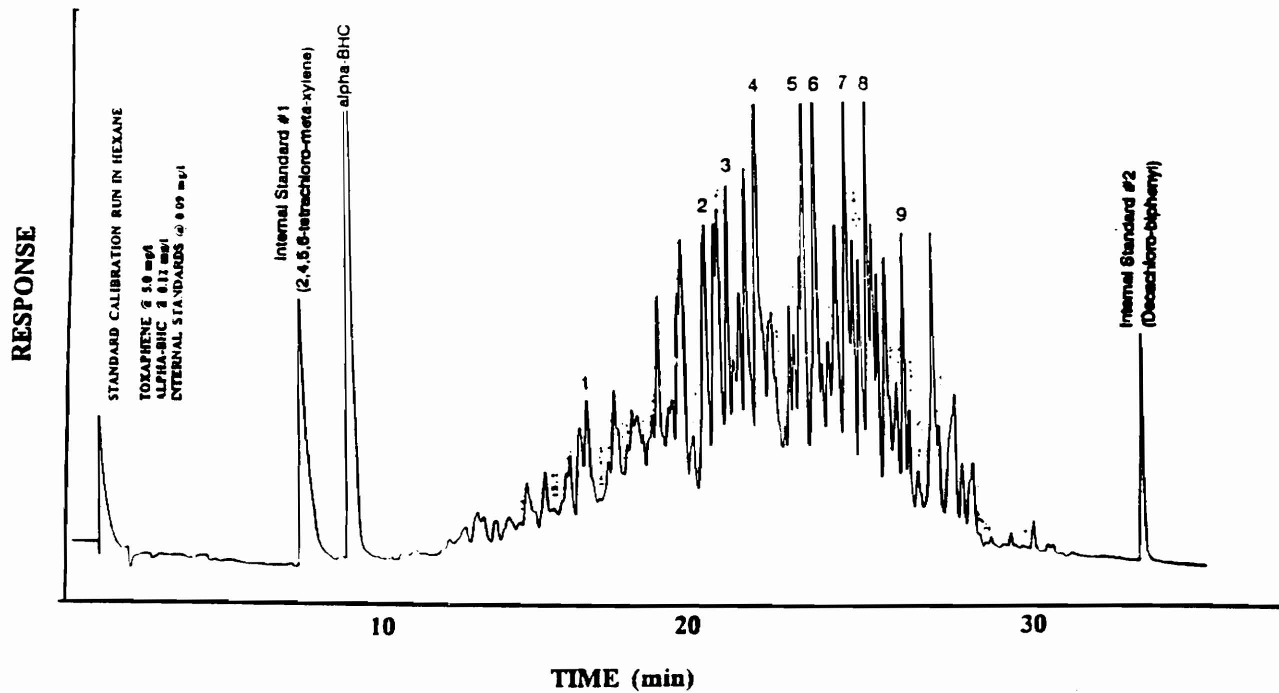


Figure 6. Capillary column gas chromatogram of 5.0 mg/L of toxaphene with target peaks 1-9 labeled.

Oxidation of the dye was determined following the method by Glenn et al. (1983). A 1.0 mL volume of the extracellular medium was removed from a microcosm and diluted ten fold with Ultrapure water. The sample aliquot was scanned from 700 nm to 200 nm; a minimum (350 nm) and maximum (484 nm) absorbance reading were determined. As dye concentrations decreased with time, samples were measured directly on the spectrophotometer without dilution. In order to distinguish between dye oxidation and absorption onto the fungus biomass, a ratio of the maximum to minimum absorbance was determined and compared to measurements taken at different points in time. The wavelengths used for the absorbance ratio were 484 nm / 350 nm (see Appendix C).

B.10 Total Chloride Analysis (TOX)

A Rosemont Dohrmann (Santa Clara, California) MC-3 model Total Organic Halide Analyzer (TOX) was used to quantify chloride concentrations in the hexane, aqueous buffer, and biomass fractions. The hexane phase was analyzed by direct injection of hexane extract into the TOX furnace at a rate of 15 μL/min with the instrument operating in the Extractible Organic halide (EOX) mode. Chloride in the aqueous phase was analyzed by passing a sample through granular activated carbon (GAC) cartridges on a Dohrmann AD-3 adsorption module and then analyzing the granular activated carbon for total chloride concentration in the Total Organic Halide (TOX) mode. The amount of chloride associated with the biomass fraction was determined by trapping extracted biomass onto fine quartz glass wool plugs and analyzing the resulting pellet in the TOX mode.

C. The Potential of *Phanerochaete chrysosporium* to Degrade Toxaphene.

The objective of this study was to determine the effect of toxaphene on the growth of *P. chrysosporium* and characterize the extent to which toxaphene could be degraded by the ligninolytic system.

Experiments were conducted in static aerobic microcosms with pure cultures of *P. chrysosporium* in liquid buffer medium to some of which toxaphene was added. Aseptic techniques were followed to maintain the purity of the fungal culture in each individual experimental microcosm and in stock cultures. To differentiate between biodegradation and abiotic reductions, toxaphene concentrations from microcosms inoculated with the fungus, also referred to as active microcosms, were compared to uninoculated control microcosms. Initially, experimental controls consisted only of toxaphene added to DMS buffer solution. Later experiments included controls with toxaphene added to pregrown mycelium that was inactived by autoclaving.

Sacrificial static microcosms were evaluated over a three to eight week period. Triplicate samples were collected on a weekly basis. Toxaphene was extracted from the microcosms using hexane and quantified by the GC analysis technique previously mentioned. Toxaphene was quantified by analyzing standards and samples with respect to the nine specified chromatographic elution times.

C.1 Effect of Toxaphene Concentration on Pesticide Degradation and Fungus Growth.

Three experimental runs were performed with concentrations of toxaphene at 3, 30, and 200 mg/L to evaluate the effect of toxaphene concentration on the growth of *P. chrysosporium* and its ability to degrade the pesticide. The microcosms were incubated at 40 °C. Samples were collected at two and four week time intervals. Active microcosms were sampled in triplicate and compared to duplicate uninoculated controls. Toxaphene concentrations in the microcosms were determined by the GC technique previously described.

C.2 Effect of Temperature on Toxaphene Degradation and Fungus Growth.

Temperature studies were performed at 20, 30, and 40 °C to determine the effect of temperature on the growth of the fungus and the degradation of toxaphene. Microcosms were prepared by adding 30 mg/L of toxaphene to DMS liquid cultures under aseptic conditions and then inoculating with *P. chrysosporium.* Samples were collected at four and eight week intervals. Active microcosms were sampled in triplicate and compared to duplicate uninoculated controls. The concentration of toxaphene in the microcosms was determined by the GC techniques previously described.

C.3 Extent of Toxaphene Degradation by *P. chrysosporium.*

Three different techniques, GC elution patterns, free chloride analyses by ion chromatography, and the distribution of chloride in the aqueous and biomass fractions of the

samples by TOX analyses were employed to assess the extent of toxaphene degradation over time. Gas chromatograms from active microcosm samples collected at 0, 7, 14, and 21 days were compared to identify any change in the early elution times and a decrease in the late eluting compounds as described by Mirasatari et al. (1987) to indicate toxaphene degradation or dehalogenation.

Ion chromatography analysis was employed to identify dehalogenation of toxaphene by the fungus resulting in an increase in free chloride concentrations in the liquid growth medium. An initial concentration of 30 mg/L of toxaphene was added to inoculated microcosms. The liquid growth medium was sampled from the microcosms on 0, 5, 9, 15, and 21 days and analyzed directly by ion chromatography analysis. Free chloride concentrations in the active microcosms were compared to uninoculated controls.

The distribution of organic chloride concentrations with time in the aqueous and biomass fractions was monitored by analyzing the different fractions using the GC and TOX analyses previously described. TOX analyses were performed on active microcosms containing an initial concentration of 30 mg/L of toxaphene after 2, 4, 10, 18, and 23 days and compared to controls. The controls contained approximately 35 mg of mycelium by dry weight. Mycelium was grown prior to the experimental run in DMS buffer solution. After fourteen days of growth the mycelium was combined, homogenized by using a sonicator, and autoclaved for thirty minutes. A 2 mL volume of the homogenized mycelium solution was determined to be equivalent to 35 mg of dry mycelium weight. Prior to adding toxaphene to the microcosms, 2 mL of the mycelium solution was aseptically transferred into the

control microcosms. After being incubated with toxaphene the active microcosms and controls were extracted with hexane for GC analysis. Following the extraction process the broken up mycelium remained dispersed in the samples aqueous fraction and was retrieved for TOX analysis by filtering the supernatant through glass wool plugs. Mycelium attached to the glass wool plugs was analyzed on the TOX to determine the mass of chloride in the biomass.

D. *P. chrysosporium* Growth and Enzyme Production

The objective of this study was to obtain an understanding of the growth and ligninolytic activity of *P. chrysosporium.* Enzyme activity was monitored using the spectrophotometric technique by Glenn et al. (1983) as discussed previously, which measured the enzyme oxidation of the azo dye Orange II, added to DMS buffer solution. The technique was used to determine the effects of temperature, pH, and additives, (nitrogen, hydrogen peroxide and veratryl alcohol) on the growth and ligninolytic activity of the fungus.

D.1 Fungus Temperature Growth Study.

Temperature growth studies were performed in liquid DMS buffer medium and on DMS solid medium plates. The growth of the fungus was determined for six temperatures, 20, 25, 30, 35, 40, 45, and 50 degrees Celsius. Liquid and solid medium cultures were

placed in temperature controlled incubators. The incubators set at the aforementioned target temperatures, fluctuated by ±2 °C throughout the study. Liquid and solid medium microcosms were aseptically prepared with DMS buffer solutions and agar plates following the procedures previously outlined. To account for temperature variations within the confines of each incubator, microcosms were randomly placed into and retrieved from different positions in each incubator .

Fungus growth in liquid cultures was determined by averaging the dry weight of six replicate samples retrieved from each of the incubators at 7 and 14 day intervals. Microcosms were removed from the incubators, filtered through pretared 0.5μ glass filter paper, rinsed with Ultrapure water, and dried in an 80 °c (± 5 °C) drying oven until replicate weight measurements indicated that a constant weight had been achieved. The average of six replicate microcosms at each temperature was recorded as the final dry weight obtained during the specified time period.

Fungus growth on solid medium cultures was determined by averaging the distance of mycelium growth from initial inoculum plugs. Mycelium growth measurements were taken at intervals of 0.8, 1.3, 1.6, 2.0, 2.5, and 2.8 days until the mycelium had covered the full length of the Petri plates. The average measurement from six replicate plates at each temperature was recorded as the total growth of the fungus obtained during the specified time period.

D.2 Ligninolytic Activity Temperature Study.

The effect of temperature on the fungus ligninolytic activity was performed in liquid DMS buffer medium and on DMS solid medium plates supplemented with 0.1% Orange II dye. Dye was added to the medium as an aqueous solution prior to being processed through an autoclave. The decoloration rate of the dye was determined for a range of six temperatures, 20, 25, 30, 35, 40, 45, and 50 °C. The incubator temperatures throughout the study fluctuated by ±2 °C at each of the aforementioned target temperatures. Liquid and solid medium microcosms were aseptically prepared with DMS buffer solutions and agar plates following the procedures previously outlined.

Dye decoloration in liquid cultures with the fungus was measured by the previously mentioned spectrophotometric technique. Microcosms were removed from the incubators, a 1.0 mL aliquot was aseptically sampled from each microcosm, diluted ten fold, scanned on a spectrophotometer, and the A_{484}/A_{350} absorbance ratio calculated. The average absorbance ratios (A_{484}/A_{350}) from six independent microcosms, incubated at each of the target temperatures, were reported as the final Orange II dye absorbance reading during the specified time interval. Absorbance readings were taken from microcosms incubated for 2, 4, 9, 13, 15, 20, 25, and 28 days at the aforementioned target temperatures. Dye decoloration was complete on day 15 for microcosms incubated at 30 °C.

Ligninolytic activity was observed on solid-medium cultures incubated at the aforementioned temperatures by decoloration of the Orange II dye in the DMS agar solid medium around the initial inoculum plug. The medium displayed a bright orange color

which turned clear upon the initiation of ligninolytic activity. Decoloration was measured at intervals of 3.8, 4.5, 7.5, and 8.5 days, until the entire plate was clear due to enzymatic oxidation of the dye. An average measurement from six replicate plates at each temperature was recorded as the final decoloration obtained during the specified time interval.

D.3 Ligninolytic Activity Amendment Study.

The effects of pH, nitrogen concentration, hydrogen peroxide addition, and veratryl alcohol addition on ligninolytic activity of the fungus was explored. Liquid DMS microcosms were prepared with 0.1% Orange II dye added to the medium. Amendment studies were performed at the previously determined optimum ligninolytic activity temperature of 30 °C. A 1.0 mL sample of supernatant was aseptically retrieved from the microcosms at the prescribed time periods. Dye decoloration in the liquid cultures with the fungus was measured following the previously outlined spectrophotometric technique. Each data point was obtained from triplicate microcosm samples.

The pH study was run at pH ranges of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. The pH of the microcosms was adjusted with a 10 N NaOH solution. Microcosms were sampled and the decoloration of the dye was analyzed at 4, 11, 13, 18, and 25 day intervals.

Four different concentrations of nitrogen (NH_4NO_3) were used to evaluate the effect of nitrogen concentration on ligninolytic activation at 30 °C. Microcosms were prepared in triplicate with 0.6, 4.5, 24, and 45 mM of nitrogen and sampled 4, 8, 13, 15, and 18 days.

The effect of hydrogen peroxide on ligninolytic activity at 30 °C was evaluated by

supplementing liquid DMS medium with 0.003, 0.03, 0.3, and 3.0 mM concentrations of hydrogen peroxide. Microcosms were sampled and dye absorbance measured at time intervals of 4, 8, 13, 18, and 25 days.

Three different concentrations of veratryl alcohol were added to DMS medium in an attempt to stimulate ligninolytic activity at 30 °C. Microcosms were prepared in triplicate with veratryl alcohol ranging between 0.2, 2.0, and 20 mM concentrations. Supernatant, amended with 0.1% Orange II dye, was sampled from each of the microcosms at time intervals of 4, 8, 11, 18, and 25 days.

D.4 Ligninolytic Reactivation Study.

Microcosms used to evaluate the ability to reactivate the ligninolytic system were prepared differently than in the previously mentioned experiments. The fungus was grown in a nonimmersed liquid culture system as outlined by Dosoretz et al. (1993). It consisted of fungal mycelium immobilized on porous pieces of polyurethane foam saturated with liquid DMS medium amended with 0.1 % Orange II dye. Inoculated microcosms were run in triplicate and an uninoculated control was run with the active microcosms to monitor any abiotic reduction of dye in the microcosms. The active microcosms were sampled, and the decoloration of the dye was measured following previously mentioned spectrophotometric techniques. The microcosms were sampled on 2, 5, 7, and 15 days. On the fifteen day the bright orange color of the supernatant disappeared. Following aseptic technique fresh DMS buffer solution amended with 0.1 % Orange II dye was added to the microcosms. The

microcosms were sampled again on the twenty-third and twenty-eighth day. By the twenty-eight day the dye was once again decolored. The control microcosms lost approximately 40% of the dye from adsorption to the polyurethane foam. Adsorption of the dye, in the control microcosms, onto the polyurethane foam reach a maximum by day six of the experiment.

4. RESULTS AND DISCUSSION

A. Degradation Potential of Toxaphene by *P. chrysosporium.*

Initial studies with toxaphene incubated with *P. chrysosporium* revealed a difference between the extractible concentration of toxaphene from the active microcosms and the uninoculated controls (Figure 7). The greatest rate for the decrease of toxaphene was observed during the first week of incubation and fungal growth. Initial fungal growth was observed within twenty-four hours after inoculation and continued at a steady rate until approximately six days after inoculation. On the sixth and seventh day of incubation white spores appeared on the mycelium giving the appearance of powder sugar sprinkled atop the mycelium mats floating on the DMS buffer solution. By day ten of the experiment the mycelium turned a light brown color indicating the initiation of cell autolysis as described by Tien et al. (1984). Toxaphene concentrations dropped 30 % in active microcosms compared to the uninoculated controls. Attempts to add amendments to the growth solution to reactivate a decrease in toxaphene concentration in the microcosms was unsuccessful. On day twenty-five a fresh 30 mL aliquot of DMS nutrient solution was added to the microcosms. The fungus initiated growth within twenty-four hours, but there was no significant change in the toxaphene concentration after three and a half weeks (Figure 7).

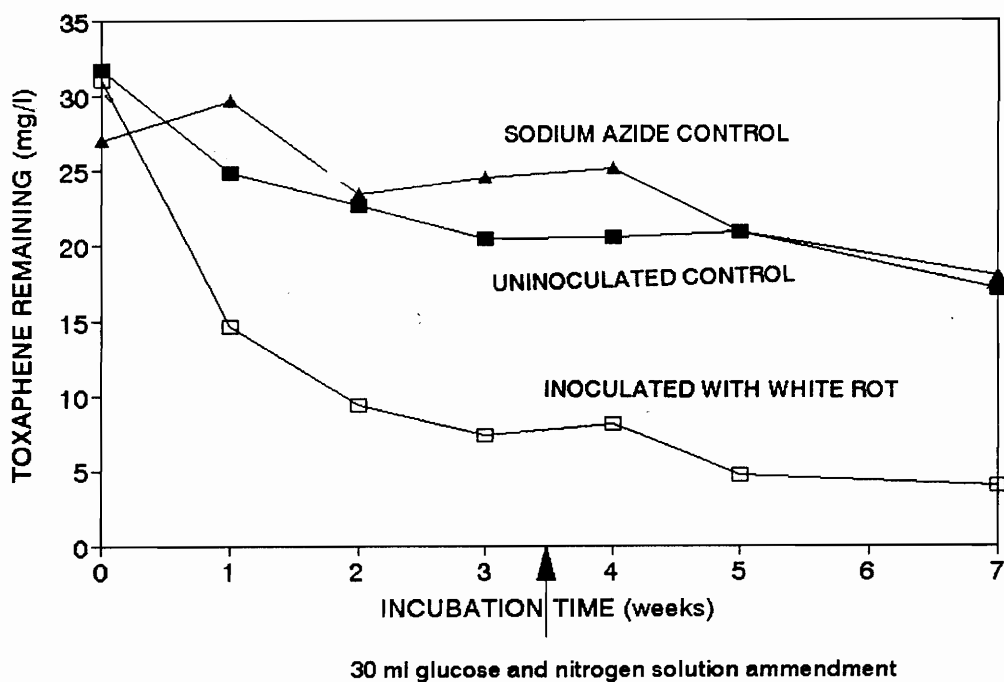


Figure 7. Toxaphene concentration in weeks at 40 °C. Initial experimental run with 30 mg/L of toxaphene incubated with *P. chrysosporium* versus uninoculated controls. Fresh DMS buffer added on day 25 of incubation.

A.1 Effect of Toxaphene Concentration on Fungus Growth.

Fungus growth was observed in all microcosms spiked with toxaphene at concentrations of 3, 30, and 200 mg/L, (Figures 8 and 9). Mycelium growth was more sparse in microcosms incubated with 200 mg/L of toxaphene, but the concentration was not lethal to the fungus. Reduction of the toxaphene concentration appeared to be complete after the initial week of incubation. The rate of toxaphene reduction in the active microcosms was equivalent to uninoculated controls between the two and four week incubation period. The decrease in toxaphene concentration during this later time period was attributed to volatilization as observed in the uninoculated controls.

A.2 Effect of Temperature on Toxaphene Degradation in Liquid Cultures.

Fungal growth was observed in microcosms incubated at 30 and 40 °C, and negligible growth was observed in microcosms incubated at 20 °C. Toxaphene concentration profiles in the microcosms incubated at 30 and 40 °C followed similar patterns after four and eight weeks of incubation (Figure 10). The concentration of toxaphene in the active microcosms incubated at 20 °C did not exhibit any difference from uninoculated controls over the eight week period (Figure 11). Growth in the microcosms incubated at 20 °C was not observed until five or six days after inoculation and then proceeded slowly with minimum mycelium biomass accumulation after eight weeks.

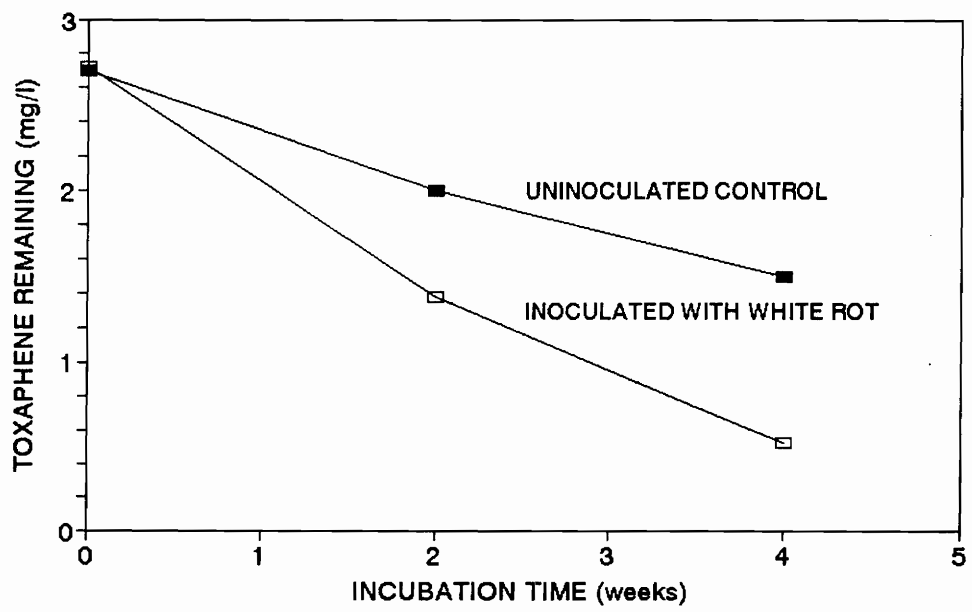
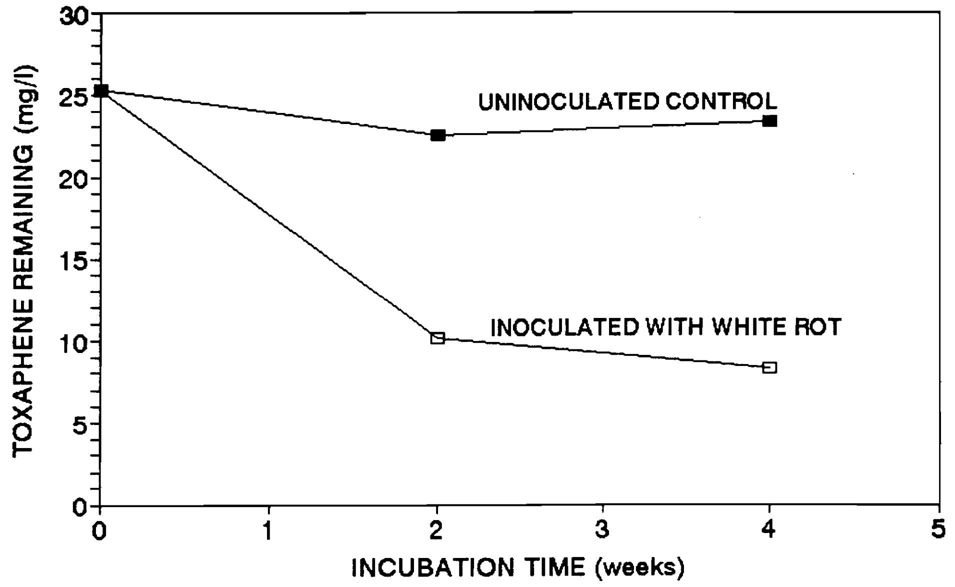


Figure 8. Concentration of toxaphene in weeks at 40 °C. Experimental run with an initial concentration of 3 mg/L of toxaphene incubated with *P. chrysosporium* versus uninoculated controls.



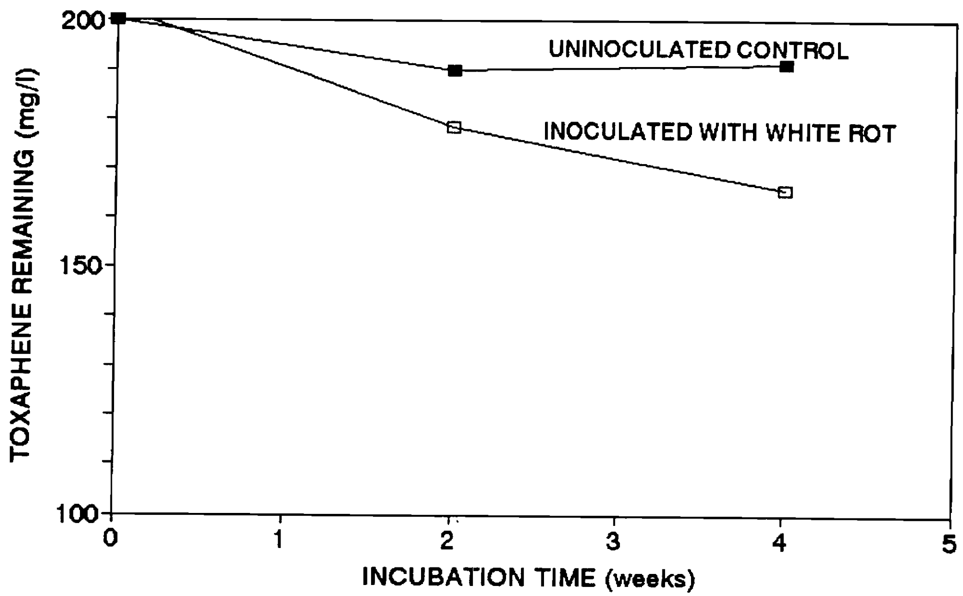
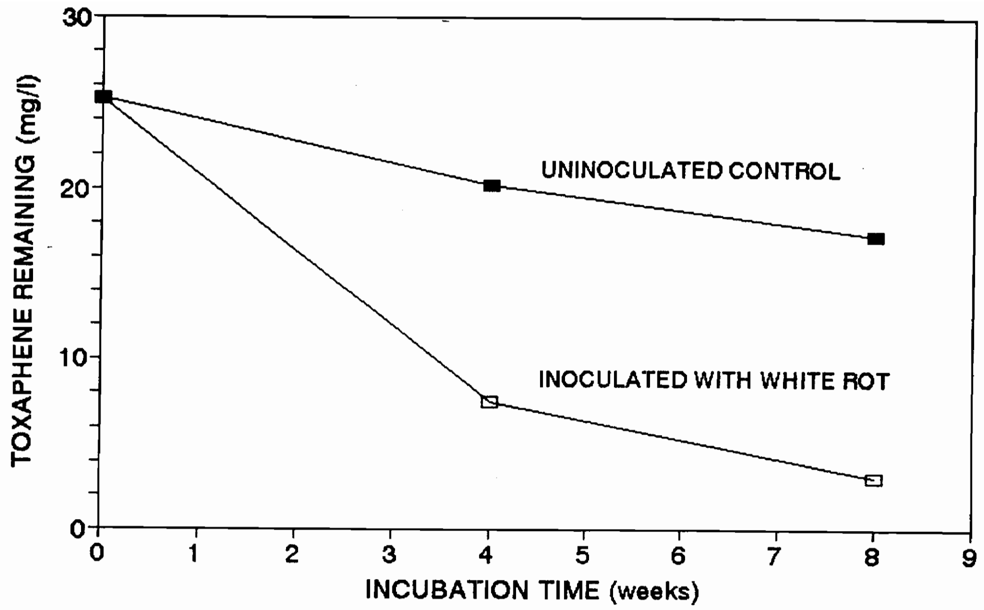


Figure 9. Toxaphene concentration in weeks at 40 °C. Experimental runs with initial concentrations 30 mg/L (top plot) and 200 mg/L (bottom plot) of toxaphene incubated with *P. chrysosporium* versus uninoculated controls.



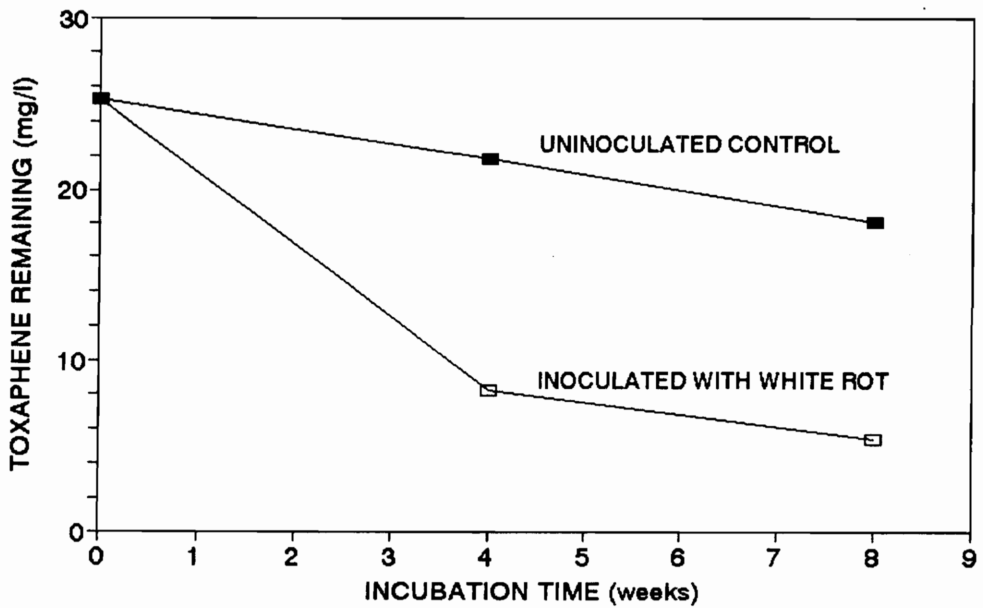


Figure 10. Toxaphene concentration in weeks at 30 °C (top plot) and 40 °C (bottom plot). Experimental run with initial concentration of 30 mg/L of toxaphene incubated with *P. chrysosporium* versus uninoculated controls.

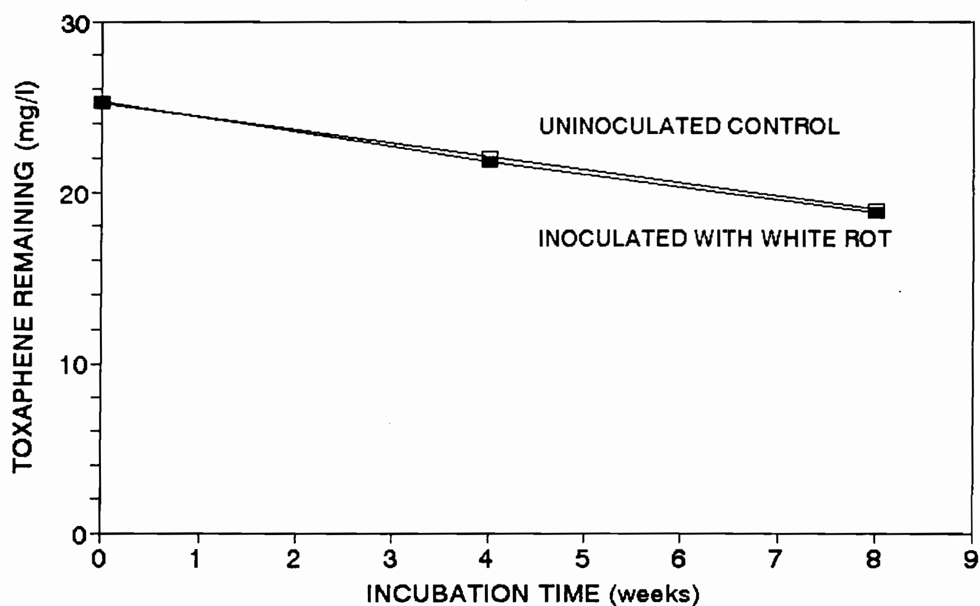


Figure 11. Toxaphene concentration in weeks at 20 °C. Experimental runs with intial toxaphene concentration of 30 mg/L incubated with *P. chrysosporium* versus uninoculated controls.

It was observed at the 30 and 40 °C incubation temperatures that the highest decrease in toxaphene occurred during the initial two weeks of incubation. A minimum temperature of 30 °C was required to obtain sufficient fungus growth to reduce the toxaphene concentration.

A.3 Assessment of Toxaphene Degradation by *P. chrysosporium*.

The chromatographic patterns of toxaphene incubated with the fungus at 40 °C for 0, 7, and 25 days are displayed in Figure 12. Overall the chromatographic peaks decreased uniformly. There was a minimal shift in late eluting compounds to early retention times. The percent peak height distribution for the nine target toxaphene elution times and four larger chromatographic groups are shown in Figure 13. Ratios calculated by the height of GC peaks 1, 2, 3, 4, 5, 7, 8, and 9 over the height of peak 6 are displayed in Table 3. Peak height ratios for the early eluting congeners increased during the twenty-five day incubation period while the ratios for later eluting peaks decreased. The wider grouped chromatographic elution times of 10-15.5 minutes, 15-20 minutes, and 20 to 25.5 minutes, displayed an increase in peak height of 2%, 7% and 1% respectively. A 6% decrease in peak height was observed for the 25.5-30 minutes chromatographic eluting peaks.

In an attempt to identify the presence of toxaphene degradation or dehalogenation, the DMS buffer solution from active microcosms was analyzed using ion chromatography to detect any increase in free chloride concentrations with time. DMS aliquots retrieved from

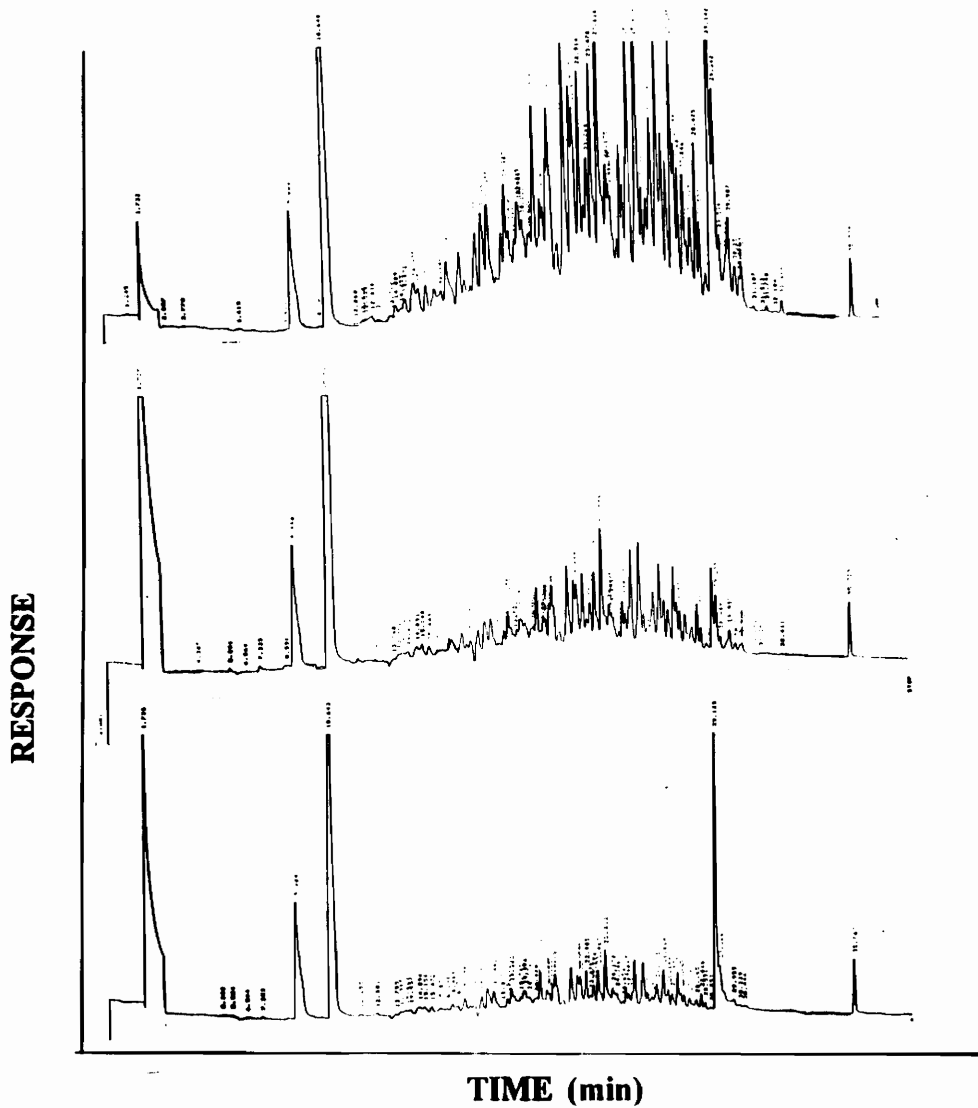


Figure 12. Chromatograms of toxaphene at 0, 7, and 25 days of incubation with *P. chrysosporium* at 40 °C.

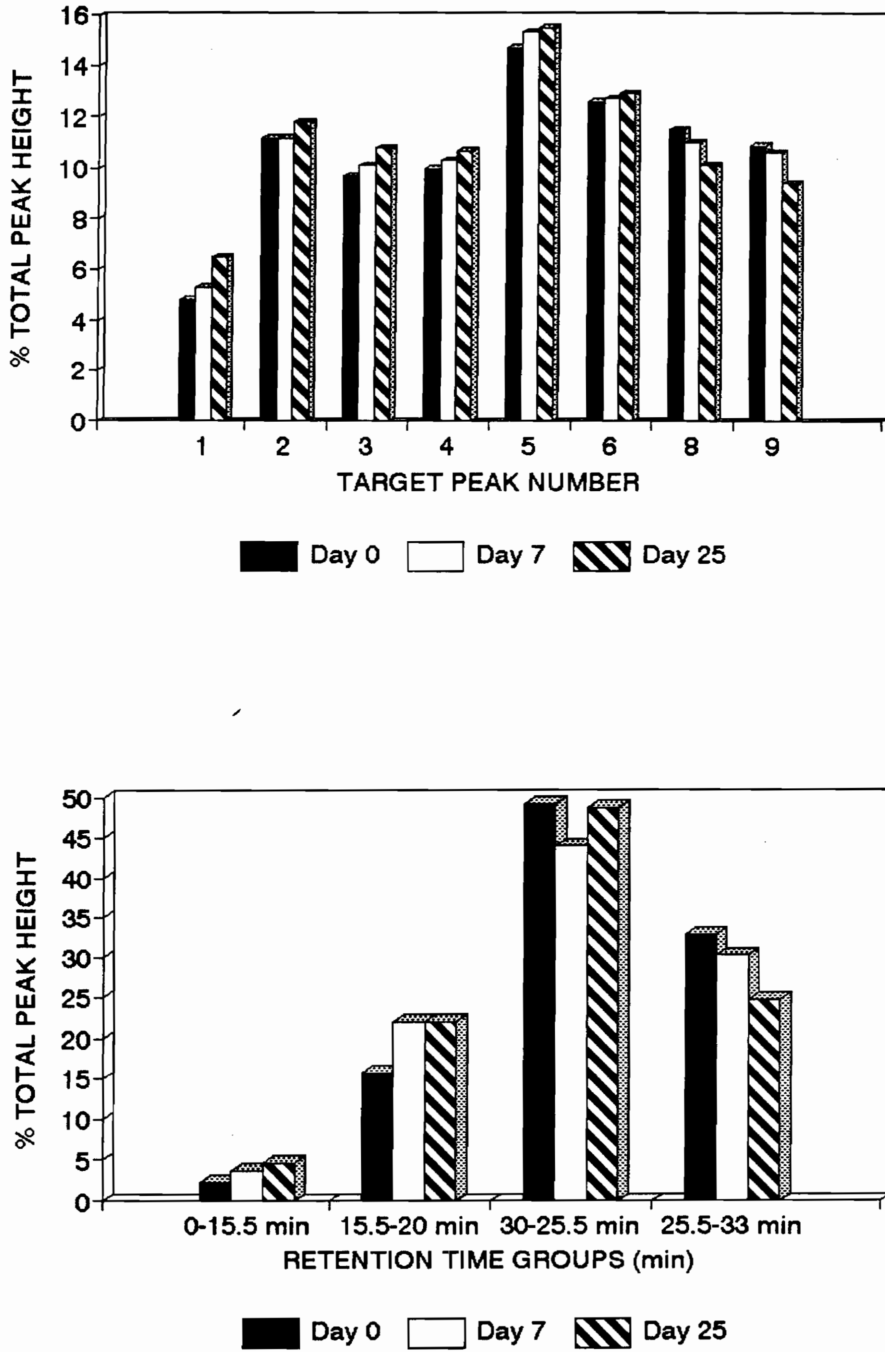


Figure 13. Chromatographic peak height distributions of toxaphene at 0, 7, and 25 days of incubation with *P. chrysosporium* at 40 °C. Peak height distribution of the nine target peaks (top plot) and retention time groups (bottom plot).

Table 3. Ratio of the height of GC peaks 1,2,3,4,5,7,8, & 9 to height of peak 6 over incubation times of 0, 7, and 25 days.

Sample	0	7	25 days
Peak #			
1	0.38	0.42	0.50
2	0.89	0.88	0.97
3	0.77	0.80	0.83
4	0.79	0.81	0.83
5	1.17	1.21	1.20
6	1.00	1.00	1.00
7	1.15	1.07	0.95
8	0.91	0.86	0.78
9	0.86	0.83	0.73

active microcosms on 0, 5, 8, 15, and 21 days after inoculation resulted in increased concentrations of free chloride concentration from controls sampled during the same time period. The average concentration of free chloride quantified in controls and active microcosms is displayed in Figure 14. A maximum concentration of 1.5 mg/L of free chloride above controls was detected seventeen days after inoculation.

An increase in the free chloride concentration in the DMS buffer was indicative of toxaphene degradation or dehalogenation with time. A total of 1.5 mg/L of free chloride detected in the active microcosms was equivalent to the release of 7% of the chloride mass originating from the initial 30 mg/L toxaphene concentration. These results were similar to findings reported by Katayama et al. (1991) who reported an 8% decrease over a one week period with an initial 1 mg/L toxaphene concentration.

A.4 Mass Balance Studies to Determine Chloride Concentrations in Biomass.

It was proposed that the uniform decrease in the toxaphene chromatographic peaks with time, as shown in Figure 12, was due to direct dehalogenation of the toxaphene congeners by the ligninolytic system. The inability to detect an equivalent increase in free chloride released to the liquid medium suggested that the unaccounted chloride mass may be released into the headspace. To determine if the ligninolytic system was directly dehalogenating the toxaphene to volatile halogenated compounds, a mass balance experiment was performed. Five 125 mL screw cap bottles with air tight septum caps were aseptically

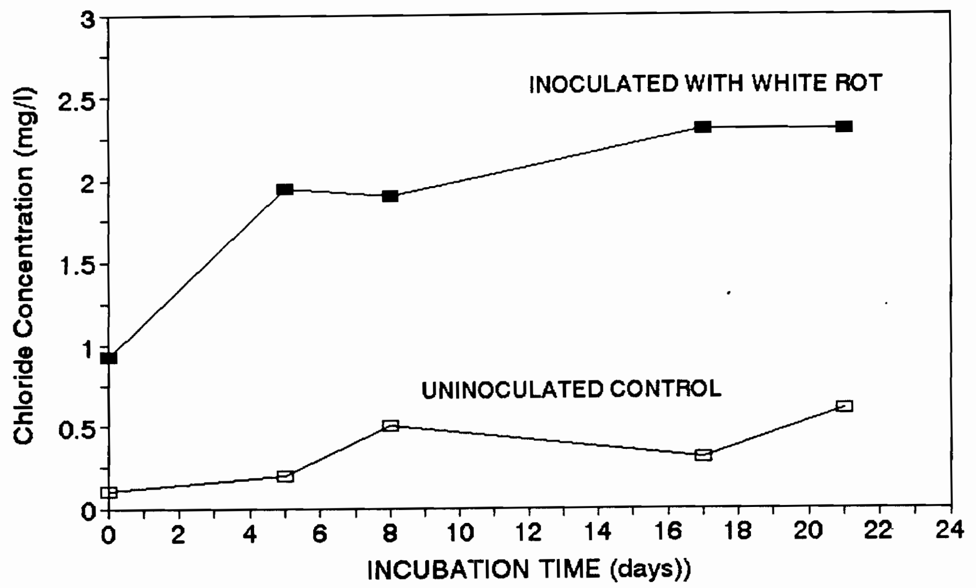


Figure 14. Free chloride concentrations by ion chromatography in days at 40 °C. Initial toxaphene concentration of 30 mg/l incubated with *P. chrysosporium* versus uninoculated controls.

prepared with 30 mLs of the DMS growth medium. One of the bottles served as a control with 35 mg of autoclaved biomass and the remaining four bottles were inoculated with the fungus. A 30 mg/L concentration of toxaphene was added to each bottle. Each bottle was purged with three volumes of 0.45 micron filtered pure oxygen and incubated at 40 °C for two weeks. On the fourteenth day approximately 20 mL of gas samples were taken from the headspace of each bottle and analyzed on a mass-spectra detector via a purge and trap apparatus. No chlorinated gas byproducts, such as mono- and poly-chlorinated methanes, were detected by the mass spectral analysis. The biomass and liquid medium fractions were extracted with hexane and then analyzed for total chlorides, using the TOX analyses previously outlined. Results from the TOX analyses for the liquid and biomass fractions are displayed in Figure 15. An average recovery of ninety percent for the chloride mass associated with the toxaphene initially added to the bottles was recovered. Results from the mass balance study indicated that a large portion of the toxaphene was retained by the biomass and no significant amount of volatile chlorinated constituents were released into the headspace.

A.5 Final Toxaphene Profiles in Microcosms Inoculated with *P. chrysosporium.*

Upon recognition by the mass balance studies that adsorption of toxaphene on to biomass was a main removal mechanism, the initial microcosm experiments were repeated using controls that contained 35 mg of autoclaved biomass. Samples were collected from

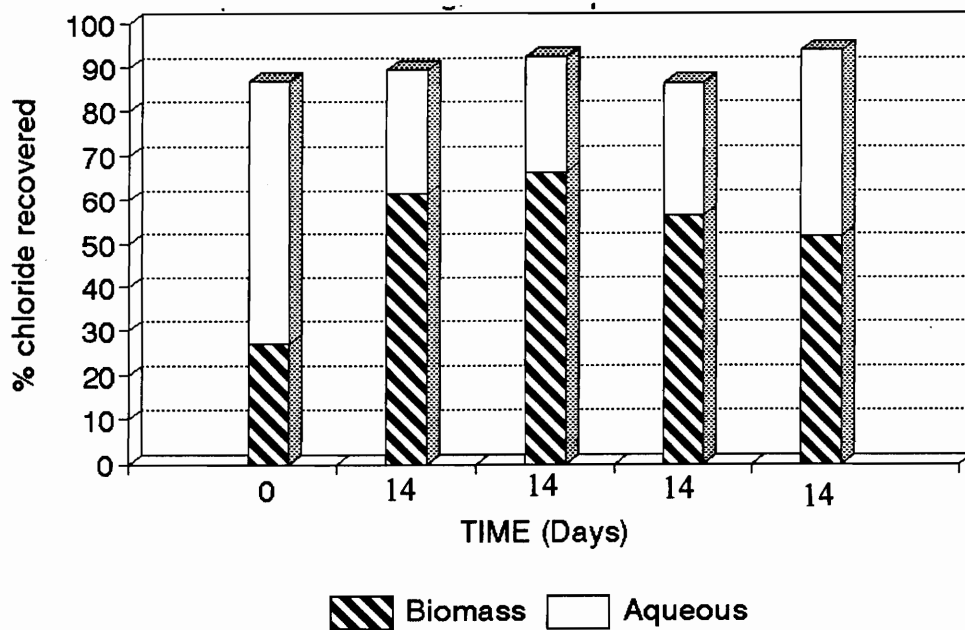


Figure 15. The percent chloride recovered in aqueous and biomass fractions of mass balance studies, based upon four active microcosms compared to an uninoculated control.

the active microcosms at 2, 4, 10, 18, and 23 days after being inoculated with the fungus. The biomass fraction was analyzed directly on the TOX instrument following the extraction process previously mentioned. Studies revealed a good correlation between aqueous toxaphene concentrations determined by GC and TOX (Figure 16). Therefore, toxaphene concentrations in the aqueous fraction were analyzed by GC.

The percent chloride recovered with time in the aqueous and biomass phases is displayed in Figure 17. A plot of toxaphene with controls that incorporated inactivated biomass via autoclaving versus active microcosms is displayed in Figure 18. It was apparent from this study that in the initial experiments, shown in Figure 7, the 30% difference in toxaphene concentration between the controls and active microcosms was due to the lack of mycelium being incorporated into the experimental controls. Therefore, as the mycelium mass grew in the active microcosms over the first two weeks the concentration of toxaphene in solution decreased as it was incorporated into the biomass. This project did not determine if the toxaphene associated with the biomass was metabolically reduced or physically adsorbed to the surface of the biomass.

A.6 Evidence and Implication for Fungal Degradation of Toxaphene.

A review of the research results shows there to be substantial evidence for toxaphene degradation by the *P. chrysosporium* ligninolytic system. Although the main removal mechanism of toxaphene from solution was determined to be adsorption to the fungus

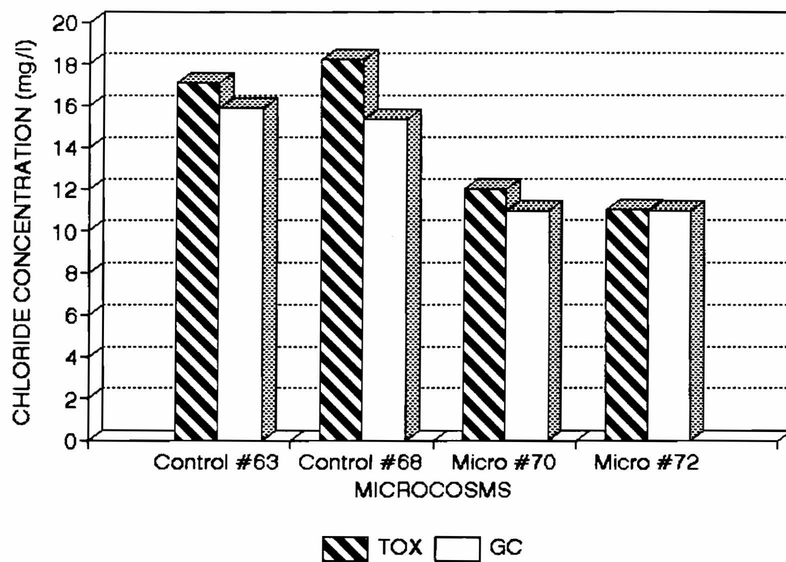


Figure 16. Comparison of TOX versus GC analyses of chloride concentrations associated with 30 mg/l of toxaphene in sample aqueous fraction.

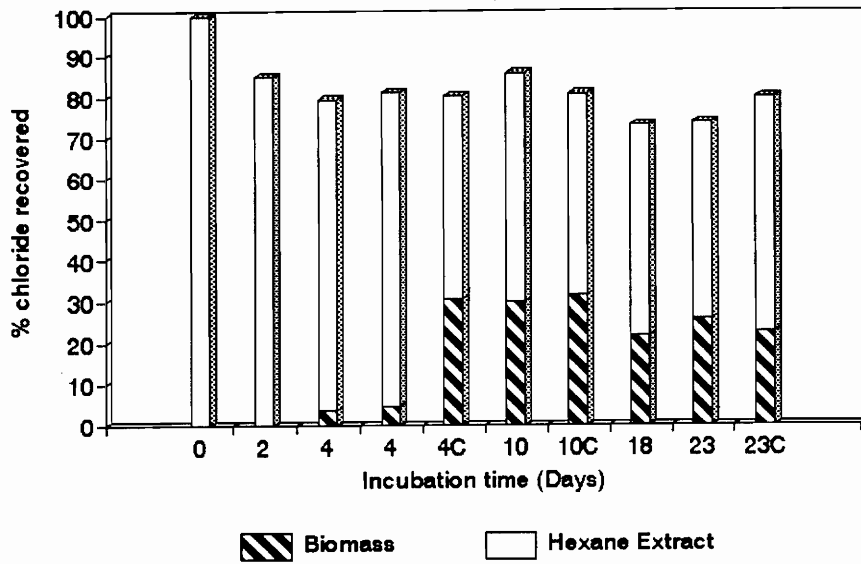


Figure 17. Percent chloride recovered with time in aqueous and biomass fractions of microcosms with an initial toxaphene concentration of 40 mg/L at 30 °C. A "C" notation indicates controls that contained 35 mg of autoclaved biomass.

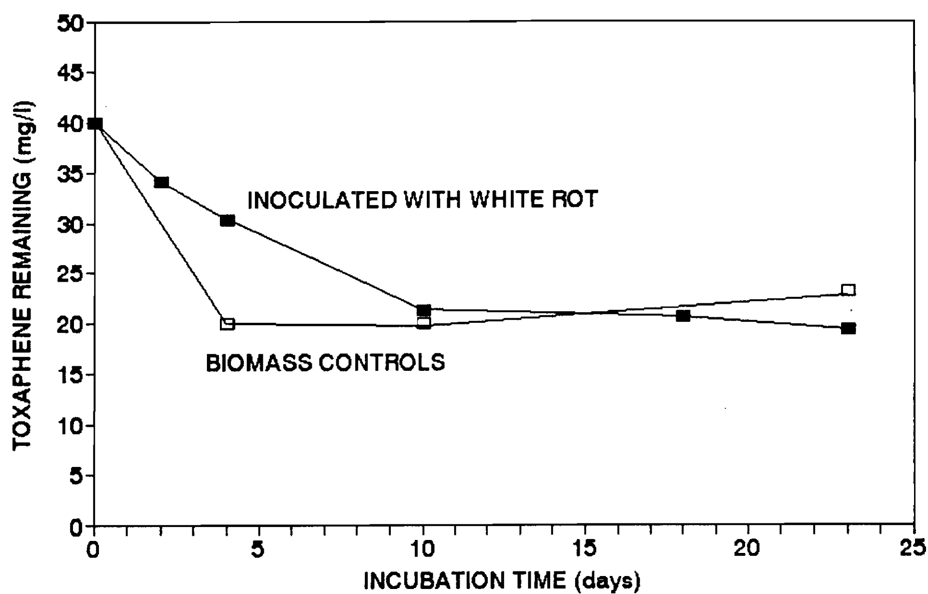


Figure 18. Toxaphene concentration in days at 30 °C. Initial concentration of toxaphene at 40 mg/L incubated with *P. chrysosporium* versus autoclaved biomass controls (35 mg).

mycelium. An increase in early eluting gas chromatographic activity, shown in Figure 13, implied that a percentage of the original toxaphene mass was reduced to smaller constituents over time. Increased production of free chloride concentrations over time, as shown in Figure 14, was also indicative of toxaphene degradation by the ligninolytic system. A 7% increase in the release of free chlorides to solution over time was consistent with research results published by Katayama et al. (1991) who concluded that *P. chrysosporium* did degrade toxaphene. Therefore, we conclude from these results that the ligninolytic system of *P. chrysosporium* has the capability to degrade toxaphene. The main obstacle observed during this research project in applying the ligninolytic system to degrade toxaphene is the inability to reactivate or produce ligninolytic conditions on a continuous basis. Published literature has also made mention of this limitation. Unless an efficient system is devised that can control ligninolytic activation and increase the production of ligninolytic enzymes then degradation rates will continue to be slow and the technology will be ineffective for large scale biotreatment of alkyl halide pesticides.

B. *P. chrysosporium* Growth and Enzyme Activation.

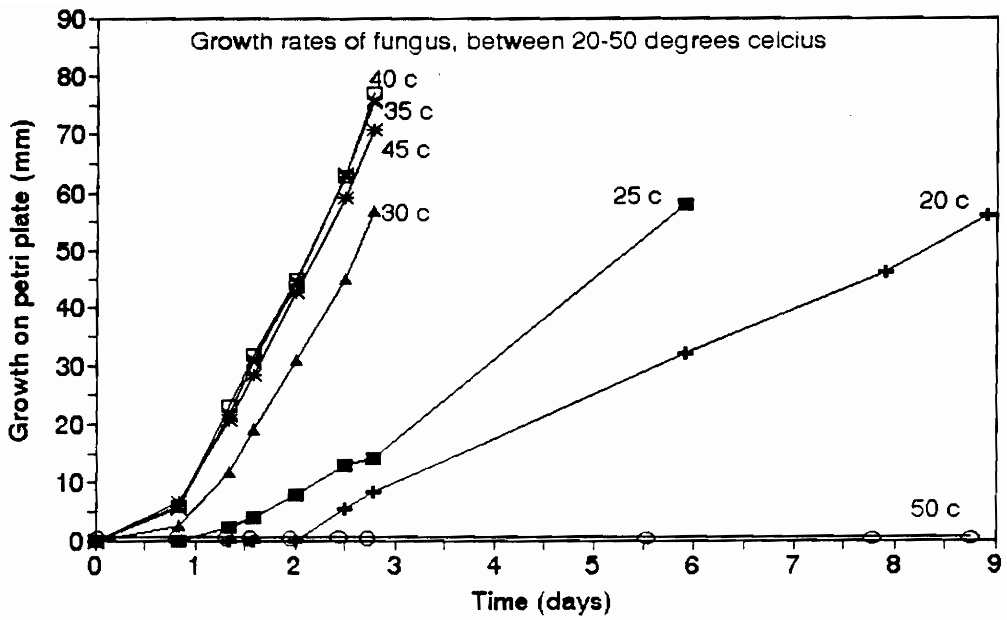
The identification of adsorption being a main component in the removal of toxaphene from solution raised the question of ligninolytic conditions being achieved in the

experimental microcosms. A series of experiments focused on the identification and activation of the ligninolytic system, and a discussion of the results of these experiments follows.

B.1 *P. chrysosporium* Temperature Growth Studies.

The growth rates at various temperatures of the fungus on DMS plates and in liquid medium, between 20 and 50 °C are displayed in Figures 19 and 20. The best results were obtained on the DMS plates by measuring the mycelium growth from inoculum plugs over time. Growth of the fungus was observed for all temperatures below 50 °C. Fifty degrees Celsius was observed to be a lethal temperature for the fungus. The maximum growth rate occurred at 40 °C. Growth of the fungus was much slower at temperatures less than 30 °C.

Results from the growth studies performed in liquid medium were more variable. The initial dry weights obtained for the fungus grown in the liquid-medium at the different temperatures concurred with the results obtained on the DMS solid-medium studies. Forty degrees Celsius (40 °C) was the temperature at which the maximum mycelium dry weight was obtained. There was a decrease in the overall dry weights of the fungus measured between the day 7 and day 14 time periods. The drop in the mass overall dry weights was most probably due to autolysis of the fungus. Kirk et al. (1978) reported a decrease in mycelium dry weight between the seventh and fourteenth day of incubation. The growth of the fungus reached a maximum after five to six days.



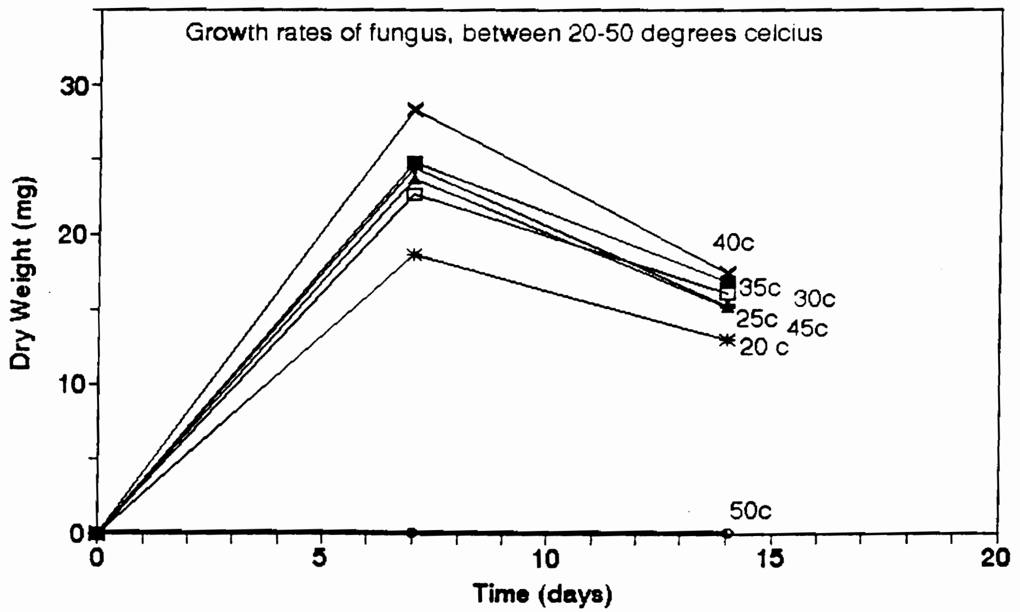


Figure 19. Fungus growth in days as a function of temperature on DMS Solid-medium (top graph) and in liquid-medium (bottom graph).

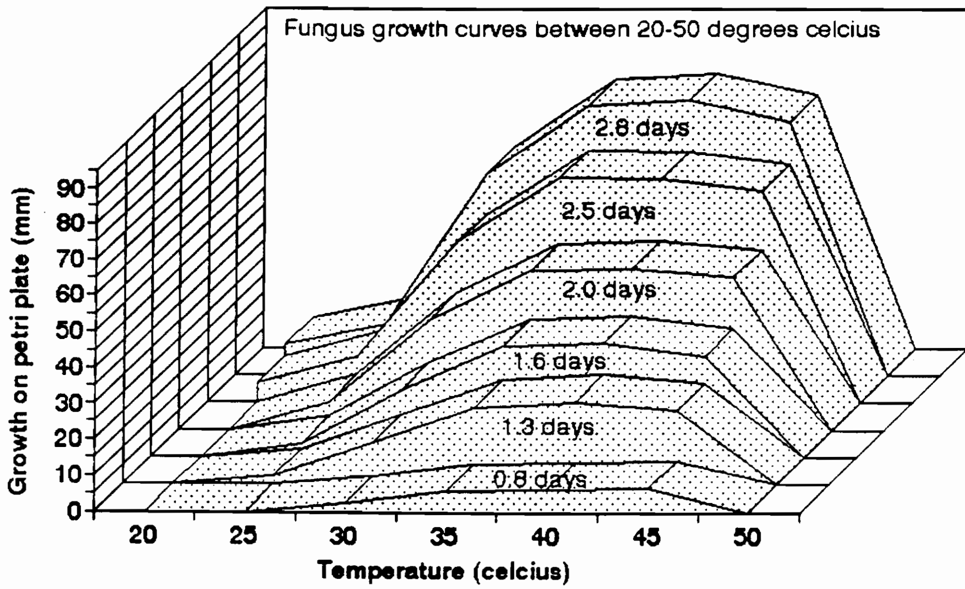


Figure 20. Fungus growth curve as a function of temperature on DMS solid-medium.

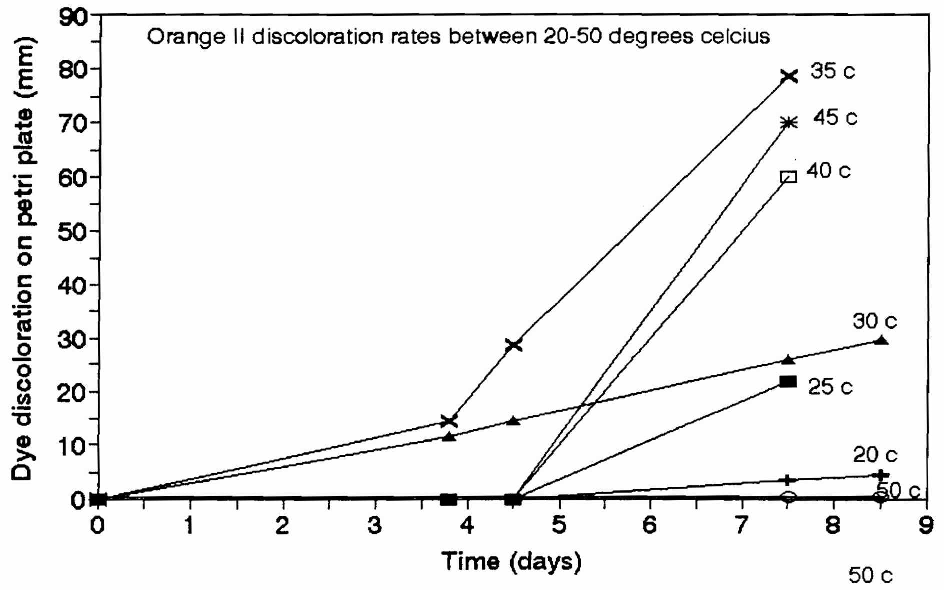
B.2 The Effect of Temperature on Ligninolytic Activity.

The results of the enzyme activity temperature studies are displayed in Figures 21 and 22. The best results were obtained from the liquid medium cultures that were spectrophotometrically measured. The optimum temperature for ligninolytic activity was 30 °C. Results did not clarify if the increased enzyme activity was due to a larger quantity of proteins being produced or if only the proteins were more active at 30 °C.

The literature suggests that enzyme activity is proportional to optimum fungal growth. These data results indicate that although the maximum growth occurred at 40 °C, increased enzyme activity was observed at 30 °C. The peak temperature for maximum fungus growth compared to maximum enzyme activity are displayed in Figure 23. The Orange II dye used as an indicator for ligninolytic activity confirmed that ligninolytic conditions were initiated approximately three days after the inoculum was introduced into the microcosms. Figure 24 displays the time sequence of growth and ligninolytic activity that occurred in the microcosms.

B.3 The Effect of pH on Ligninolytic Activity.

The decoloration of Orange II by the fungus enzyme system at pH between 4.0 and 7.0 are plotted in Figure 25. Over all there did not appear to be any major stimulation or inhibition of the enzyme system with the range of pHs used in the study. Kirk et al. (1978)



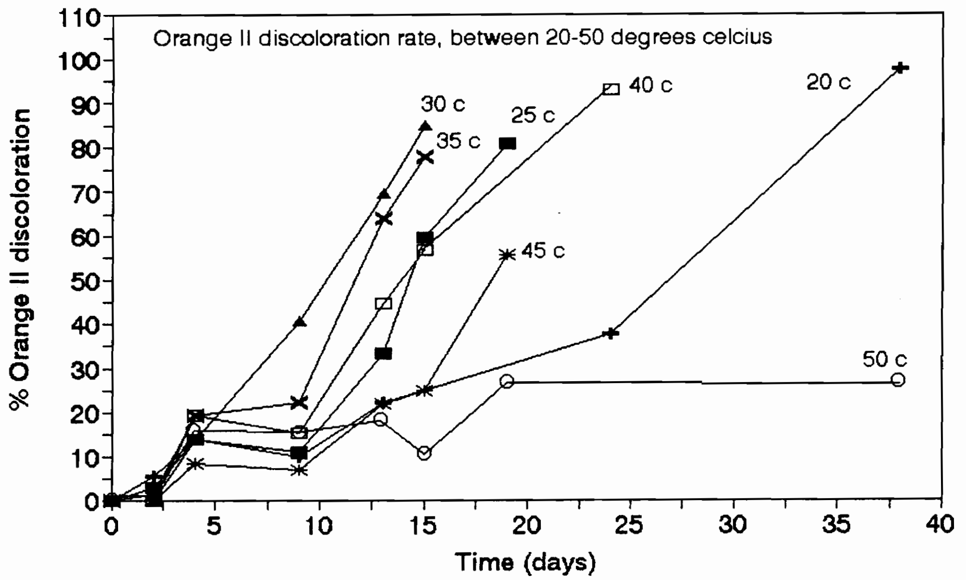
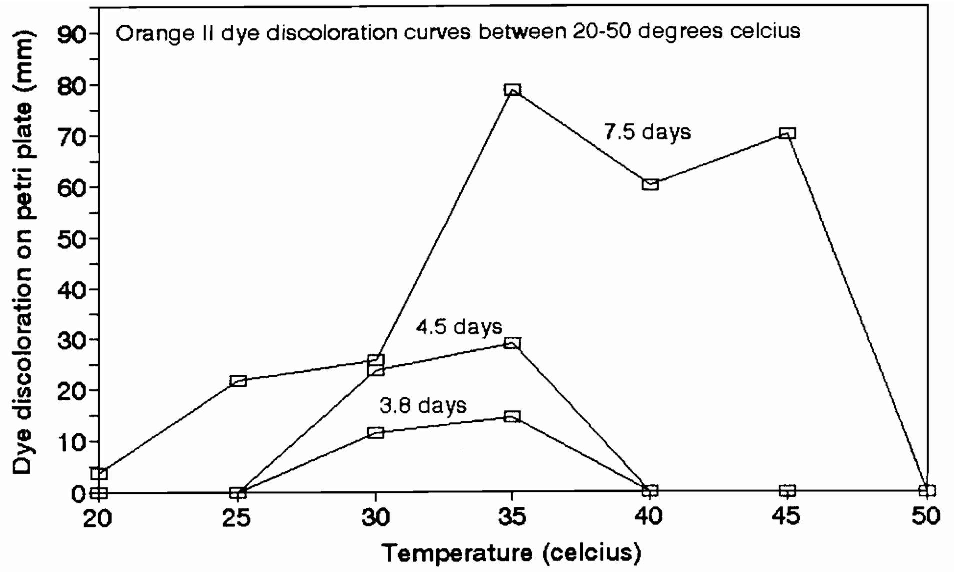


Figure 21. Orange II dye decoloration in days by *P. chrysosporium* enzymes as a function of temperature on DMS solid-medium (top graph) and in liquid medium (bottom graph).



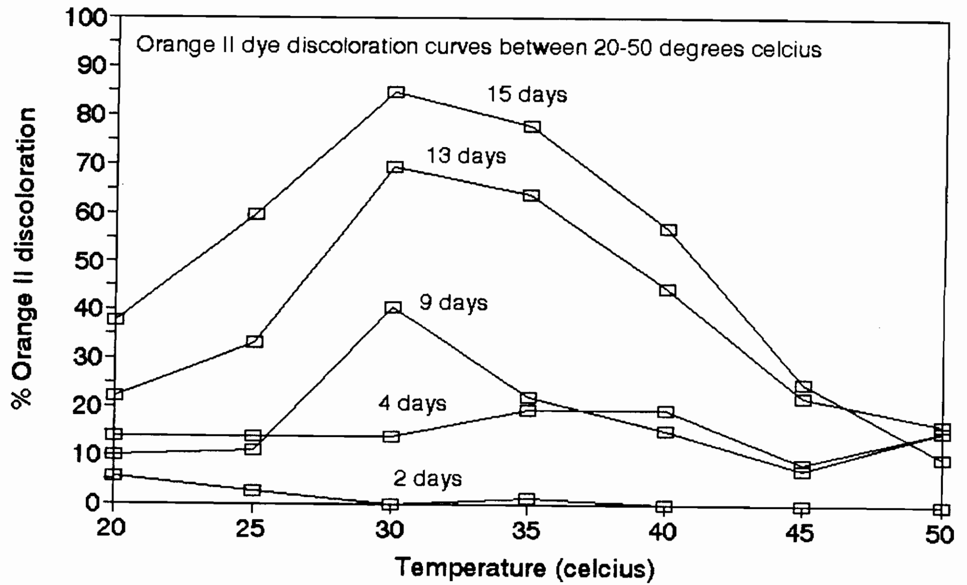


Figure 22. Orange II dye decoloration curves by *P. chrysosporium* enzymes as a function of temperature on DMS solid-medium (top graph) and in liquid-medium (bottom graph).

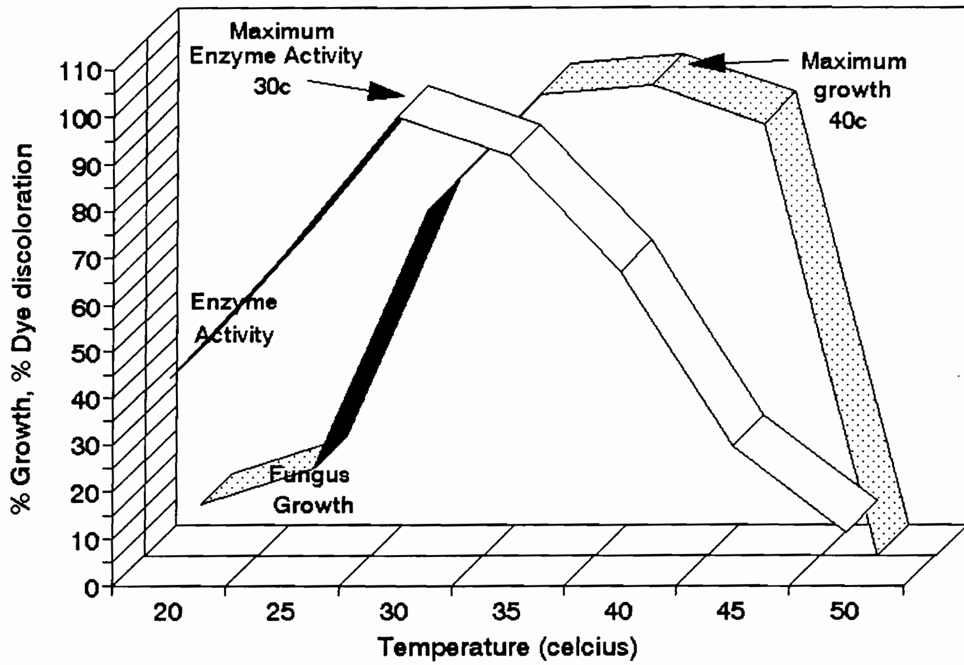


Figure 23. Optimum fungus growth curve and enzyme activity curve with respect to temperature.

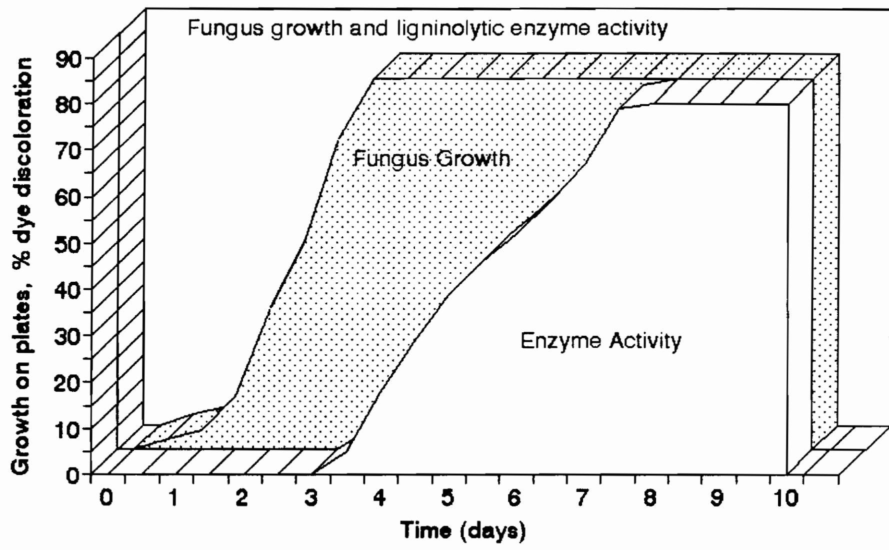


Figure 24. *P. chrysosporium* mycelium growth and enzyme activity in days at 40 °C.

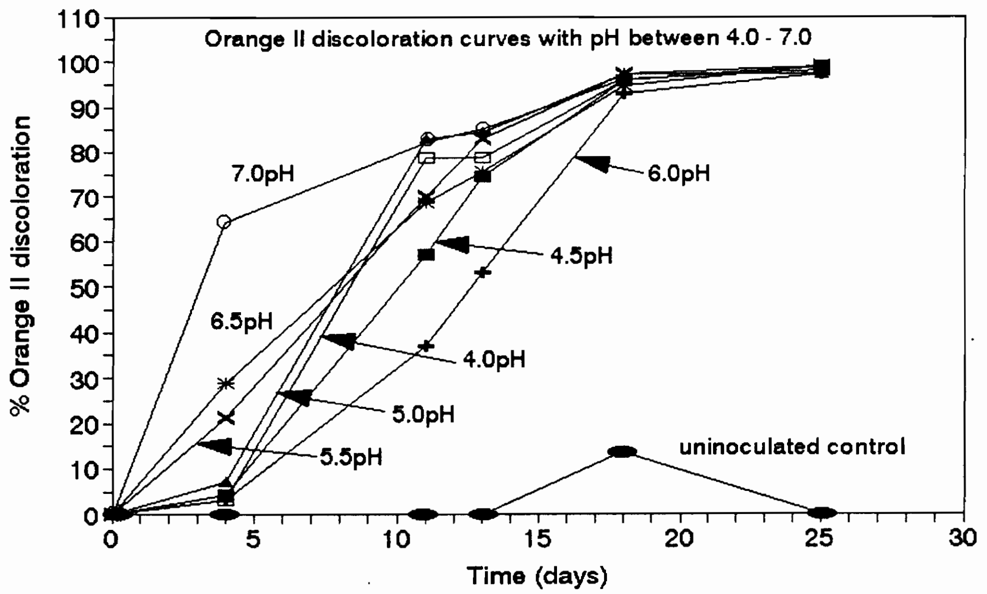


Figure 25. Orange II dye discoloration by *P. chrysosporium* enzymes at 30 °C and pH between 4.0-7.0, as days.

indicated that the optimum pH for growth was 4.5. Results from our research indicate that the enzyme system is not greatly affected by more neutral pH ranges encountered in soils.

B.4 The Effect of Nitrogen on Ligninolytic Activity.

Enzyme activity versus nitrogen concentration is displayed in Figure 26. The enzyme activity of the fungus was dependent upon the concentration of nitrogen in the medium. This comes as no surprise due to many references in the literature that have previously reported that the enzyme system is nitrogen limited. The highest enzyme activity was observed in cultures with 4.5 mM of nitrogen. Generally the microcosms used in these studies contained only 0.6 mM of nitrogen. Nitrogen concentrations above 10 mM caused inhibitory effects to the ligninolytic system.

B.5 The Effect of Hydrogen Peroxide on Ligninolytic Activity.

Addition of hydrogen peroxide to the liquid culture medium did not appear to increase the enzyme activity of the fungus (Figure 27). Hydrogen peroxide at four different concentrations, 0.003, 0.03, 0.3, and 3.0 mM, had similar effects on enzyme activity. However, the concentrations of hydrogen peroxide had an overall inhibiting affect to the system compared with microcosms that had no hydrogen peroxide in the liquid medium.

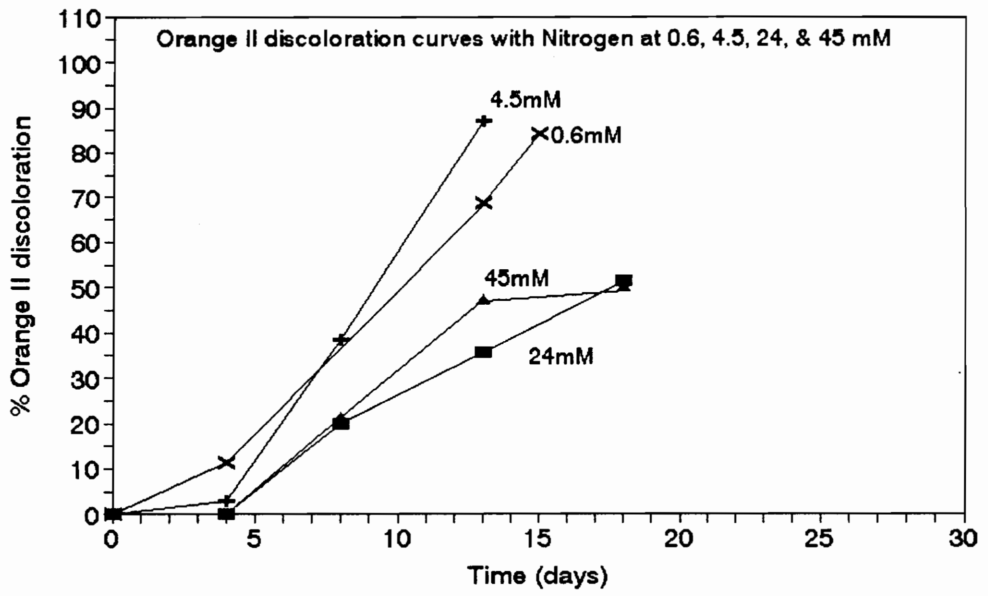


Figure 26. Orange II dye discoloration by *P. chrysosporium* enzymes at 30 °C and nitrogen concentrations between 0.6-45 mM, as days. Note the inhibiting effect of 24 and 45 mM nitrogen concentrations.

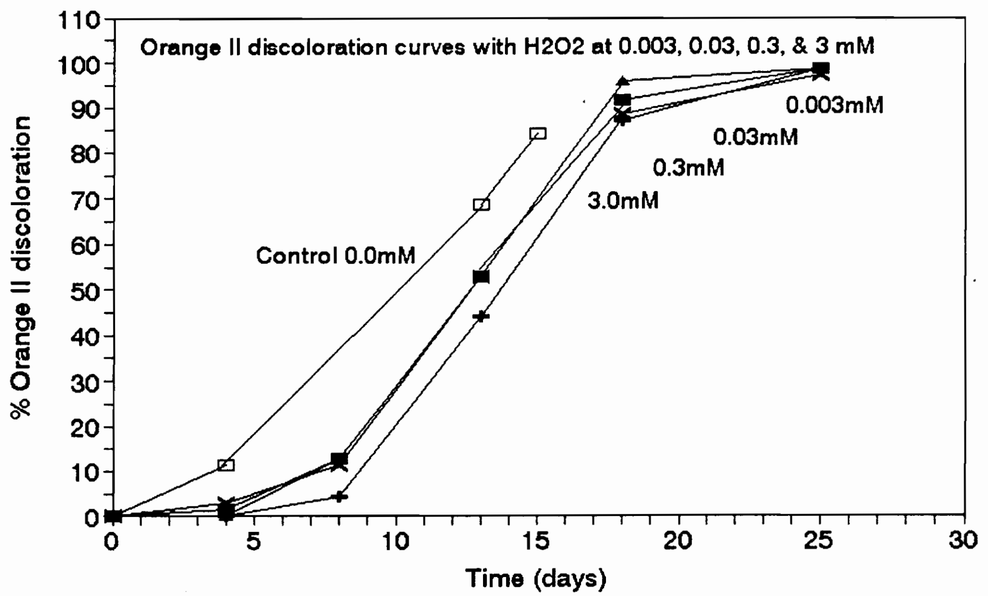


Figure 27. Orange II dye discoloration by *P. chrysosporium* enzymes at 30 °C and hydrogen peroxide concentrations between 0.003-3 mM, as days.

B.6 The Effect of Veratryl Alcohol on Ligninolytic Activity.

Addition of veratryl alcohol to the liquid culture medium did not increase the enzyme activity of the fungus. Three different concentrations of veratryl alcohol, 0.2, 2.0, and 20 mM were added to liquid DMS medium as shown in Figure 28 and did not stimulate enzyme activity over microcosms with no veratryl alcohol. Veratryl alcohol concentration at 20 mM displayed an increased inhibitory affect on the system whereas the effects of lower concentrations were similar to one another.

B.7 Reactivation of the Ligninolytic System

The ability to reactivate the ligninolytic system of the fungus grown in a nonimmersed liquid culture, (fungal mycelium immobilized on porous pieces of polyurethane foam saturated with liquid DMS medium) was successful. Reactivation of the ligninolytic system is shown in Figure 29. Decoloration of the dye was complete on day 15. At that time, the old DMS medium was removed from the microcosms with sterilized pipettes and a new batch of liquid DMS medium, amended with 0.1% Orange II dye was added to the microcosms to regenerate the dye in the system. The second batch of dye was decolored by the twenty-ninth day, displaying the ability of the ligninolytic system to be reactivated by addition of fresh liquid growth medium.

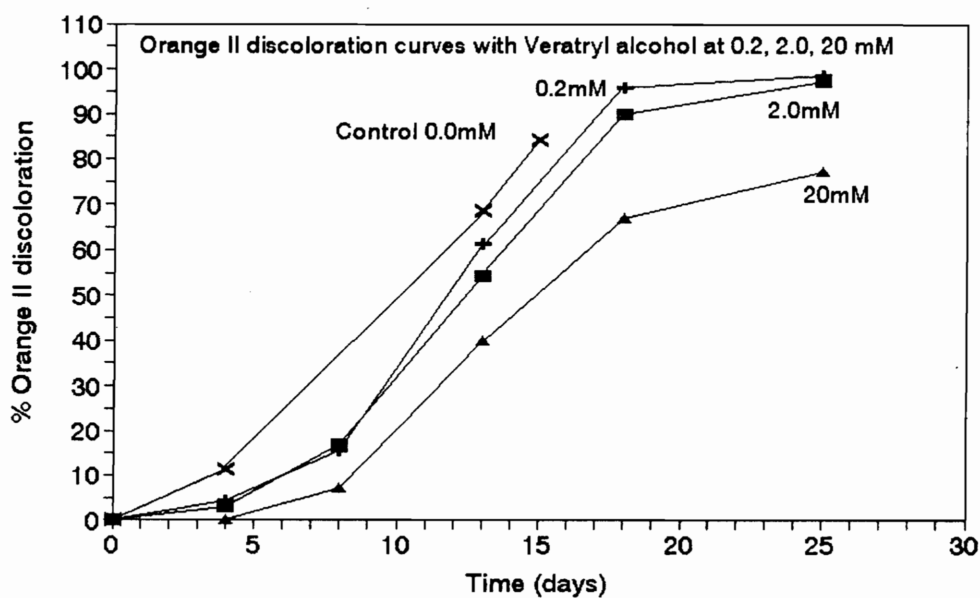


Figure 28. Orange II dye discoloration by *P. chrysosporium* enzymes at 30 °C and veratryl alcohol concentrations between 0.2-20 mM, as days.

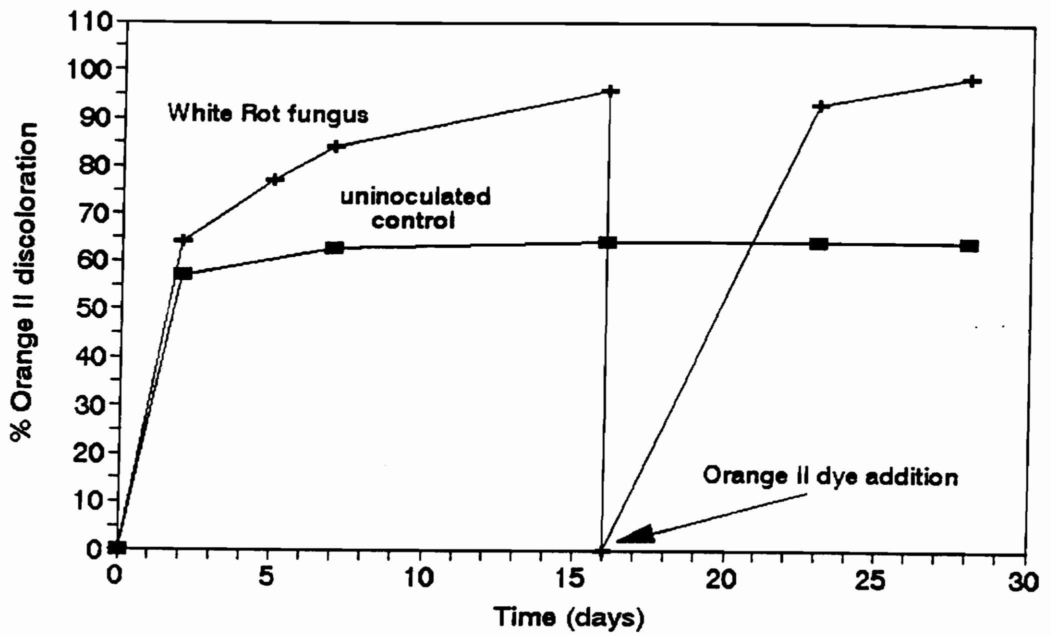


Figure 29. Orange II dye decolorized by the *Phanerochaete chrysosporium* enzyme system in immobilized microcosms, as a function of time, showing reactivation of the enzyme system.

5. CONCLUSIONS

The overall objective of the research project was twofold; to evaluate the ability of *P. chrysosporium* to degrade toxaphene and to determine culture conditions that may suppress or enhance the activity or reactivation of the ligninolytic system.

The ability of *P. chrysosporium* to degrade toxaphene was evaluated in liquid medium cultures. The effects of culture temperature and initial toxaphene concentration on the growth of the fungus and the degradation of the pesticide were explored. Three different techniques, GC elution patterns, free chloride analyses by ion chromatography and the distribution of chlorides in the aqueous and biomass fractions of the cultures by total organic halide (TOX) analysis, were employed to assess the extent of toxaphene conversion over time. Based on the results, the following conclusions were made.

1.) There is evidence that toxaphene undergoes biological degradation by the *P. chrysosporium* ligninolytic system. An increase in early eluting gas chromatographic activity implies that a fraction of the original toxaphene mass is reduced to smaller constituents with time. Increased production of free chloride concentrations with time also indicate toxaphene degradation by the ligninolytic system. Seven percent of the original mass of toxaphene was released as free chloride, as measured by ion chromatography.

2.) The main removal mechanism of toxaphene from solution was observed to be adsorption onto the mycelium or conversion of toxaphene to nonextractible fractions bound to the biomass material.

3.) Limited growth of the fungus was observed at 20 °C and therefore toxaphene concentrations were not observed to be reduced in these cultures. Cultures incubated at 30 °C and 40 °C supported growth of the fungus and toxaphene concentrations were reduced.

4.) The fungus was observed to grow in cultures with toxaphene concentrations of 3, 30, and 200 mg/L, which indicated that cultivation of the fungus was not limited by high concentrations of the pesticide.

The effects of culture conditions and reactivation of the ligninolytic system was explored in liquid cultures and in an immobilized liquid culture system. Ligninolytic activity was monitored employing a spectrophotometric technique which measured the ligninolytic oxidation of the azo dye Orange II amended to the growth solutions. The spectrophotometric technique was used to determine the effects of temperature, pH, and additives (nitrogen, hydrogen peroxide, and veratryl alcohol) on the activity and reactivation of the ligninolytic system. Based on the results obtained during this portion of the study the following conclusions were made.

5.) The ligninolytic system was successfully activated in liquid medium experimental cultures as evidenced by the oxidation of the azo dye Orange II.

6.) The optimum growth rate of the fungus was observed to occur at 40 °C, but the ligninolytic system was observed to be most active at 30 °C. It has been reported in the literature that the optimum temperature for the ligninolytic system is equivalent to the optimum growth requirement of the fungus. Results indicate that ligninolytic activity is equally active at a reduced temperature of 30 °C, which implies that the ligninolytic system can be as effective with reduced temperature requirements.

7.) The ligninolytic system was activated in liquid cultures that ranged in pH from 4.0 to 7.0, which indicates that the ligninolytic system is active at higher more neutral pHs which are more characteristic of soil properties.

8.) Reactivation of the ligninolytic system was accomplished using a nonimmersed liquid culture system which consisted of the mycelium immobilized on porous pieces of polyurethane foam. The immobilized configuration appeared to be an effective system for cultivating the ligninolytic system on a continuous basis.

9.) Nitrogen concentrations above 4.5 mM were shown to inhibit the ligninolytic system, which confirmed previously published reports.

10.) The amendment of liquid cultures with veratryl alcohol concentrations did not increase the ligninolytic activity, and cultures amended with an excess of 20 mM of veratryl alcohol inhibited activity.

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APPENDIX-A

Extraction efficiencies and gas chromatography analysis

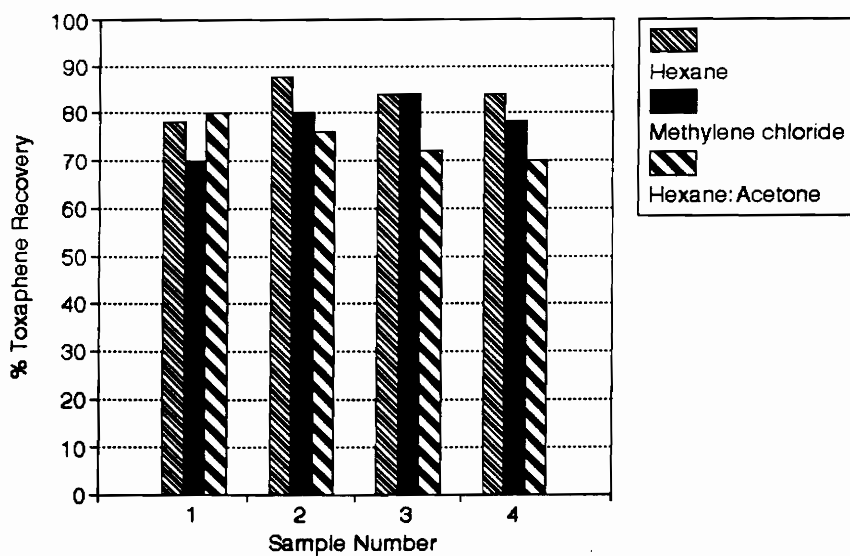


Figure A1. Extraction efficiency for 10 mg/L of toxaphene extracted with three different solvents; hexane, methylene chloride, and hexane:acetone (4:1).

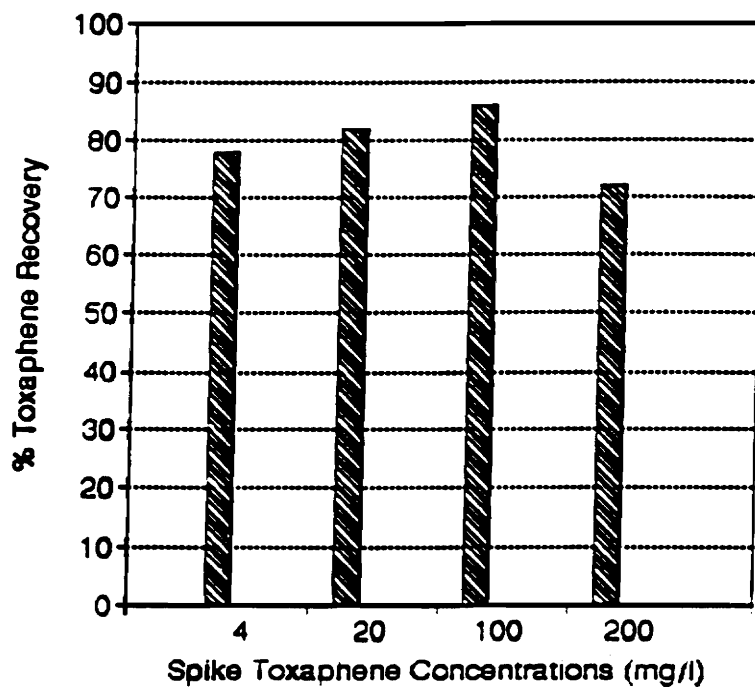


Figure A2. Extraction efficiency for toxaphene at 4, 20, 100, and 200 mg/L, with hexane solvent.

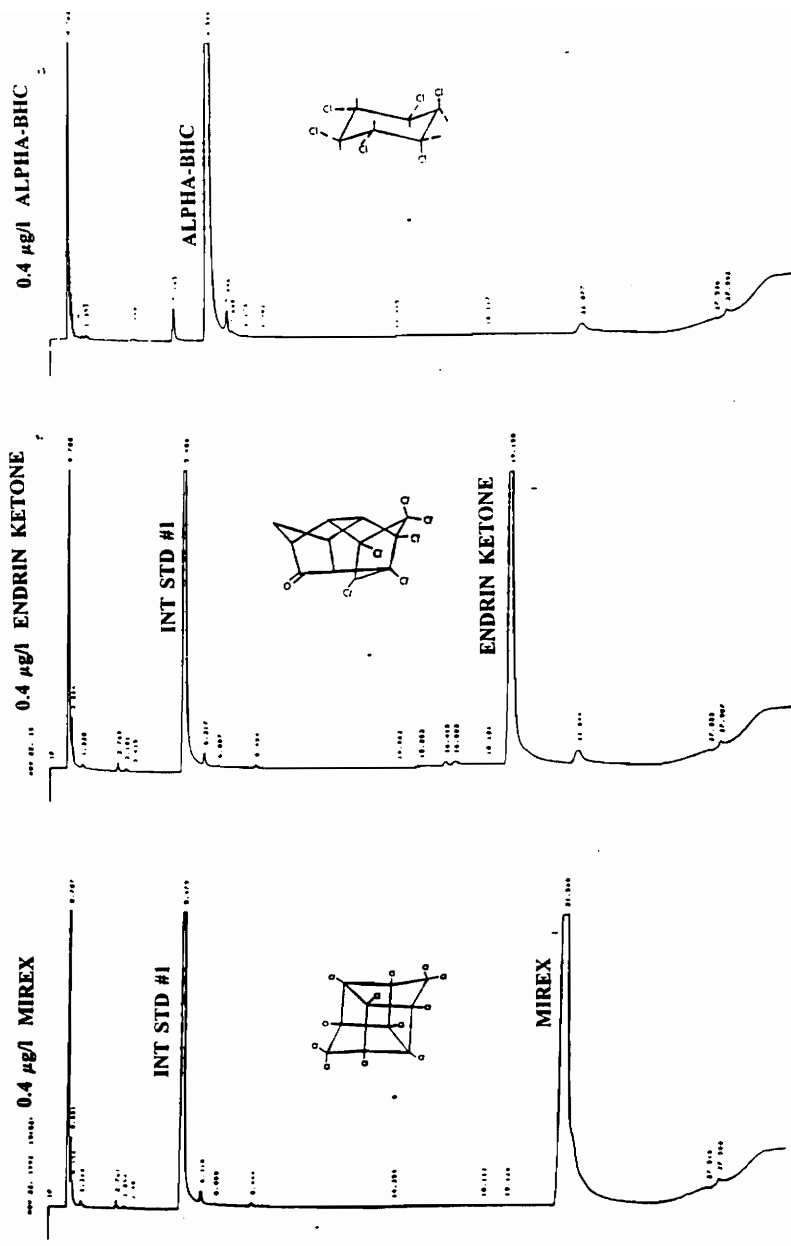


Figure A3. Gas chromatograms of potential surrogate compounds; mirex, endrin ketone, and alpha-BHC at 0.4 μg/L.

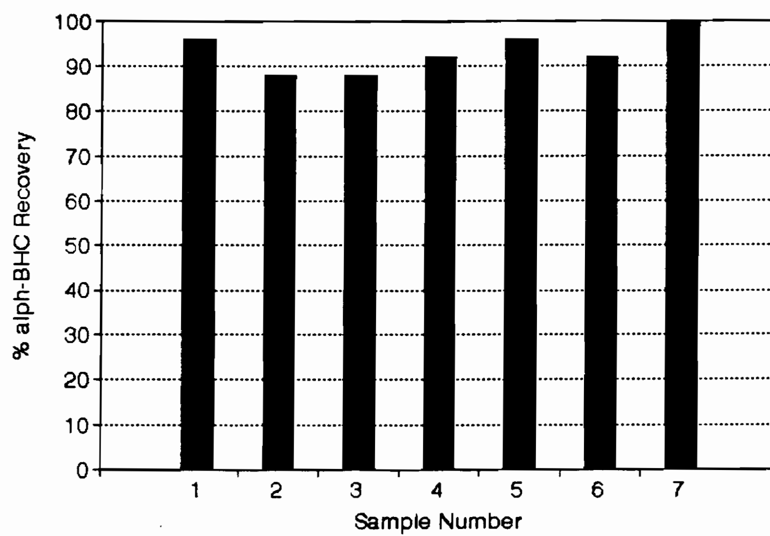


Figure A4. Extraction efficiency for 0.8 mg/L of alpha-BHC in aqueous solution, extracted with hexane.

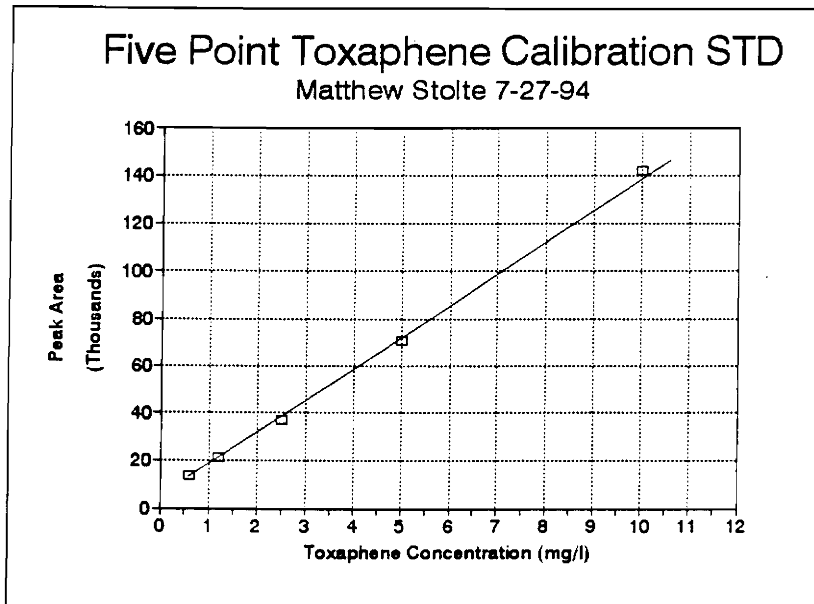
Table A1. Instrument operating conditions for gas chromatography analysis.

<u>ECD detector operating parameters:</u>
N_2 gas flow rate: 60 mL/min
Detector temperature: 315 °C

<u>Column and purge valve configurations:</u>
Volume of sample injected: 2μL
Column carrier gas flow rate: 100 mL/min
Column back pressure: 10 psi
Purge valve flow rate: 5.4 mL/min
Purge valve off: 0.00 min
Purge valve on: 0.75 min

<u>Oven temperature program and injector settings:</u>
Initial oven temperature: 150 °C
Oven temperature ramp: 4 °C/min
Final oven temperature: 300 °C
Final hold time: 1 min
Injector temperature: 310 °C

<u>Integrator settings:</u>
Chart speed: 0.5
Attenuation: 3.0
Peak height



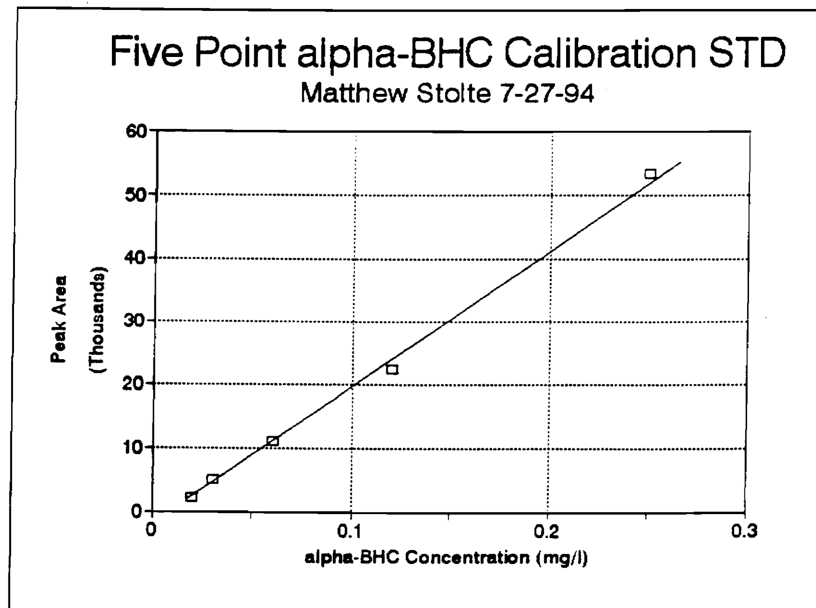


Figure A5. Toxaphene and alpha-BHC five point standard calibration curves.

APPENDIX-B

Ion chromatography analysis

Microcosm w/ White Rot Fungus
1.0 mg/L Br^- surrogate

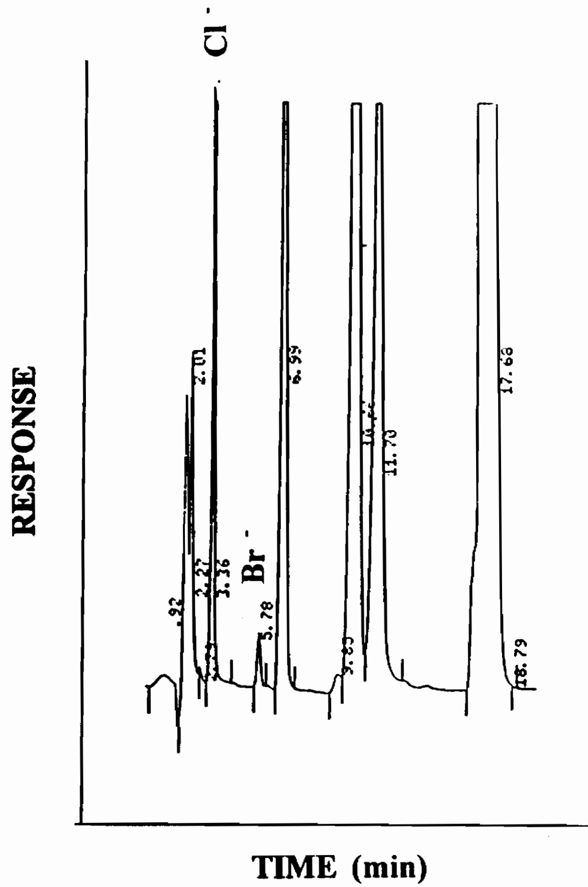


Figure A6. Ion chromatogram of DMS nutrient solution from microcosm inoculated with *P. chrysosporium* and 1.0 mg/L of Br^- surrogate.

APPENDIX-C

UV-Vis spectrophotometry analysis

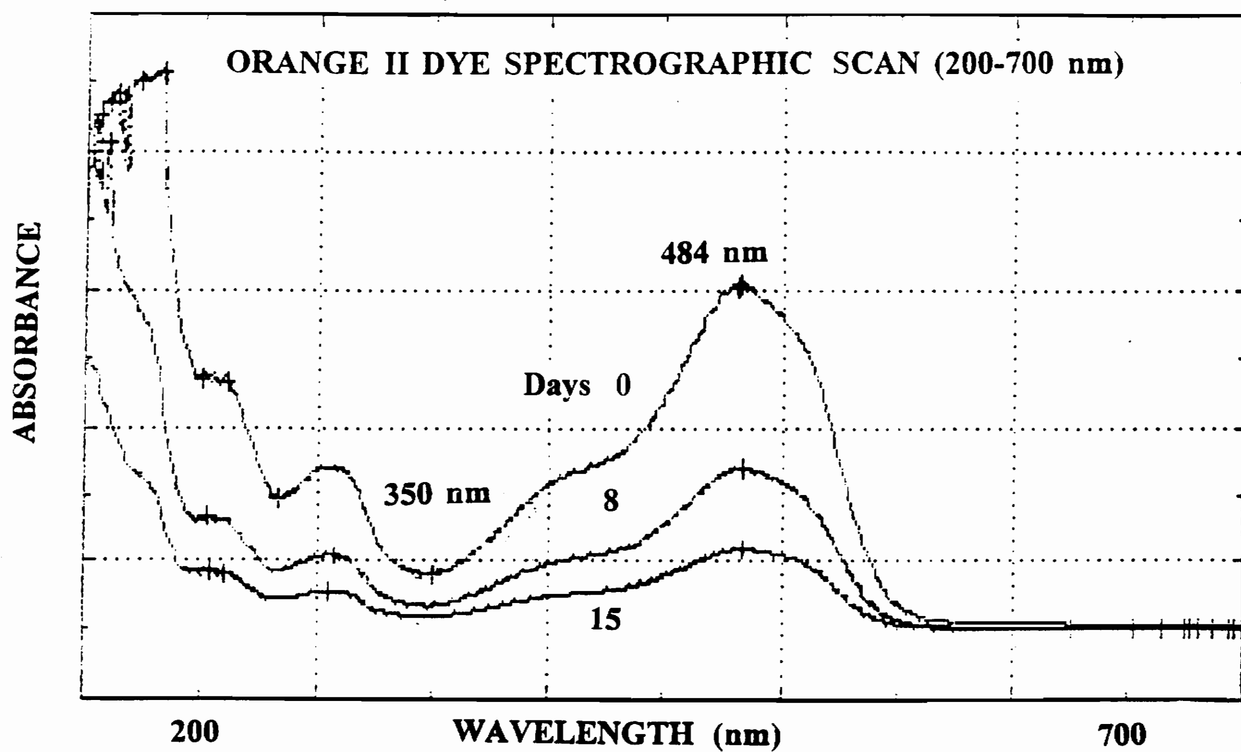


Figure A7. Spectrophotogram of 0.1% Orange II dye in DMS buffer solution with minimum and maximum absorbance wavelengths at 350 nm and 484 nm.

VITA

Matt Stolte was born in Bluefield, West Virginia on June 13, 1965. He was raised in Berea, Kentucky where he obtained a B.A. degree in Chemistry from Berea College. Mr. Stolte currently works as a staff engineer in the Environmental Engineering team for Draper Aden Associates in Blacksburg, Virginia. He has previously worked as a chemist for the Merrell Dow Pharmaceutical Company in Cincinnati, Ohio and the Tracer Research Corporation in Tucson, Arizona.