BIOCHEMISTRY OF CHLOROLIGNIN AND CHLOROPESTICIDE DEGRADATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE IN
THE DEPARTMENT OF BIOCHEMISTRY

MAY 1996

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

BIOCHEMISTRY of CHLOROLIGNIN AND CHLOROPESTICIDE DEGRADATION

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May 1996, 94 Pages

White-rot fungi are a group of organisms that participate in lignin degradation, as well as the degradation of organochlorine pesticides resembling the chlorolignin structure, such as lindane. Phanerochaete chrysosporium and Trametes versicolor, which are belonging to the white-rot fungi, were examined in solid-state and shake flask experiments, to remove an organic pollutant, namely lindane. During the course of solid state experiments, it was observed that P. chrysosporium and T. versicolor were able to grow in the presence of solid lindane and lindane-impregnated coal particles. The fungi were cultured on lindane-containing malt agar plates and growth was recorded. When the cultures on lindane-containing malt agar plates were transferred onto shake flasks, containing Saboraud medium and lindane solution, the gas chromatography analysis indicated a significant amount of degradation of lindane.

Keywords: White-rot fungi, Phanerochaete chrysosporium, Trametes versicolor, chlorolignin, lindane degradation.

ÖZ

KLOROLİGNİN VE KLOROPESTİSİT PARÇALANMA BİYOKİMYASI

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Mayıs 1996, 94 Sayfa

Beyaz çürükçül funguslar, ligninin ve lindan gibi kloroligninin yapısına benzeyen organoklorlu pestisitlerin parçalanmasında yer alan bir organizma grubudur. Beyaz çürükçül funguslara dahil olan Phanerochaete chrysosporium ve Trametes versicolor organizmaları katı fazda ve sıvı fazda çalkalamalı erlenlerde üretilenler olmak üzere iki gurupta incelenmiştir. Katı faz üremesi esnasında P. chrysosporium ve T. versicolor katı lindanın bulunduğu alanlarda ve lindan emdirilmiş kömür parçacıkları üzerinde üreyebilmiştir. Bu üremeler için lindan içeren malt agarlı petrilerde inkübasyon yapılmış ve üreme kaydedilmiştir. Lindan içeren malt agarlı petrilerdeki kültürler, Saboraud agar ve lindan çözeltisi içeren çalkalamalı erlenlere aktarıldığında, gaz kromatografisi analizi ile önemli miktarda lindanın parçalandığı görülmüştür.

Anahtar Kelimeler: Beyaz çürükçül fungus, Phanerochaete chrysosporium, Trametes versicolor, Kloroligninin, Lindan parçalanması

TO MY PARENTS

ACKNOWLEDGEMENTS

I express sincere appreciation to Prof. Dr. Celal F. Gökçay for his careful supervision, guidance and suggestions.

I offer sincere thanks to the personnel of Environmental Engineering Department, especially to Mr. Kemal Demirtaş for Gas chromatography.

Thanks are also due to Mrs. Zinnur Özkan for her careful typing.

The author thanks to all his friends, especially Mohammad Reza Mozafari and E. Türker Baran for their interests and moral support throughout the study.

Consequently the author is grateful to his family members for their endless support during the study.

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LIST OF ABBREVIATIONS

ABTS: 2,2' - Azinobis (3-ethylbenz-thiazolinesulfonic acid)
AMP : Adenosine Monophosphate
AOX: Adsorbable organic halogens
B.C: Before Christ
BHC: Benzene hexachloride
BPE: Bleach Plant Effluent
BSA: Bovine Serum Albumin
C : Elemental Carbon
COD: Chemical Oxygen Demand
DDT: 1,1-bis (p-Chlorophenyl)- 2,2,2,- Trichloroethane
ECD: Electron Capture Detector
ESR: Electron Spin Resonance
GC: Gas Chromatography
Kd: Kilodalton
KTBA: 2 -keto-4- thiomethylbutyric acid.
MyCoR: Mycelial Color Removal
NADH: Reduced Nicotinamide Adenin Dinucleotide.
N: Elemental Nitrogen
n.m: Nanometers
O.D.: Optical Density
PAH: Polyaromatic Hydrocarbons
PCB: Polychlorinated Biphenyls
RBC: Rotating Biological Contactor
RNA: Ribonucleic acid
S: Elemental Sulfur
TOCI: Total Organic Chlorine
UV: Ultraviolet light
VAO: Veratryl alcohol oxidase

CHAPTER 1

INTRODUCTION

1.1 LIGNIN AS A SUBSTRATE

1.1.1 Definition

Lignin, a 600-1000 kD polymer, is found in higher plants, including ferns, but not in liverworts, mosses or plants of lower taxonomic ranking (Brauns et al, 1960). Wood, the most important raw material for the production of chemical pulp, is composed of 20-30% by lignin. Most lignin is found within the cell walls, where it is intimately interspersed with the hemicellulose forming a matrix that surrounds the orderly cellulose microfibrils. In wood, lignin in high concentration acts as a glue that binds contiguous cells, forming the middle lamella. (Kirk et al., 1987; Kringstad et al., 1984)

Lignin is formed in wood by an enzyme-initiated dehydrogenative polymerization of a mixture of three different 4-hydroxyarylpropenyl alcohols. One of the precursor alcohols is p-hydroxycinnamyl (coumaryl) alcohol, which gives rise to p-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, leads to the guaiacyl units, and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, from which the syringyl units are formed. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked, and highly polydisperse polymer. (Kirk et al., 1987, Kringstad et al., 1984)

According to Brauns and Brauns (1960) lignin may also be defined as the plant component which, when refluxed with ethanol in the presence of catalytic amounts of hydrogen chloride gives a mixture of ethanolysis products; - Hibbert's monomers- such as α-ethoxypropioguaiacone, vanillin and vanilloylmethylketone from coniferous woods, and in addition, the corresponding syringyl derivatives from decidous woods.

The proportions of these alcohols vary with different wood species. Softwood lignin is largely a polymerization product of coniferyl alcohol, and most gymnosperm lignins contain primarily guaiacyl units. Angiosperm lignins contain approximately equal amounts of guaiacyl and syringyl units. (Kirk et al., 1987; Kringstad et al., 1984)

According to Kirk and Obst(1985) lignin is a natural plastic containing carbon, hydrogen and oxygen. Composed of phenylpropane units, lignin is heterogeneous and chemically complex. Over ten interphenyl propane linkage types occur, including four that predominate. The dominant linkage of lignin polymer is the β-0-4 type, seen between units 1 and 2,2 and 3,4 and 5, 6 and 7,7 and 8, and 13 and 14. (Fig.1) β-1 type linkages are also important in the structure as well. (Kirk et al., 1987; Boominathan et al., 1990)

Kringstad et al. (1984) have proposed that softwood lignin is a branched molecule in which the phenylpropane-based units are linked by different types of bonds. These include ether bonds of alkyl-aryl, alkyl-alkyl and aryl-aryl configurations. Various types of carbon-carbon bonds are also found. The aromatic content, expressed as monomeric phenol, is approximately 51%.

In quantitative determination, research has been greatly aided by the introduction of ^{14}C- labeled lignins, produced either in vivo or in vitro. Conversion of labeled lignins to $^{14}CO_2$ has served as a useful assay for biodegradation. (Kirk et al., 1984)

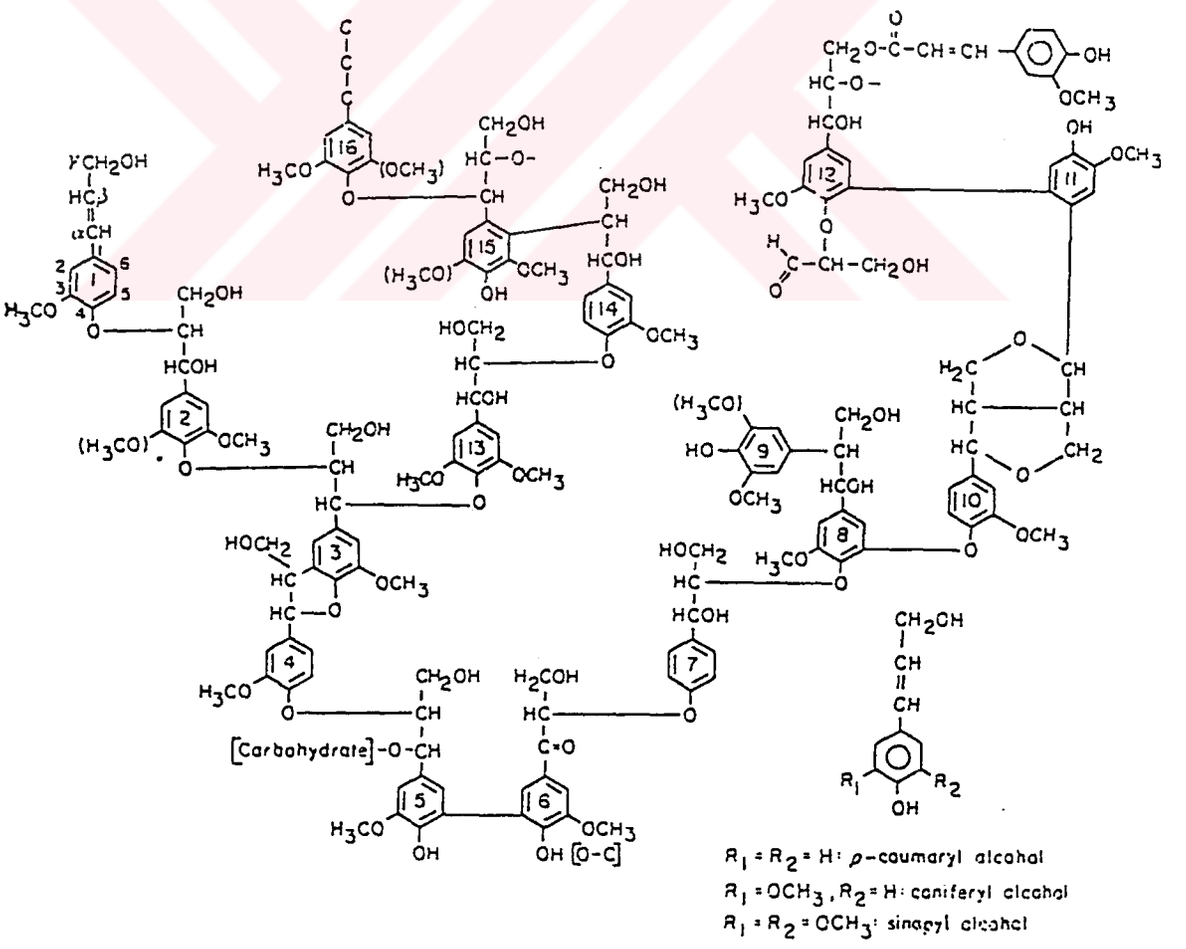


Figure 1.1. Schematic structural formula for lignin, adapted from Adler.

1.1.2. Degradation of Lignin by White-Rot Fungi

Several ascomycetes, fungi imperfecti and phycomycetes that are not associated with soft rot of wood, including 12 marine fungi, 18 Trichoderma strains, and Trichoderma horzianum failed to degrade lignin significantly. An isolate of Fusarium solani released only 4-5 % of the ^{14}C in labeled synthetic lignins as $^{14}CO_2$ in 30 days. (Kirk et al., 1984)

White-rot fungi, a limited group of basidiomycetes, possess an active ligninolytic system which is able to degrade protolignin as well as heavily modified lignins, such as kraft lignin and chlorolignins. White-rot basidiomycetes degrade lignin more rapidly and extensively than other studied microbial groups. Fungi differ from bacteria in that they have complicated growth cycles and grow by hyphal extension and not by binary fission. Like the brown rot fungi, they invade the lumens of wood cells, where they secrete enzymes that degrade lignin and the other wood components. (Galeno et al., 1990; Kirk et al., 1987; Leisola et al., 1984)

During its mineralization by white-rot fungi, lignin undergoes a number of oxidative changes, including aromatic ring cleavage. Recent studies have shown that a progressive depolymerization occurs, which are extracellular, and the further metabolism of fragments released from the polymer which is likely to be intracellular. Fragments of smaller than 1 kD seem to predominate. The production of ethylene from α-keto- γ-methylthiobutyric acid, a measure of fungal. OH radical production has been proposed as a measure of ligninolytic activity. For total degradation of lignin in lignocellulosic materials, the hemicellulose-cellulose structure must be at least partially degraded. (Gleen et al., 1983; Kirk et al., 1987; Livernoche et al; 1983)

Eriksson (1985) have postulated that the white-rot fungi need polysaccharides and/or low molecular weight sugars in order to degrade lignin, the sugar being necessary partly to provide the energy for growth and metabolism and partly to produce hydrogen peroxide, which is an important requirement for lignin degradation.

1.1.3. Species Involved in Lignin Degradation

One species of white-rot fungus, Phanerochaete chrysosporium, also called Sporotrichum pulverulentum, has been studied widely. Culture conditions for lignin degradation have been optimized, and P. chrysosporium exhibits the highest reported rates of lignin degradation. This allows P. chrysosporium in bioconversion of lignin,widely used in paper industry. (Kirk et al.; 1987 and 1988)

There are many white-rot fungi that are not selective for lignin degradation and large losses of polysaccharides also are removed. Coriolus versicolor is an example of a white-rot fungus that causes a simultaneous degradation of all cell wall components. It is a species that has been repeatedly used in assays as a representative of all white-rot fungi. However, there is a great deal of variation among the white-rots. (Blanchette and Burnes, 1988)

Other types of white-rot attack also have been reported, e.g. Phellinus pini, Phlebia tremellosus, Poria medullapanis and Scytinostroma galactinum was selective for lignin degradation. A white-rot fungus, Ganoderma australis selectively degrades lignin in the ecosystem, palo podrido, which means rotten wood in English, mentioned by Philippi (1893), and first described in detail by Knoche et al. (1929), occurs in the rain forests of southern Chile. Also delignification by P. chrysosporium was significantly different from the process with C. subvermispora and D. squalens. Some strains of P. chrysosporium are also highly selective, likewise BKM-F-1767. (Blanchette et al; 1988; Rios et al., 1992; Srebotnik et al., 1994)

1.2 PHYSIOLOGY AND BIOCHEMISTRY OF DEGRADATION

1.2.1. Physiology of White-Rot Fungi in Brief

According to Kirk et al. (1987) research during the late 1970's demonstrated that several nutritional and cultural parameters are important for lignin degradation by P. chrysosporium:

a) Presence of a cometabolizable substrate

b)High oxygen tension

c) Growth as mycelial mats rather than as submerged pellets in agitated cultures

d) Correct choice of buffer

e) Correct levels of certain minerals and trace elements

f) Growth-limiting amounts of nutrient nitrogen.

1.2.2. Nutritional Requirements for White-Rot Fungi

1.2.2.1. Nitrogen Source Limitation

Kirk et al. (1987) have reported that in P. chrysosporium, lignin is degraded only during secondary (idiophasic) metabolism, which is triggered by limiting cultures for nutrient N,C,S and several other species, but not by all white-rot fungi, is stimulated by N-limitation. N-limited conditions are natural for the white-rot fungi, because wood is N-poor. The N-regulated transitions from primary to secondary metabolism and vice versa are associated with increased and decreased levels of cyclic AMP.

Joyce et al. (1988) have concluded that the Mycelial Color Removal (MyCoR) process, which is based on P. chrysosporium, immobilizing on a Rotating Biological Contactor (RBC), proposed and developed by Eaton et al. (1982) also requires nitrogen as well. Keyser et al. (1978) implied that addition of NH_4, a nitrogen source, resulted in an eventual, temporary decrease of ligninolytic activity, which was obtained in stationary batch cultures of P. chrysosporium var. Burds .

1.2.2.2. Carbon Source Requirement

One of the significant aspects of P. chrysosporium is that a co-substrate, such as glucose, cellulose or even the organic matter in primary sludge, is required for lig-

ninolytic activity, as well as for growth (Eaton et al., 1982). Jeffries et al. (1981) concluded that, in carbohydrate-limited cultures, ligninolytic activity appeared when the supplied carbohydrate was depleted. For the decolorization process, Gökçay and Dilek (1994) concluded that P. chrysosporium cultures containing high concentrations of glucose supplies 75% color removal efficiency. Also in the presence of sucrose in cultures of Coriolus (Trametes) versicolor, tested by Livernoche et al. (1983), removed over 60 % of the color of the effluents in the bleaching kraft process as well.

1.2.2.3. Growth Mineral Salts

According to Jeffries et al. (1981) the inorganic nutrient, Mg^{2+}, exerted a strong influence on both growth and lignin degradation. Ca^{2+} had little effect alone. Kern et al. (1989) suggested the addition of $Mn0_2$ to P. chrysosporium cultures provides an intention to protect ligninases against inactivation and damage by hydrogen peroxide via catalytic decomposition of H_2O_2 by MnO_2, Gökçay et al. (1994) have suggested that for phosphorus and potassium requirement KH_2PO_4 supplies both two and Mn^{2+} can be supplied as $MnSO_4$, Ca^{2+} can be supplied as $CaCl_2$.

1.2.2.4. Effect of Tween 80

Tween 80 (Sorbitan polyoxyethylene monooleate), a non-ionic surfactant, is a detergent. Jager et al. (1985) tried to obtain ligninolytic enzyme activity in an agitated culture but in the absence of Tween 80, no activity was detected. A conclusion, reached by Vasudevan (1984) declares the addition of a small amount (0,1 %). The usage of Tween 80 in the decolorization stage, not only enhanced the decolorization rate, but also extended the fungal lifetime. In fact, the additive effect of nitrogen and trace elements (growth mineral salts) can only be seen when Tween 80 is present. Jager et al. (1985) established that the total ligninolytic system ([^{14}C] - lignin $^{14}Co_2$) was active in cultures supplemented with surfactant, where Tween 80 presumably enhanced ligninolytic activity. (Ashter et al., 1987; Joyce et al., 1988)

1.2.3. Biochemistry of Degradation

The discovery of several enzymes that are thought to have roles, has projected lignin biodegradation research into the realm of biochemistry. These enzymes include ligninases, manganese peroxidases, phenol-oxidizing enzymes, and H_2O_2-producing enzymes. (Kirk et al., 1987)

1.2.3.1. The enzyme Ligninase

In 1983, two groups announced discovery in P. chrysosporium of an extracellular H_2O_2-requiring enzyme activity that catalyzes several of the reactions formerly seen with intact cultures. According to Kuwahara et al. (1984), this extracellular preparation is able to generate ethylene from 2-keto-4-thiomethylbutyric acid (KTBA), to oxidize a variety of lignin model compounds, to oxidize a variety of lignin model compounds, to depolymerize lignin and to decolorize the polymeric dye Poly-R. Besides ligninolytic activity and model compound degradation, ethylene production from KTBA is also expressed as secondary metabolic events in P. chrysosporium. Lignin degradation in P.chrysosporium takes place only during secondary (idiophasic) metabolism. (Glenn et al., 1983; Kirk et al., 1987)

Tien and Kirk (1984) was purified an extracellular lignin degrading enzyme, from the basidiomycete P. chrysosporium var. Burdsall and 42.000 dalton ligninase contains one protoheme IX per molecule. It catalyzes, non-stereospecifically, several oxidations in the alkyl side chains of lignin-related compunds: Cα -Cβ cleavage in lignin model compounds of the type aryl - Cα HOH-Cβ HR-Cγ H_2OH (R=-aryl or-0-aryl), oxidation of benzyl alcohols to aldehydes or ketones, intradiol cleavage in phenylglycol structures and hydroxylation of benzylic methylene groups. It also catalyzes oxidative coupling of phenols, and all reactions require H_2O_2.

Ligninase is a generic name of a group of isozymes that catalyze the oxidative depolymerization of lignin. Ligninases exhibit a high degree of homology. The major isozyme, ligninase H8, has been extensively characterized and is the protein initially

isolated by Tien and Kirk. Based on kinetic and spectroscopic data, this ligninase has been characterized as a peroxidase containing one high-spin ferric heme per enzyme molecule. Like horseradish peroxidase, the ligninases are capable of catalyzing a wide range of one-and two-electron oxidations. (Tien et al., 1988)

Tien and Kirk (1983) reported that lignin peroxidase is able to catalyze Cα -Cβ π cleavage of β-0-4 and β -1 lignin substructure models; in the presence of added H_2O_2. Kirk et al. reported that the major consequence of ligninase catalyzed oxidation of veratryl-glycerol- guaiacyl ether is Cα -Cβ cleavage. Also lignin-model amino acid adducts are degraded by the enzyme ligninase. (Tien et al., 1987; Umezawa et al., 1986)

Subsequent spectroscopic studies have shown that the ligninase is distinct from P_{450} oxygenases, shares some properties with oxygen-carrying heme proteins, and is a true peroxidase. ESR spectral studies showed that the iron is high-spin Fe (III). (Kirk et al., 1987)

1.2.3.2. Manganese Peroxidase

Another participant in lignin degradation is an extracellular peroxidase that unlike ligninase, requires. The enzyme has a molecular weight of 46 kD and requires H_20_2, Mn^{2+} and lactate. (Brown et al., 1990; Higson et al., 1991, Kirk et al., 1987)

Manganese peroxidase differs from lignin peroxidase in that Mn^{2+} serves as the reducing agent for the compound I and compound II, which will be reported in the section of Pollutant Oxidation Mechanisms of Peroxidase Enzymes. The Mn^{3+} produced during the cycle, can diffuse away and promote the subsequent oxidation of other chemicals. (Barr and Aust, 1994)

Activity of manganese peroxidase depends on H_20_2 using either phenol red or ABTS as a substrate. According to Glenn and Gold (1985), the peroxidase is capable of oxidizing NADH and a wide variety of dyes, including Poly B-411 and Poly R-481.

1.2.3.3 Pollutant Oxidation Mechanisms of Peroxidase Enzymes

Peroxidases, including lignin peroxidase and manganese peroxidase, use hydrogen peroxide to promote the one-electron oxidation of chemicals to free radicals. In the resting state the heme iron of the peroxidase is in the ferric state. Hydrogen peroxide oxidizes the ferric enzyme by two electrons to a form of the enzyme known as compound I, a ferryl (FeIV)π-porphyrin cation radical. A chemical can then be oxidized by one electron to a radical, and compound I can be reduced by one electron to compound II. A subsequent oxidase to its ferric resting state. (Barr and Aust, 1994)

Barr and Aust (1994) have proposed that the white-rot fungus P. chrysosporium produces veratryl alcohol (3,4- dimethoxybenzyl alcohol) from either lignin or glucose. Veratryl alcohol is an excellent substrate for lignin peroxidase and is oxidized to a cation radical, which can then oxidize other chemicals that are not directly oxidized by lignin peroxidase. Veratryl alcohol appears to increase the rate and extent of chemical degradation by white-rot fungi. Certain chemicals like polymeric dyes or PAH's are efficient substrates for compound I of lignin peroxidase but not compound II. If veratryl alcohol were not present, compound II would react with H_20_2 to form the inactive compound III.

According to Barr and Aust (1994) veratryl alcohol may serve as an electron mediator to facilitate the oxidation of pollutants in some cases, oxidation of veratryl alcohol to its cation radical is catalyzed by compound I and/or compound II. Electron transfer between the veratryl alcohol cation radical and the pollutant would then occur resulting in oxidation of the pollutant, termed as indirect oxidation by lignin peroxidase.

The secondary metabolite of P.chrysosporium, veratryl alcohol, is synthesized from phenylalanine, via 3,4-dimethoxycinnamyl alcohol, which is oxidized to 1-(3,4-dimethoxyphenyl) glycerol; this is then oxidized to veratrylaldehyde. Veratryl alcohol causes an increase in ligninase activity by increasing the amount of certain

ligninase proteins, in a concentration dependent fashion of PSBL-1, a mutant of P. chrysosporium. (Faison et al., 1986; Kirk et al., 1987; Orth et al., 1991)

Tien et al. (1986) mentioned that the ligninase-catalyzed oxidation of veratryl alcohol to veratrylaldehyde exhibits a stoichiometry of 1 H_2O_2 utilized per aldehyde formed in P.chrysosporium var. Burds ligninase.

For Trametes hirsuta and Trametes gibbosa no activity of ligninase was detected in the absence of veratryl alcohol. They were strongly dependent of veratryl alcohol. Besides P. chrysosporium and Coriolus versicolor, another lignin-degrading basidiomycete Pleurotus sajor-caju oxidizes veratryl alcohol rapidly to veratrylaldehyde by VAO, and also veratryl alcohol is subject to a two-electron oxidation with concomitant reduction of O_2 to H_2O_2. (Bourbonnais et al., 1988; Nerud et al., 1991)

Alternative to veratryl alcohol assays, a new assay, based on the oxidation of micromolar concentrations of the polymeric dye, introduced by Archibald in 1992, is as simple and rapid as the veratryl alcohol assay, it overcomes the interference from UV and short-wavelength visible-light -absorbing materials.

1.2.3.4 Laccase and Glucose Oxidase

Laccase, also named p-diphenol oxidase, has received considerable attention as lignin-degrading enzyme together with lignin and manganese peroxidase enzymes. (Higuchi, 1990; Kirk and Chang, (1990). According to Higuchi (1990) and Kirk and Shimada (1985), laccase is commonly excreted by lignin-degrading fungi induced by phenols. Laccase is able to catalyze the oxidative breakdown of lignin substructure model compounds. Reinhammar and Malmström (1981) postulated that laccase contains four copper atoms per molecule and these copper atoms are present in three distinct environments named as type 1,2 and 3. (Kawai and Ohashi, 1993)

Malmström et al. (1975) reported that laccase is a blue copper oxidase that catalyses the four -electron reduction of 0_2 to H_20 during its oxidation of phenolics, aromatic amines, ascorbate and metal cyanides. 1,2,4,5-tetramethoxybenzene is oxidized by HRP and laccase in the same way as it is by lignin peroxidase. (Kersten et al., 1990)

Glucose oxidase, another enzyme found in ligninolytic cultures of P. chrysosporium, catalyzes the oxidation of D-glucose to α -D gluconolactone and H_20_2 in the presence of molecular oxygen. (60) The addition of glucose oxidase to a glucose-oxidase negative mutant did not enhance its capacity to degrade lignin. (Joyce et al., 1988)

1.3. ENVIRONMENTAL APPLICATIONS OF WHITE-ROT FUNGI

1.3.1 Pulp and Paper Industry Process

Wastewaters from the forest industries often contain colored, high-molecular mass lignin residues. Most of the organic compounds in the wastewater of pulp mills producing bleached chemical pulp are chlorinated degradation products of lignin. According to Pellinen and Salonen (1980), approximately 150.000 tons of chlorinated lignin derivatives, or about 15.000 tons of organic chlorine, are discharged annually in Finland, and about 250.000 tons of organic chlorine throughout the world reported by Kringstad and Lindström in 1984. (Eriksson, 1985; Pelinen et al., 1988; Sjöström et al., 1985)

Kringstad and Lindström (1984) identified a number of chlorinated organic compounds in spent bleach liquors like chlorinated acids, phenols, alcohols, aldehydes, ketones, thiophenes and aliphatic and aromatic hydrocarbons. Approximately 70% of organically bound chlorine is found as chlorolignin. Small quantities of polychlorinated dibenzo p-dioxins and polychlorinated dibenzofurans was established in

effluents and in sometimes bleached chemical pulp. (Faison et al., 1986; Kringstad et al., 1988; Sjöström et al., 1985)

Carlberg et al. (1986) reported that over 200 chlorinated compounds have been identified in effluents from pulp mills. About 80 of them are present in the spent chlorination liquor from the bleaching of sulfite pulp. They concluded that the chlorinated compounds in spent bleach liquors from the bleaching of sulfite and kraft pulp are similar (Haggblöm et al., 1991)

Bergbauer and Eggert (1992) have reported that in the conventional bleaching of pulp, the effluent was collected at a commercial kraft pulp mill using the CEH-DED bleaching sequence. C represents the chlorination stage, as a bleaching agent of pulp fibers chlorine is applied. The residual lignin is chlorinated so it becomes soluble in alkali and can be extracted from the pulp in the subsequent alkali extraction (E1 step). Hypochlorite (H), a strong bleaching agent follows E1 step. D stage is the proceeding one, in which chlorine dioxide oxidizes the lignin residues. Second alkalination and consequently, second chlorine-dioxide treatment is involved in CEH-DED process.

The pulp industry has adopted many measures to reduce the discharge of spent bleach liquors and associated chlorinated organic matter into receiving waters. Extensive use of aerated lagoons, introduction of oxygen bleaching and partial replacement of chlorine by chlorine dioxide are important examples of such measures. Replacement of chlorine by chlorine dioxide will reduce the highly chlorinated phenolic compounds, mutagenicity, acute toxicity and degree of chlorination of the high molecular mass material. (Kringstad et al., 1988)

The advantage of using chlorine is simply that it is cheap and effective, since chlorine reacts specifically and very effectively with lignin, the efluents of the bleaching process contain various chlorinated lignin derivatives which can be determined as adsorbable organic halogens (AOX). Gergov et al . (1988) concluded that

the amount of AOX in the bleach plant filtrates depends mainly on the total elemental chlorine charged with the bleaching chemicals. The use of chlorine results in an effluent with high COD (Chemical Oxygen Demand), color and TOCI (Total organic Chlorine) due primarily to the presence of chlorinated lignins known as chlorolignins. The total organic chlorine was determined by subtracting the amount of inorganic chloride from total chlorine by using Schöniger combustion method. (Bergbauer et al., 1992; Joyce et al., 1988; Pellinen et al., 1988)

According to Eriksson and Kolar (1985), a commonly used pulp making process also used commonly is sulfate pulping. In the kraft process, the wood is treated at elevated temperature with a mixture of sodium hydroxide and sodium sulfide. It removes approximately 90% of the lignin, leaving a fibrous residue, which, for bleaching purposes, is further delignified in chlorination and alkaline extraction stages.

1.3.2 Toxicity of Pulp and Paper Industry Wastewaters

Some of these compounds in the wastewaters of pulp mills are known to be stable in receiving water systems, reported by Salkinoja- Salonen et al. (1984) and are in part toxic (Hardig et al.,1988), mutagenic (Eriksson et al.,1979) and may accumulate in vertebrates (Landner 1979). According to Leach and Thakore (1975), especially chlorinated guaiacols have been shown to be toxic to juvenile rainbow trout (Salmo gairdneri). These effects are displayed at concentrations of 1mg/liter or less, so the persistence of such compounds in the aquatic environment is a matter of serious concern. (Bergbauer et al., 1992; Neilson et al., 1988)

Galli et al. (1987) proposed that the color, generated from these toxic discharges, has at least two negative effects on receiving waters. It is aesthetically objectionable and it blocks the sunlight, reducing photosynthetic production of oxygen. Organic chlorine compounds can be responsible for toxicity to fish and other aquatic organisms. Low molecular-weight chlorolignins are presumably responsible for acute toxicity. Fate and effects of high molecular weight compounds are not known.

1.3.3 Potential Applications for White - Rot Fungi

The degradation of lignin by white-rot fungi, especially those that selectively degrade lignin from wood, is a characteristic that makes them ideally suited for industrial applications where lignin or various phenolic compounds must be altered or removed. According to Tien and Myer (1990) there are numerous potential applications for ligninolytic fungi and the isolated ligninases. These applications include biopulping waste treatment, and coal desulfurization and cracking, and decolorization. (Archibald et. al., 1990, Blanchette et al., 1988)

Roy-Arcand et al. (1991) have concluded that similar to P. Chrysosporium , T. versicolor is able to decrease COD, as well as visible color and non-dialyzable (majority) organochlorine fractions were selectively attacked, similar to P. chrysosporium.

The white-rot basidiomycete P.chrysosporium is reportedly capable of at least partially degrades a wide range of organic pollutants including polychlorinated biphenyls (PCB), DDT and lindane, chlorinated anilines and many mono- and polychlorinated phenolics, including pentachlorophenol (PCP) and chlorolignins. Bumpus et al. (1985) reported that the ability of P.chrysosporium to degrade lignin and to metabolize halogenated aromatics suggested them that more recalcitrant organohalides such as DDT, polychlorinated biphenyls, polychlorinated dibenzo(p)dioxins and lindane, a non-aromatic compound that is chlorinated on every carbon atom to $C0_2$; nomenclatured as 1,2,3,4,5,6,- hexachlorocyclohexane, might also be degraded by this organism. More information about the pesticide degradation will be reported in the next section of this chapter. (Roy-Arcand et al., 1991)

Sasek et al. (1993) tested five strains of white-rot fungi. Two strains alone and other three in combination with specific yeasts or bacteria degraded PCB's in polluted soil within interval of 20-30 %. When all organisms were applied together by a fungal-bacterial consortia, PCB degradation achieved was about 50%.

Other applications of white-rot fungi are, biodegradation when inoculated into soil, by P. chrysosporium ATCC 24725, Chrysosporium lignorum CL 1, and Trametes versicolor, mineralizes 3,4-dichloro aniline and benzo(a)pyrene in soil. (Morgan et al., 1993). White-rot fungi are applied to olive-mill waste waters, containing phenols (Sayadi and Ellouz, 1993), polymeric dyes like Poly B-411, Poly R-481 and Poly Y-606, azo and heterocyclic dyes including Orange II, Tropaeolin O, Congo Red and Azure B, widely by P. chrysosporium. (Cripps et al., 1990; Glenn et al., 1983; Gold et al., 1988; Platt et al., 1985).

1.3.4 Pesticides

1.3.4.1. A Brief History

Costa et al. (1987) mentioned that pesticides have been used to a limited degree since ancient times. For example, The Eberys Papyrus, written about 1550 B.C., lists preparations to expel fleas from the house. Even in this century until the mid 1930's, pesticides were mainly of natural origin or inorganic compounds. The period between 1935 and 1950 was characterized by the development of DDT and other chlorinated hydrocarbon insectisides. During World War II, the practical value of DDT was demonstrated when, with the aid of it, a severe epidemic of typhus in Naples was successfully stopped. This disease is transmitted by body lice, and after powdering 1,3 million people successful uses of DDT has been in the malaria eradication programs.

After Carson (1962) implied the bioaccumulation of DDT and its effects on bird reproduction, the federal government of U.S.A. took action agains water and air pollution as well as against some persistent pesticides. As a consequence, the manufacture and sale of DDT was banned in Sweden in 1970 and in the U.S.A in 1973. (Costa et al., 1987)

Agricultural uses (68 %) dominate the total pesticide consumption. Other uses include Industrial and Commercial (17%), Home and Garden (8%) and Government

(7%). Furthermore, the control of vector-borne diseases, such as Malaria, Filariasis and Schistomiasis, account for more than 650 million cases per year and they are controlled, in part by pesticides. On the other hand, the illness related to pesticide exposure in California, U.S.A. were 1,355 in 1985 with two deaths. (Costa et al., 1987)

1.3.4.2. Persistence of Organochlorine Pesticides in the Environment

The major source of environmental contamination by pesticides, is the deposits resulting from application of these chemicals to control agricultural pests and pests causing public health problems. Misplacement and leakage during the transportation, distribution, or storage processes related to the manufacture of pesticides could result in environmental contamination. If certain species are severely affected by absorption or accumulation of pesticides through the food web so that the balance of the ecosystem in nature is directly or indirectly disturbed. Toxicity is of prime importance when considering effects on humans and both chronic and acute toxicities must be taken into account (Croll, 1974; Matsumura, 1985)

According to Croll (1974) the organo-chlorine insectisides are extremely important because of the persistence and chronic toxicity of its members. DDT can persist for longer than 20 years in soil and any problems associated with these materials could last for many years. Their ability to accumulate in body fat means that very low intake levels can have toxicological significance. The maximum acceptable levels of the various compounds in potable waters have been quoted as lying between 0.001 mg/l for Endrin and 0,6 Mg/l for Lindane, where mammalian toxicity was the prime concentration.

The very persistence of the organo-chlorine insectisides in the soil and their low water solubilities indicates that only very low concentrations are likely to be found in water percolating through soil treated them. The persistence of DDT is longer than 20 years in soil.The persistences of other materials, apart possibly from Endrin, are shorter, with Lindane being probably the shortest. (Croll, 1974)

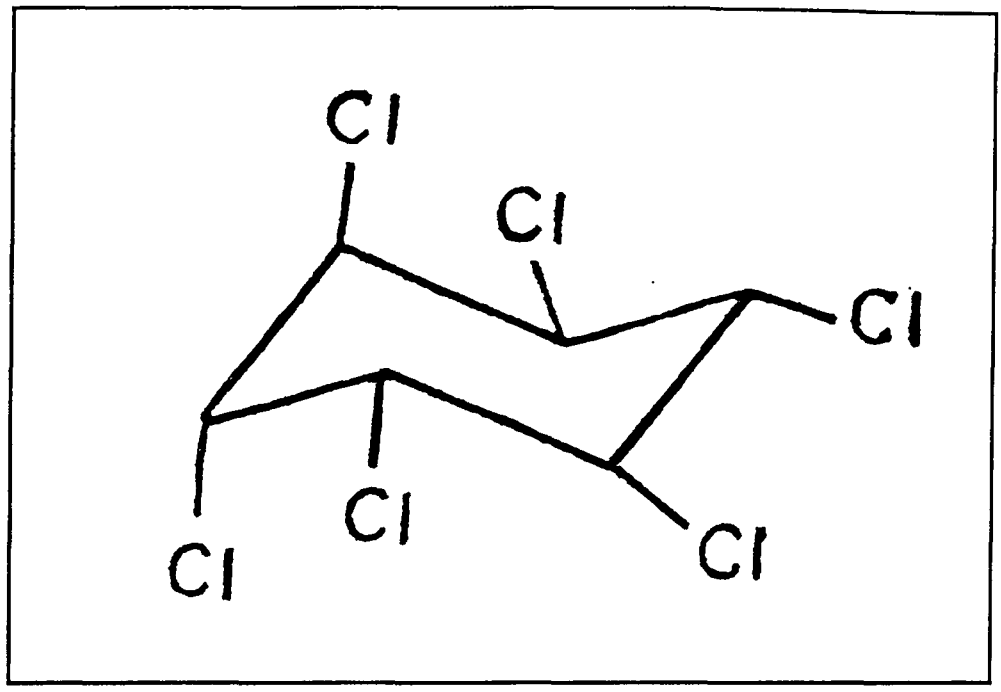


Figure 1.2. Schematic structural formula for lindane (From Higson)

In surface waters in Kent, England, three organo-chlorine insectisides, namely α-BHC, γ-BHC (Lindane) and Dieldrin were detected in all of some 200 water samples at average levels of about 20,60 and 20 ng/l respectively by using ECD gas chromatography. (Croll, 1974)

Matsumura (1985) mentioned that despite their low water solubilities, some of the chlorinated hydrocarbon insectisides do migrate into plant tissues. For instance, BHC and , to a lesser extent, aldrin are picked up by plants.

1.3.4.3. Lindane

The γ-BHC (-hexachlorocyclohexane), commonly konwn as lindane, is the toxic principle of BHC, an insectiside , as well as used in the treatment of scabies and pediculosis. Because of the specific toxicity of the γ - isomer as compared with other stereoisomers, the problem of the action mechanism of lindane has attracted the attention of many researchers and has led to many excellent research studies on structure-toxicity relationships. The structure of lindane is shown in Fig. 1.2. (Matsumura, 1985; Tusell et al., 1987)

The most intriguing aspect of the chemistry of BHC, is the large differences in biological activity, found among the closely related isomers. This strongly suggests that a rigid spatial arrangement of the molecule is necessary for strong insectisidal activity. By using Stuart models, Mullins (1955) estimated the molecular diameters in the plane of the ring and discovered that only the γ - isomer has a value of less than 8,5 A° for all three maximum diameters. According to Mullins, when lindane enters the interspace the membrane could be thrown out of equilibrium by the attractive force of chlorine atoms applied against the membrane constituents, Thereby it would become excited because of untimely ion leaks due to distortion of the lipoprotein molecules. They concluded that the spatial-as well as symmetrical-(but not absolute symmetrical) requirements seem to be an absolute basis for lindane toxicity. (Matsumura, 1985)

It is clear that the only toxic isomer is, which is bisymmetrical and compact. Another hypothesis based on lindane's structure-toxicity relationships with other structural isomers has been proposed at 1965 by Soloway . According to him, the presence of two electronegative centers positioned evenly acrosses the plane of symmetry is a very important factor in addition to the general molecular shape, in deciding the insectisidal potency of all chlorinated cyclodiene compounds and lindane. Since all chlorine atoms are electrophilic, the center carbon atoms of three equatorial and the opposing three axial chlorines should become electronegative. (Matsumura, 1985)

1.3.4.4. Removal and Degradation of Organochlorine Pesticides From Environment

According to Faust, Aly (1964) and Robeck et al. (1965) most organochlorine insectisides are not affected by normal water treatment methods, Robeck et al. (1965) concluded that DDT may largely be removed by coagulation. Treatment with activated carbon is the only effective method of removing the majority of these materials from water. Standard enrichments tend to favor rapidly growing, versatile

gram-negative bacteria, and fungi as a group have consequently received less attention as candidates for waste treatment studies. A microbiological approach to bioremediate the recalcitrant waste still holds promise, however, as an economical and efficient alternative to such methods as burial or incineration. (Croll, 1974; Higson, 1991)

Croll (1974) proposed that more than 100 forms (congeners) of PCB's , differing in the number and position of chlorine substituents were commonly used over the last half-century. The more highly chlorinated PCB's , those with four or more chlorines, have generally been considered resistant to biodegradation in the environment.

According to Unterman et al. (1988), traditional methods for the remediation of PCB-contaminated sites involve incineration of the material or its removal and burial in secure landfills. Both of these techniques are very expensive and often encounter public opposition. A diverse group of PCB-degrading bacterial was isolated by Unterman et al., including a gram-positive Corynebacterium species, Pseudomonads belonging to all three ribosomal RNA homology groups: Pseudomonas putida, P.cepacia and P. testosteroni, an Alcaligenes faecalis and an A. eutrophus. Thus, diverse environmental microorganisms have developed the ability to degrade PCB's.

One such method of bioremediation involves the white-rot fungi, providing the unique, non-specific degradative system for lignin degradation. This system supplies the effectivity at degrading a wide variety of hazardous environmental pollutants. The enormous structural diversity of the pollutants that are degraded by these fungi has made their potential use for bioremediation extremely intriguing. Recalcitrant pollutants, such as chlorinated pesticides (Kennedy et al., 1990), polycyclic aromatic hydrocarbons (Bumpus, 1989), polychlorinated biphenyls, and nitroaromatic explosives (Fernando et al.,1990) are all effectively degraded to CO_2 by white-rot fungi. (Barr and Aust, 1994)

The enzymes involved in lignin degradation by white-rot fungi, also involved in pesticide degradation. Degradation of a xenobiotic should be initiated like that of lignin by nutrient limitation. Xenobiotics often have very limited solubility in water and are not readily available in soil to intracellular metabolism. Cleavage occurs at a variety of C-C and C-0 bonds regardless of the configuration of chiral centers. (Barr and Aust, 1994; Higson, 1991)

Logan et al. (1994) reported that several species of fungi, including P. chrysosporium, T. versicolor and all four Ganoderma sp. removed more than 50% of the PCP within one day. A biphenyl mixture, Aroclor 1254, is degraded by P. chrysosporium, at an extent of 7,1%. Bumpus and Aust (1987a) concluded the extensive degradation of DDT by P. chrysosporium, 2,4,5-T in non-sterile soil can be mineralized by P. chrysosporium. (Eaton, 1985; Higson, 1991)

According to Ohisa and Yamaguchi (1987) and Sethuran et al. (1969) under anaerobic conditions, a rapid and reductive dehydro- dechlorination of lindane takes place during the microbial degradation. In the degradation of lindane by P. putida, Matsumara et al. (1976) found that ring opening only occured on dechlorination products bearing two chlorines or less. Kennedy et al. (1990) observed significant mineralization of [^{14}C] chlordane and lindane over 60 days from both a corncob-amended silt loam soil (14,9 % and 22,8 %) or liquid culture (9,4 % and 23,4 %) by nitrogen-limited P. chrysosporium, respectively. (Higson, 1991)

The rates of pollutant degradation are proportional to the concentration of the chemical. This is related to the pseudo-first-order kinetics that are observed for the free radical mechanisms used by these fungi. As the carbon skeletons of many of these pollutants are found within the structure proposed for the lignin polymer, and as the radioactive labeling in these compounds was restricted to the ring carbons, P. chrysosporium is able to degrade halogenated aromatic rings and also able to dechlorinate alkyl chlorides, like lindane, a non-aromatic compound that is chlorinated on every carbon atom. (Barr and Aust, 1994; Bumpus et al, 1985)

1.4. AİMS AND SCOPE OF THE STUDY

The aims of this study were restricted to biodegradation of a chlorinated pesticide, namely lindane, by using white-rot fungi, P. chrysosporium and T. versicolor, in laboratory conditions. The scope of the project was expanded to include strain selection observing for higher removal efficiencies. Chlorolignin degradation was also implied in this work as bleached kraft lignin was used as substrate in some of the experiments.

CHAPTER 2

MATERIALS AND METHOD

2.1. MATERIALS

2.1.1. Organisms and Cultivation

The organisms used in this study were the white-rot fungi, Phanerochaete chrysosporium and Trametes versicolor, obtained from Kirk et al., Forest Products Laboratory, Forest service-U.S. Department of Agriculture, Madison, Wisconsin U.S.A. For the experiments, the white-rot fungi were grown in petri dishes containing Malt Agar (OXOID) at 37°C. The hyphal suspension for inoculum was homogenized by a glass homogenizer and OD was adjusted to 0.5 absorbance at 550 nm using distilled water.A 2 ml fungal suspension at 0.5 OD was added to 150 ml medium in 500 ml Erlenmayer flasks.

During the course of solid-phase experiments, Malt Agar (OXOID) plus lindane (SIGMA) was used. For the fourth cycle experiment, Saboraud medium (DIFCO) was used during the liquid cultivation in 500 ml Erlenmayer flasks.

For all cycles of experiments, incubation was carried out at 35° C. In shake flask experiments agitation speed was 180 rpm. Static cultures in Erlenmayer flasks were maintained at room temperature without agitation.

2.1.2. Growth Medium for Organisms

2.1.2.1 Nutritional Factors

The growth medium ingredients, which were prepared according to Gökçay and Dilek (1994), were as follows: (in g/l) KH_2PO_4 (PANREAC) 2.0; Mg SO_4 (PANREAC), 0.5; $CaCl_2$. $5H_2O$ (MERCK), 0.1; thiamin, 0.001; and in agitated cases, supplementation with Tween 80 (BDH ltd), 0.005 % (w/v), was employed. The pH of the medium was adjusted to 4.5; which was optimal for growth of white-rot fungi. The varying principle carbon sources participating, were glucose, purchased from BDH Ltd. and acetate, as sodium salt (MERCK) (CH_3 C00Na. 3 H_2O). The elemental nitrogen was supplied as NH_4CL (MERCK), prepared by dissolving 5.35 g anhydrous salt in 100 ml distilled water A 10 ml of this solution supplied 100 % N and 1 ml supplies 10% N of N demand by the fungi.

2.1.2.2. Lindane

Lindane, which was purchased from SIGMA, was dissolved in 5 ml methanol (BDH) (20 mg lindane/ 5 ml methanol) and diluted to 1 liter for the first cycle experiments. In the second cycle experiments., 3.3 mg lindane was dissolved in 5 ml methanol and diluted to 500 ml. For the third cycle experiments, for each petri plate used in the first stage of this cycle, solid lindane crystals were placed on the petri plates. For preparing malt agar plus lindane plates 20 mg lindane was dissolved in 5 ml Methanol (BDH) and added in to 150 ml molten malt agar.

2.1.2.3. Alkaline Effluent

The bleach plant effluent, (E1 stage- Alkaline) was provided from Selüloz ve Kağıt Sanayii (SEKA) Afyon plant, located in Çay, Afyon. This effluent was used in the first and second cycle experiments.

2.1.2.4. Lignite Coals

The coals used in second and third cycle experiments were supplied from Tür-

kiye Kömür İşletmeleri (TKİ) Beypazarı and Elbistan lignite mines. The size of the coal particles were 1-2 mm.

2.2. METHOD

2.2.1. Glucose Utilization

Glucose utilization was observed according to the dinitrosalicylic acid (DNS) method. (Anonymous, 1970) and absorbances were detected by Pharmacia-LKB-Novaspec II spectrophotometer.

2.2.2 Protein Determination

Protein determinations were carried out according to the method of Bradford (1976); and absorbances were detected at Pharmacia LKB- Novaspec II.

2.2.3. Analysis of Aromatics with UV Spectroscopy

UV absorbance were detected by a Secomam S-750 spectrophotometer, based on two wavelengths, 254 and 280 nm. The 280 nm' readings' were used for determining the phenolics, and 254 nm' were for the aromatic ring structures.

2.2.4. UV-Scanning Analysis

UV - scanning was done in a Shimadzu UV-2100 S UV-visible recording spectrophotometer.

2.2.5. Lindane Analysis

The lindane analysis was carried out by a Chrompack Gas Chromatograph. The oven temperatures for this analysis were maintained as INPUT: 80° C, OUTPUT: 190°C, HEATING CYCLE: 5°C/Min; and the injection temperature was ranging between 200°C and 220°C. For each cycle of experiment the following procedure was applied.

(a) 1 ml sample is taken and diluted to 50 milliliters by distilled water, with

addition of 0,5 ml Methanol (BDH)

(b) The pH of the solution is brought to 2,0 by H_2SO4 (MERCK)

(c) A C-18 column (VARIAN BOND-ELUT) was initially solved by passing not less than 5 ml of methanol (BDH) through the column, with the help of syringe (SET) and a pressure-vacuum pump (MILLIPORE) under 10 atm. pressure.

(d) The 50 ml solution was passed through C-18 column, providing the same conditions reported in step (C).

(e) After solution was passed, the column was dried with air by the help of the pressure-vacuum pump.

(f) After drying, the column was eluted with 1 ml of chloroform. (Baxter-B and J Brand High Purity Solvent) The collected chloroform was dried by passing through anhydrous Na_2SO_4 column and applied in 0,2-0,3μl by using a Hamilton syringe to a SE-30 capillary column. The separated peaks were then detected by the ECD detector employing^{63} Ni as electron source.

A chromatogram of standards mixture is shown in Appendi A-3, where Lindane peak is indicated by an arrow on this figure. Slight shift in retention times in differing chromatograms was due to manual operation of the integrator.

2.2.6. Growth Measurements

The mycelium dry weights were determined after collecting and drying mycelia on 45-mm diameter Millipore filters, (0,45 m) For growth measurement, the following procedure is applied:

(a) A filter paper was dried at 105°C for 1 hour in an incubator.

(b) After drying, the filter paper was cooled in a desicator for 15 minutes.

(c) A tare of the filter paper was obtained by weighing.

(d) A known amount of fungal suspension was passed through the filter paper under suction.

(e) Filters were dried at 105°C for 1 hour.

(f) After cooling for 15 minutes in a desicator, filters were re-weighed. Subtracting tare provided the dry-weight of mycelia.

In all the experiments, the non-static flasks were incubated at 35°C and shaking at 180 rpm. All static flasks were maintained at room temperature without agitation.

2.2.7. Experimental Culture Conditions

The flask contents involved in all experimental cycles (1, 2 and 4) are tabulated in Tables 2.1, 2.2 and 2.3. The third cycle experiments was carried out in petri plates, and the composition of the plates were mentioned in Results section.

Table 2.1. The Flask Compositions of The First Cycle Experiments

Flask no	Composition	Static/Shake
1	10 g/L Glucose, 100%N Tween 80, Growth Mineral Salts medium, Thiamin, 20 mg/L Lindane, 2 ml P. chrysosporium	Shake
2	2 g/L Glucase, 10%N Tween 80, Growth Mineral Salts medium Thiamin, 20 mg/L Lindane, 2 ml P. chrysosporium	Shake
3	2 g/L Glucose, 10%N, Alkaline BPE, Tween 80, Growth Mineral Salts Medium, Thiamin, 20 mg/L Lindane, 2 ml P. chrysosporium	Shake
4 (control)	Distilled Water + 20 mg/L Lindane	Shake

Table 2.2. The Flask Compositions of The Second Cycle Experiments

Flask no	Composition	Static/Shake
1	2 g/L Glucose, 10%N, 40 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake
2	2 g/L Glucose, 100%N, 10 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake
3	2 g/L Glucose, 10%N, 10 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake
4	2 g/L Glucose, 10%N, 10 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Static
5	10 g/L Acetate, 10% Elbistan Coal, 100%N 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake
6	10 g/L Acetate, 10% Beypazarı Coal, 100%N 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake
7	10 g/L Acetate, 10% Elbistan Coal, 10%N 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake
8	6,6mg/L Lindane,Distilled Water	Shake
9	10% Beypazarı, 6,6mg/L Lindane,Distilled Water	Shake
10	10% Elbistan, 6,6mg/L Lindane,Distilled Water	Shake

Table 2.3. The Flask Compositions of The Fourth Cycle Experiments

Flask no	Composition	Static/Shake
1	Saboraud Broth, 40 mg/L Lindane, 2 ml *P. chrysosporium*	Shake
2	Saboraud Broth, 40 mg/L Lindane, 2 ml *T. versicolor*	Shake
3	Saboraud Broth, 40 mg/L Lindane, 2 ml *P. chrysosporium*	Static
4	Saboraud Broth, 40 mg/L Lindane, 2 ml *T. versicolor*	Static
Control 1	Saboraud Broth, 40 mg/L Lindane	Shake
Control 2	Saboraud Broth	Shake
Control 3	40 mg/L Lindane	Shake

CHAPTER 3

RESULTS

3.1. THE FIRST CYCLE EXPERIMENTS

3.1.1. Experimental Procedure

For the first cycle of experiment, the following flask contents were prepared as summarized below.

Erlenmayer flask 1: was composed of 10 g/l Glucose +100 %N + Min. Salts + Thiamin + Tween 80+20 mg/L Lindane + fungal suspension (P. chrysosporium).

Erlenmayer flask 2: 2 g/l Glucose+10% N+ Min. Salts + Thiamin + Tween 80+20 mg/l Lindane + fungal suspension (P.chrysosporium).

Erlenmayer flask 3: 2 g/l Glucose+ 10% N+ Min. Salts + thiamin + Tween 80+ Alkaline BPE + fungal supension (P. chrysosporium)

Control Flask: 20 mg/L Lindane + Distilled water.

In this cycle of experiments, 500 ml Erlenmayer flasks contained an aliquot of 150 ml. Except control flask, 10 ml aliquots were taken from each flask during 0,2,4,7 and 9^{th} days, and centrifuged at 4000 rpm for 15 min by an MSE centrifuge. For the control flask, 9^{th} day a 10 ml aliquot was collected and used in lindane analysis . Glucose determination was carried out at 0,2,4^{th} days. When glu-

cose was depleted in the medium, analysis was stopped. The protein assay and UV-absorbance measurements were carried out at 0,2,4,7 and 9th days. UV-absorbances were measured at 254 and 280 nanometers.

For the growth curve, a 500-ml Erlenmayer flask was used with a combination of 10 g/l Glucose + 10% N + 2 ml fungal suspension, added into 150 ml growth mineral salts medium. Thiamin was also present in mineral salts medium. At 0,2 and 4th days, mycelial dry weights were measured.

The lindane analysis was carried out, for the first flask on 9th day, and for the second and third flasks on first and 9th days. The results were expressed as percent removal calculated on the basis of the control flask.

3.1.2. Glucose Utilization

This experiment involved the fast depletion of the carbon source glucose, by P.chrysosporium, during the first four days. For the first flask, glucose analysis indicated complete consumption of glucose by P. chrysosporium on 4th day from an initial 10 g/l concentration of glucose. The glucose results of the first cycle experiments are shown in Fig. 3.1.1., and data is presented in Appendix section.

3.1.3. Protein Determination.

The protein determinations in the course of the first cycle experiments are presented in Fig. 3.1.2. From this figure it can be seen that aliquot protein concentration increased in flask 2 and 3 on the 4th day of the experiment implying induction of extracellular ligninases on this day. However the protein content exhibitted a sharp decline after this day. This result is quite consistent with those of Gökçay et al. (30) but the cause of decline after 4th day is obscure.

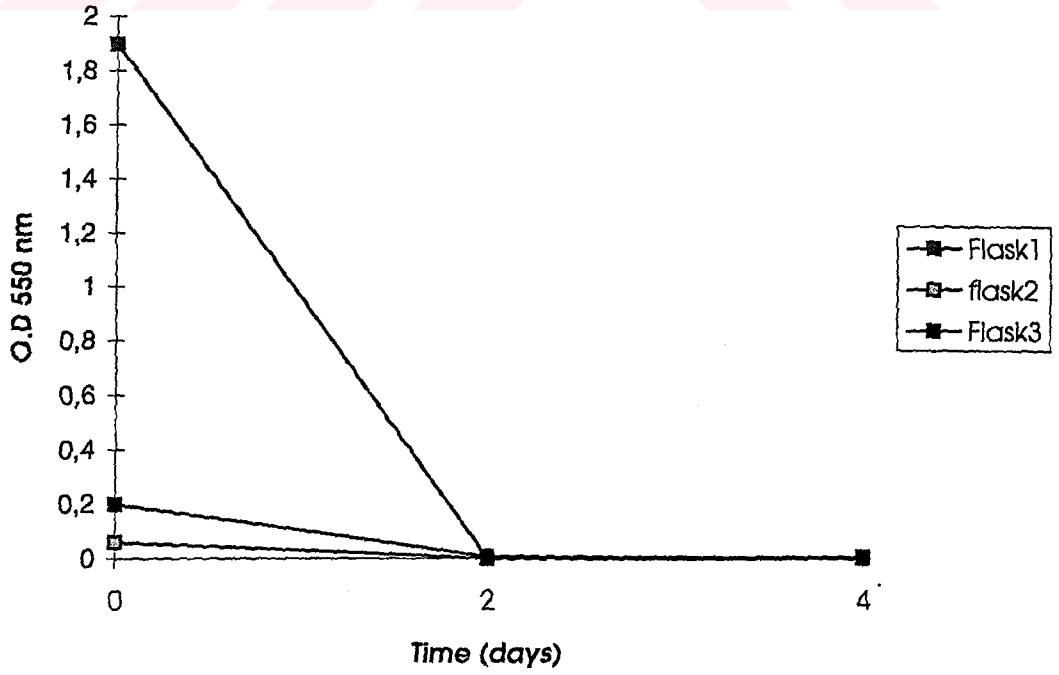


Figure 3.1.1. Results of Glucose Analysis for Flasks 1,2 and 3.

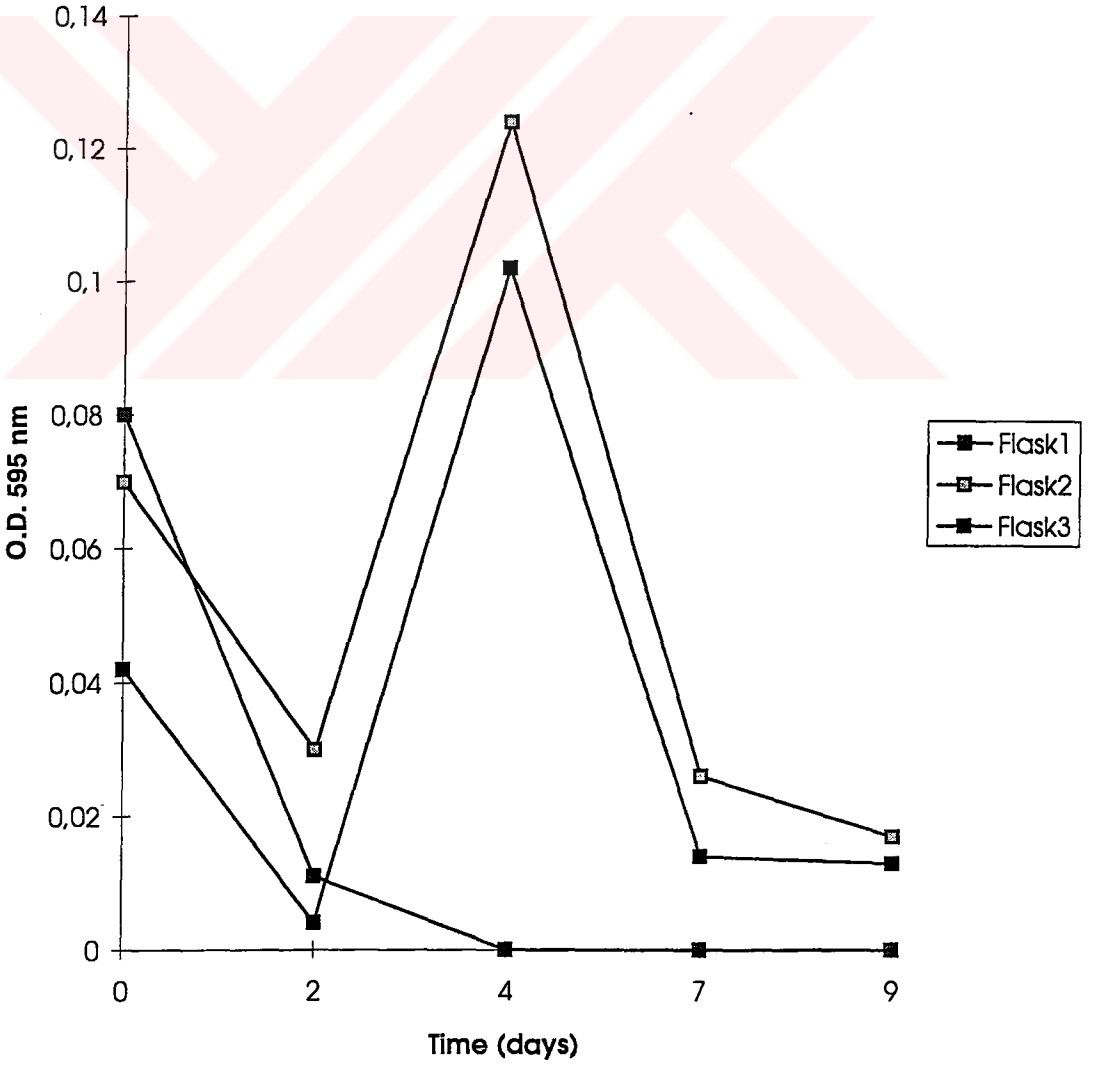


Figure 3.1.2. Results of Protein Determination for Flasks 1,2 and 3.

Table 3.1. Results of the lindane degradation for the first cycle experiments

Flask no	Flask Composition	Static/ Shake	For 150 ml Lindane Initial	Lindane Last (for 150 ml)	%Removal 0 (day)	%Removal 9 (day)
1	10 g/L Glucose, 100%N Tween 80 Growth Mineral Salts medium, Thiamin, 20 mg/L Lindane, 2 ml P. chrysosporium	Shake	3 mg	2,13 mg	0	29
2	2 g/L Glucose, 10%N Tween 80, Growth Mineral Salts medium Thiamin, 20 mg/L Lindane, 2 ml P. chrysosporium	Shake	3 mg	2,964 mg	0	1,2
3	2 g/L Glucose, 10%N, Alkaline BPE, Tween 80, Growth Mineral Salts Medium, Thiamin, 20 mg/L Lindane, 2 ml P. chrysosporium	Shake	3 mg	2,034 mg	0	32,2
4	(Control Flask) Distilled Water + 20 mg/L Lindane	Shake	3 mg	3 mg	0	0

3.1.4. UV-Absorbance Measurements

Results of UV-absorbance is presented in Fig 3.1.4.,3.1.5 and 3.1.6. As shown in Fig. 3.1.4, 3.1.5 and 3.1.6., the absorbance values showed a sharp decline from the very beginning of the first cycle of experiments as from 2nd day onwards, and the decline continued until the fourth day reached, coinciding with the secretion of extra-cellular enzymes by P. chrysosporium.

3.1.5. Growth Measurements

At the start of the experiment the biomass concentration of P.chrysosporium was 1.70 mg/ml sample. The concentration increased until day 4 and no further increase could be observed after this day as shown in Figure 3.1.3.

3.1.6. Lindane Degradation

Results of the lindane degradation are presented in Table 3.1.. GC Chromatograms associated with these experiments are presented in Appendix A-4.

3.2. THE SECOND CYCLE EXPERIMENTS

3.2.1. Experimental Procedure

In this experiment, Glucose and Acetate, as sodium salt, were used as varying carbon sources. The following flask contents were prepared as summarized below.

Erlenmayer flask 1: 2 g/l Glucose + 10 % N + Alkaline BPE + 3,3 mg / 500 ml lindane + Min. salts + fungal suspension + Tween 80.

Erlenmayer flask 2: 2 g/l Glucose + 100% N + Alkaline BPE + 3,3 mg/500 ml lindane + Min. salts + fungal suspension + Tween 80.

Erlenmayer flask 3: 2 g/l Glucose +10% N+ Alkaline BPE + lindane (3,3 mg / 500 ml) + Min. salts + fungal suspension + Tween 80.

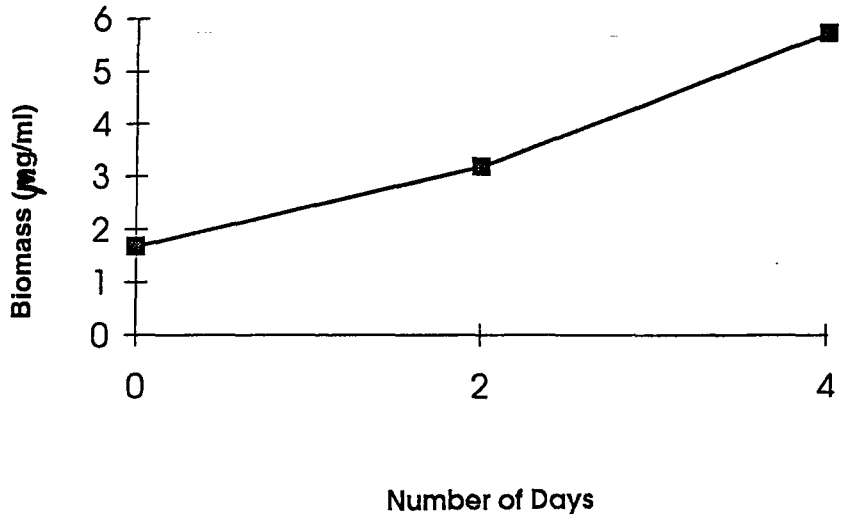


Figure 3.1.3. Growth Curve for P. Chrysosporium.

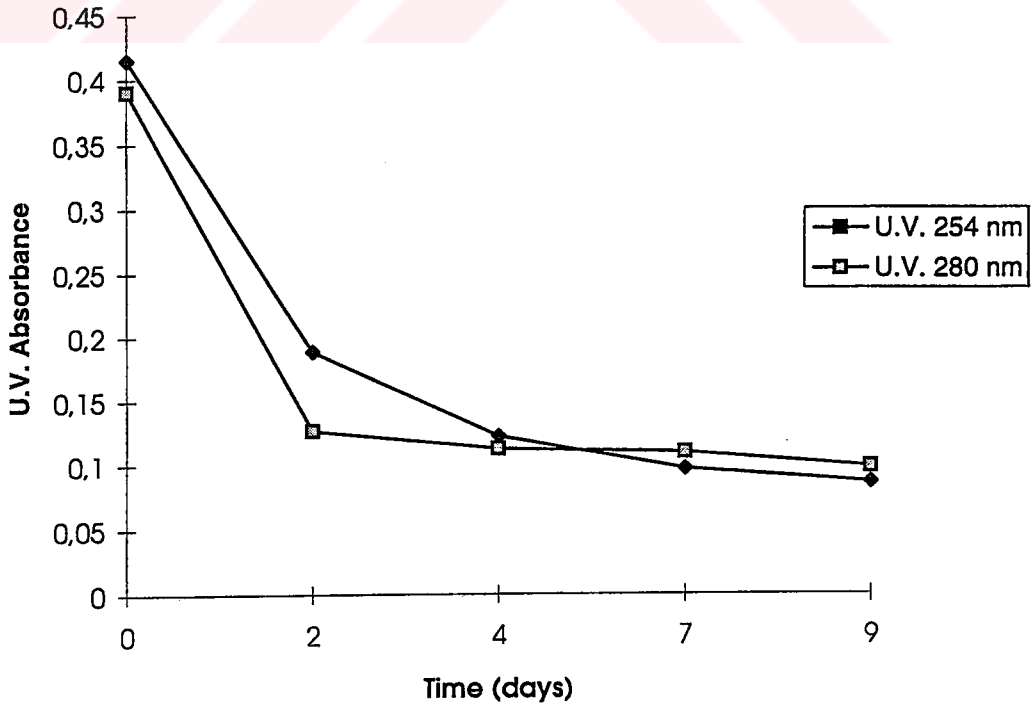


Figure 3.1.4. UV-Absorbances at 254 and 280 nm for Flask 1.

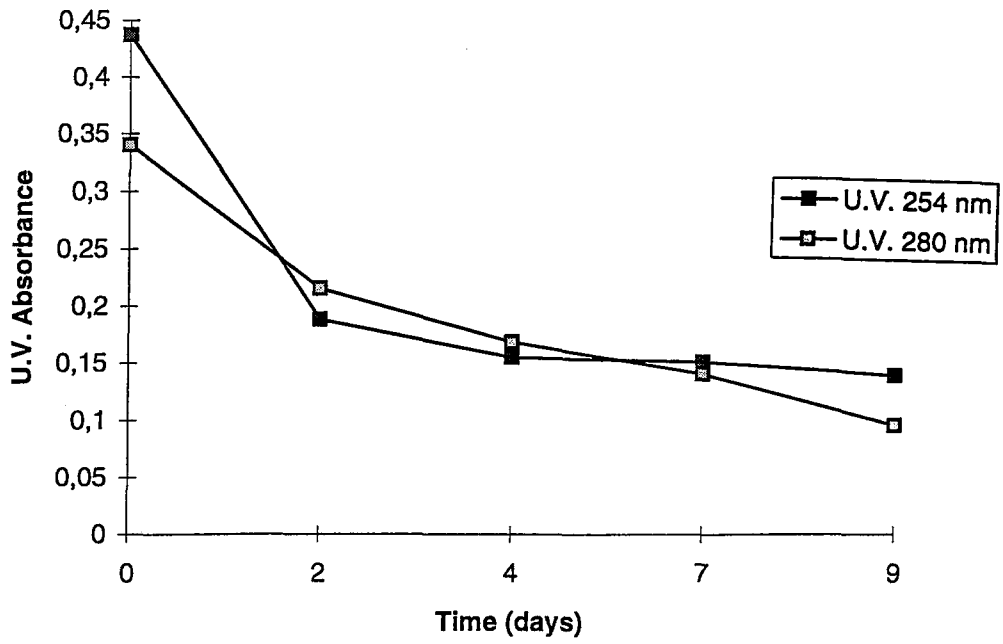


Figure 3.1.5. UV-Absorbances at 254 and 280 nm for Flask 2.

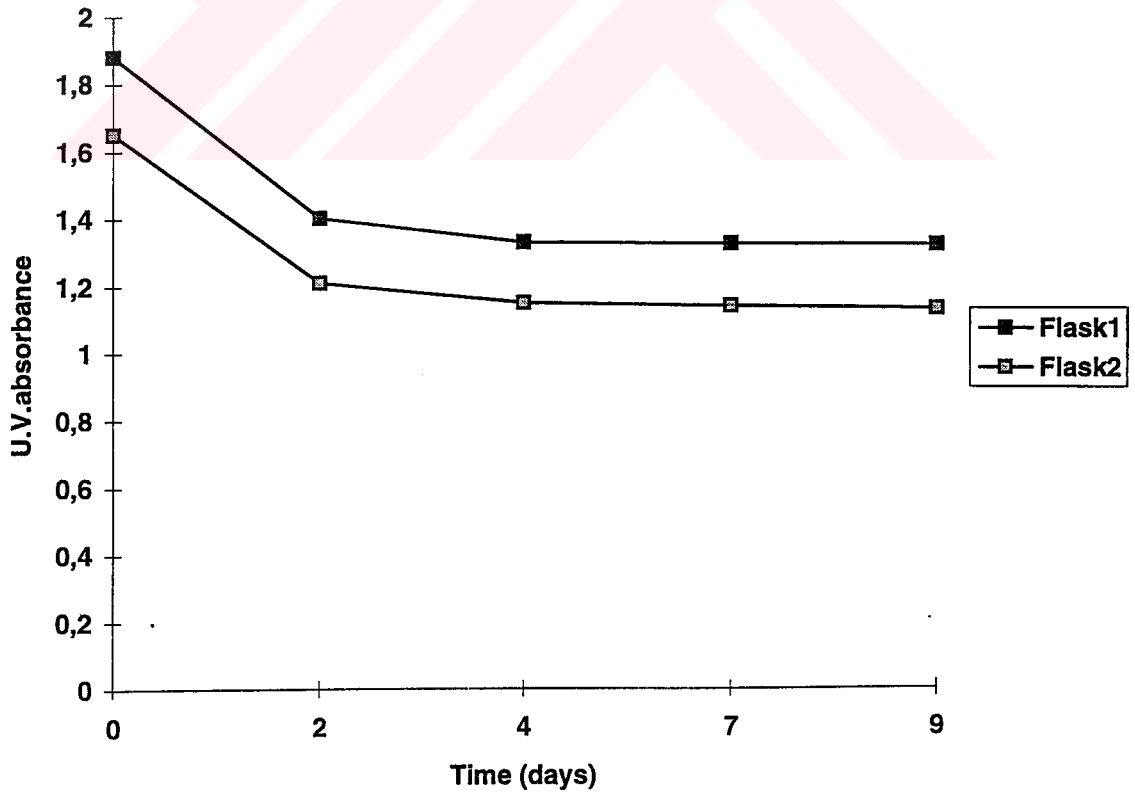


Figure 3.1.6. UV-Absorbances at 254 and 280 nm for Flask 3.

Erlenmayer flask 4: Static flask, of 2 g/l Glucose +10 % N+ Alkaline BPE + lindane (3,3 mg/500 ml) + Min. salts +fungal suspension.

Erlenmayer flask 5: 10 g/l Acetate (sodium salt) +10% 1-2 mm Elbistan lignite coal + Min. salts + lindane (3,3 mg/ 500 ml) + 100 % N + fungal suspension.

Erlenmayer flask 6: 10 g/l Acetate + 10% 1-2 mm Beypazarı lignite coal + Min. salts + 100% N+ 35 ml lindane (3,3 mg/500 ml) + fungal suspension + Tween 80.

Erlenmayer flask 7: 10 g/l Acetate as sodium salt + 10% 1-2 mm Elbistan lignite coal + Min. salts + 10 % N+ lindane (3,3 mg/500 ml) + fungal suspension + Tween 80.

Erlenmayer flask 8: Control flask, lindane (3,3 mg / 500 ml) + Distilled water.

Erlenmayer flask 9: Second control flask, 10% 1-2 mm Beypazarı lignite coal + Distilled water + lindane (3,3 mg/500 ml).

Erlenmayer flask 10: Third control flask 10 % 1-2 mm Elbistan lignite coal + Distilled water + lindane (3,3 mg/500 ml).

The experiment was carried out for 15 days, and after the experiment was finished, 10 ml aliquots were taken, centrifuged at 4000 rpm for 15 minutes at MSE centrifuge, and lindane analysis was carried out by GC. The flasks were contained 150 ml aliquot, containing 15 grams of each kind of lignite coal. The fungal suspension used in this experiment was P. chrysosporium. All flasks were incubated at 35 oC, 180 rpm whereas the fourth was excluded.

3.2.2. Lindane Degradation

The replacement of glucose with acetate showed unconclusive results as shown in Table 3.2.

Table 3.2. Results of the lindane degradation for the second cycle experiments

Flask no	Flask Composition	Static/ Shake	For 150 ml Lindane İnitial	Lindane Last (for 150 ml)	%Removal 0 (day)	%Removal 9 (day)
1	2 g/L Glucose, 10%N, 40 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake	0,99 mg	Unconclusive	–	-
2	2 g/L Glucose, 100%N, 10 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake	0,99 mg	0,715 mg	0	27,72
3	2 g/L Glucose, 10%N, 10 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake	0,99 mg	0,879 mg	0	11,2
4	2 g/L Glucose, 10%N, 10 ml Alkaline BPE, 6,6mg/L Lindane, 2 ml P. chrysosporium	Static	0,99 mg	Unconclusive	–	-
5	10 g/L Acetate, 10% Elbistan Coal, 100%N 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake	0,99 mg	Unconclusive	–	-
6	10 g/L Acetate, 10% Beypazarı Coal, 100%N 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake	0,99 mg	Unconclusive	–	-
7	10 g/L Acetate, 10% Elbistan Coal, 10%N 6,6mg/L Lindane, 2 ml P. chrysosporium	Shake	0,99 mg	Unconclusive	–	-
8	6,6mg/L Lindane,Distilled Water	Shake	0,99 mg	0,99 mg	0	0
9	10% Beypazarı, 6,6mg/L Lindane,Distilled Water	Shake	0,99 mg	0,99 mg	0	0
10	10% Elbistan, 6,6mg/L Lindane,Distilled Water	Shake	0,99 mg	0,99 mg	0	0

3.3. THIRD CYCLE EXPERIMENTS

This cycle of experiments were solid-state culture experiments, using P. chrysosporium and T.versicolor.

3.3.1. First Phase

Petri plates, containing malt agar, were inoculated from P.chrysosporium and T.versicolor malt agar slants, grown at 35°C. After a few hours of incubation, three lawn cultures of P.chrysosporium and T.versicolor were separately prepared. When slight growth of organisms occurred, pre-weighed lindane crystals were placed onto the growing areas of the lawn organisms. The cultures were allowed to grow for 10 days at 35°C in an incubator.

Lindane crystals, which were placed onto a lawn culture of P. chrysosporium were degraded after some time with only small crystals being left behind (Fig. 3.3.1). Lindane-placed areas on P. chrysosporium cultures were invaded by new micelles of P. chrysosporium. A lighter zone of growth was noticed around lindane crystals. For T. versicolor, growth was very much slower when compared with P. chrysosporium. T. versicolor was able to degrade a large lindane crystal which was placed onto the growth area of the organism.

3.3.2. Second Phase

In a prior experiment it was found in this laboratory that some of the Turkish lignites effectively sorb organic chlorine compounds and remove them from waters and waste-waters. This effect was also observed and reported earlier in Britain. However, disposal of spent lignites with sorbed chlorinated organics pose a problem, as burning these in a conventional burner may be extremely dangerous as combustion products may contain highly toxic and carcinogenic chlorinated compounds such as chlorodioxins etc. Therefore an attempt here was made to see whether lignites sorbed with lindane are amenable to degradation by white-rot fungi.

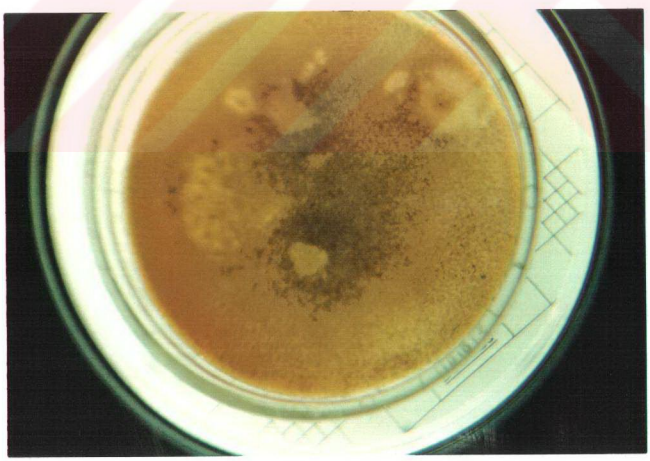


Figure 3.3.1. Phanerochaete chrysosporium, grown on a malt agar plate, which was containing powdered lindane onto it.

This phase of experiments was initiated by using lindane impregnated lignite coals. For lindane impregnation onto lignite, 5 mg lindane was weighed, and dissolved in 1 ml Methanol (BDH). This was made up to 100 milliliters by distilled water and 1,5 g 1-2 mm size Elbistan lignite coal particles were added, and agitated for 2 days in shaker. After then, coal was filtered out and dried at 105°C for an hour.

Three lawn cultures of P. chrysosporium and T. versicolor were prepared. The lindane- impregnated pre-weighed (20 mg) coal particles were placed on petri plates where slight growth has occurred. Some of the petri plates also received Elbistan lignite coal particles untreated with lindane to serve as control. The amount of coal again was 20 mg for each plate.

After 10 days of incubation, growth was observed on lignite particles impregnated with lindane and on the control particles which were not impregnated with lindane. P. chrysosporium was observed to be faster grower than T. versicolor. (Figs. 3.3.2 and 3.3.3)

3.3.3. Third Phase

In the third phase of the solid state culture experiments 20 mg lindane was dissolved in 5ml Methanol and was added into molten agar. Six petri plates were prepared by pouring the molten agar and leaving it to set containing two petri plates which were inoculated by P. chrysosporium, which was taken from plates containing lindane crystals. Inoculum was taken from nearby lindane crystals where only a weak growth could be noticable. Two other petri plates were inoculated by P. chrysosporium mycelia which were grown over coal particles. Consequently, the remaining two plates were incubated by T. versicolor, grown nearby a degraded lindane crystal in the lindane-containing malt agar. A 10 - day incubation period was allowed for the growth of the organisms. After 10 days growth was observed on lindane-maltose agar.

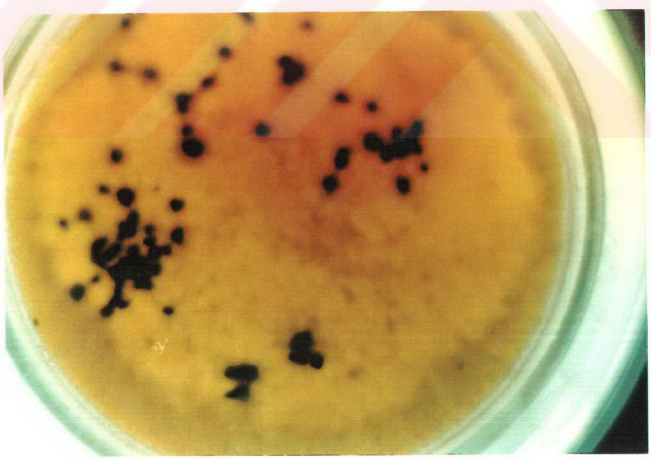


Figure 3.3.2. Phanerochaete chrysosporium, grown on a malt agar plate, which was containing lindane-impregnated Elbistan coal on it.

Figure 3.3.3. Phanerochaete chrysosporium, grown on a malt agar plate, which was containing lindane-impregnated Elbistan and non-impregnated Elbistan coal on it.

3.4. THE FOURTH CYCLE EXPERIMENTS

This cycle of experiments involved the transfer of the fungi, that were grown on lindane-maltose agar in the preceeding phase of experiments, onto Erlenmayer flasks containing Saboraud liquid broth. During the transfer, P.chrysosporium, grown nearby lindane crystals and that which was grown over coal particles were mixed together and inoculated onto lindane-maltose agar and incubated until slight growth was observed. For T. versicolor, the incubation was carried by inoculating lindane-maltose agars by mycelia which was taken nearby a degraded-lindane crystal.

When growth was recorded in petri plates, four erlenmayer flasks were prepared; as summarized below. Except static flasks, all flasks were incubated at 35° C, 180 rpm in a shaker.

Erlenmayer flask 1: Saboraud broth + Lindane + P. chrysosporium suspension.

Erlenmayer flask 2: Saboraud broth + Lindane + T. versicolor suspension.

Erlenmayer flask 3: Saboraud broth + Lindane + P. chrysosporium suspension.

Erlenmayer flask 4: Saboraud broth + Lindane + T. versicolor suspension.

In this experiment the growth was measured at zero and 10^{th} days. For the UV-scanning experiment, the 10 - day aliquots of the four flasks were used. As for the controls a zero day aliquot, only containing Saboraud broth + Lindane solution, and aliquots containing the broth and lindane solution, separately, were used.

The control flask combinations used in the fourth cycle experiments were as follows:

Control flask 1: Saboraud broth + Lindane.

Control flask 2: Saboraud broth.

Control flask 3: 500 ml lindane.

A 20 mg of Lindane, for this cycle of experiment, was dissolved in 5 ml methanol and diluted to 500 ml with distilled water.

For the other flasks, a 2 ml suspension was prepared and added onto the flasks, containing Saboraud medium and lindane. After 10 days of incubation, the lindane-analysis were carried out by GC. With reference to the control flask containing both lindane and Saboraud agar but no microorganisms, the incubated T. versicolor containing flask, showed approximately a 89,75 % loss of lindane. The static T. versicolor indicated a 95,25 % loss of lindane as summarized in Table 3.3. Whereas, P. chrysosporium produced even higher removals, approaching 99,1 % in shake flasks and 100 % in static flask. Growth in static flask appeared more confluent than was in shake flask. The growth measurements. shown in Appendix B-10 also confirmed this observation. The UV-scan was carried out to determine the absorption wavelengths of the compounds present in the medium. These findings were not conclusive.

As for the GC chromatograms, except for lindane, other peaks were probably due to the contamination of syringe or impurities in the medium. Slight shifts of the peaks were due to manual operation of the integrator. (Figs 3.4.1 to 3.4.7)

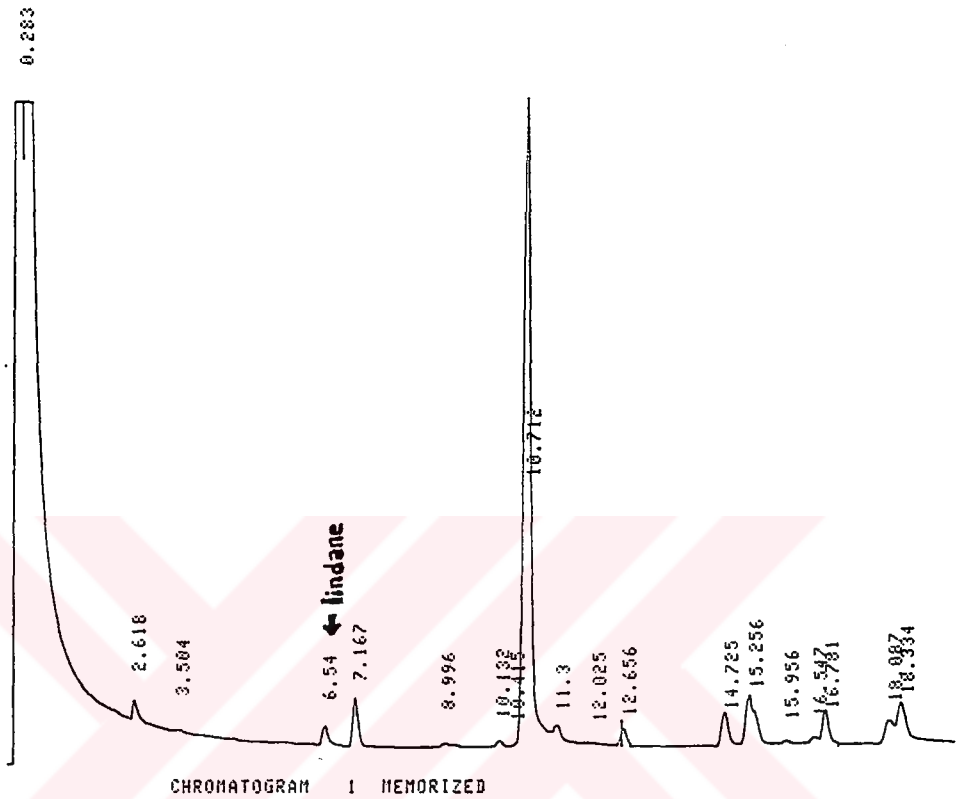
CHROMATOGRAM 1 MEMORIZED

C-RSA CHROMATOPAC
CHANNEL NO 1
SAMPLE NO 0
REPORT NO 10

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.317	13659144	S E		99.212	
2	2.611	405	T		0.0029	
3	3.525	224	T		0.0016	
4	6.556	361			0.0026	
5	7.176	12252			0.089	
6	10.144	1113			0.0081	
7	10.421	768	V		0.0056	
8	10.724	32237	V		0.2342	
9	11.308	3945	V		0.0287	
10	12.046	827			0.006	
11	12.65	3292			0.0239	
12	14.743	6253			0.0454	
13	15.258	13431	V		0.0976	
14	16.555	1298			0.0094	
15	16.796	6148	V		0.0447	
16	18.092	4144			0.0301	
17	18.343	21804	V		0.1584	
	TOTAL	13767633			100	

Figure 3.4.1. The GC analysis for the flask no. 1, in the fourth cycle experiments. Flask contains 115 ml Saboraud broth + 35 ml lindane solution + 2 ml P. chrysosporium suspension, incubated at 35°C, involved in fourth cycle experiments.

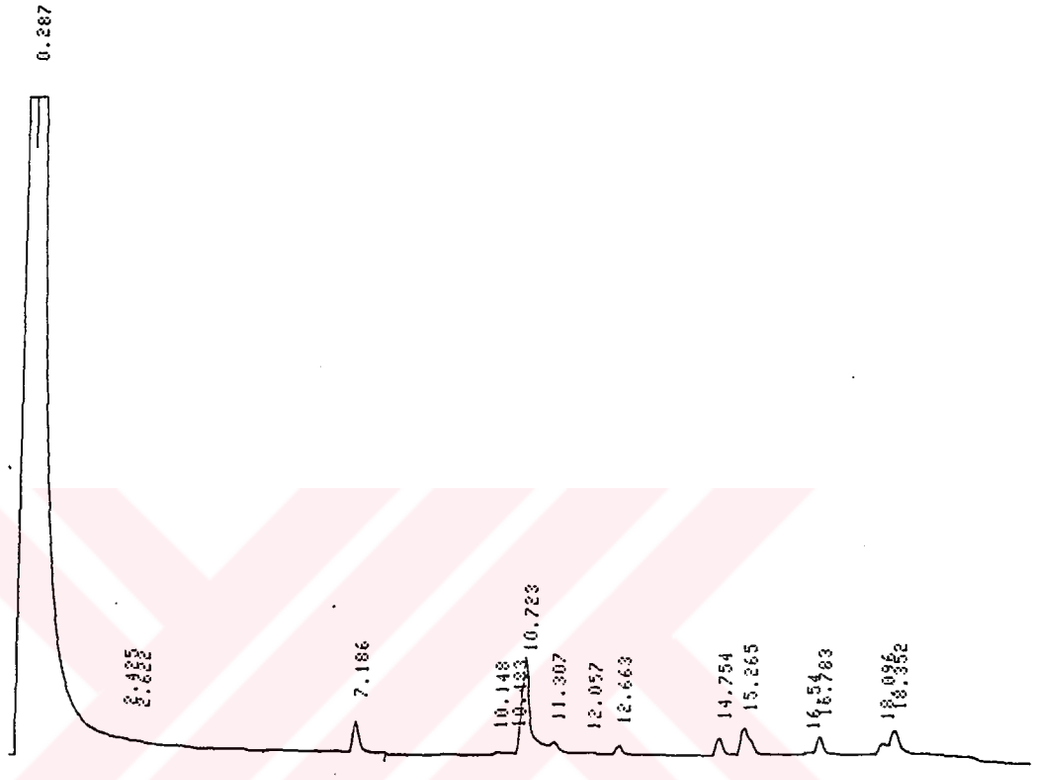


CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 8

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.283	10200718	S E		97.5827	
2	2.618	4102	T		0.0392	
3	3.504	369	T		0.0035	
4	6.54	4039			0.0386	
5	7.167	9301			0.089	
6	8.996	734			0.007	
7	10.132	1700			0.0163	
8	10.415	608	V		0.0058	
9	10.712	157807	V		1.5096	
10	11.3	11630	V		0.1113	
11	12.025	3292	V		0.0306	
12	12.656	5910	V		0.0565	
13	14.725	8204			0.0785	
14	15.256	16282	V		0.1558	
15	15.956	932	V		0.0089	
16	16.547	1500			0.0144	
17	16.781	7622	V		0.0729	
18	18.087	6549			0.0626	
19	18.334	12205	V		0.1168	
	TOTAL	10453405			100	

Figure 3.4.2. The GC analysis for the flask no. 2, in the fourth cycle experiments. Flask contains 115 ml Saboraud broth + 35 ml lindane solution + 2 ml T. versicolor suspension, incubated at 35°C, involved in fourth cycle experiments.



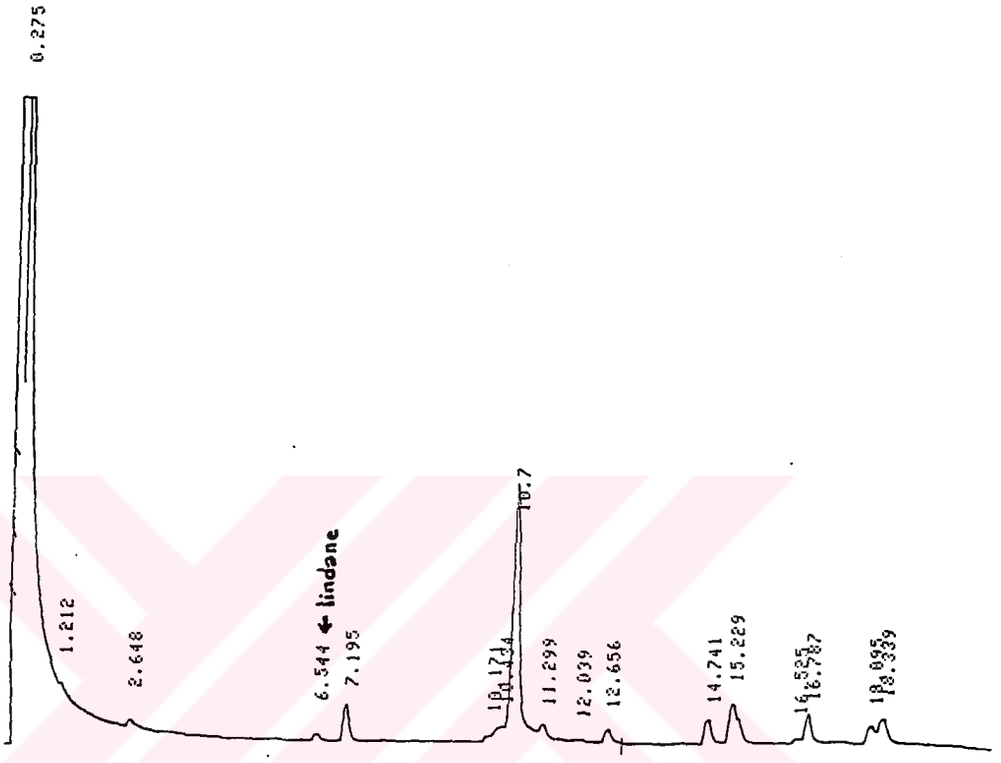
CHROMATOGRAM 1 MEMORIZED

C-RSA CHROMATOPAC
CHANNEL NO 1
SAMPLE NO 0
REPORT NO 11

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.287	10539619	S E	1	99.3258	
2	2.425	130	T		0.0012	
3	2.622	305	T		0.0029	
4	7.186	7002			0.066	
5	10.148	827			0.0078	
6	10.483	454	V		0.0043	
7	10.723	27906	V		0.263	
8	11.307	2867	V		0.027	
9	12.057	497			0.0047	
10	12.663	2194			0.0207	
11	14.754	4261			0.0402	
12	15.265	9169	V		0.0864	
13	16.54	851			0.008	
14	16.783	4054	V		0.0382	
15	18.096	3136			0.0295	
16	18.352	7895	V		0.0744	
	TOTAL	10611160			100	

Figure 3.4.3. The GC analysis for the flask no. 3, in the fourth cycle experiments. Flask contains 115 ml Saboraud broth + 35 ml lindane solution + 2 ml P. chrysosporium suspension, static at room temperature, involved in fourth cycle experiments.



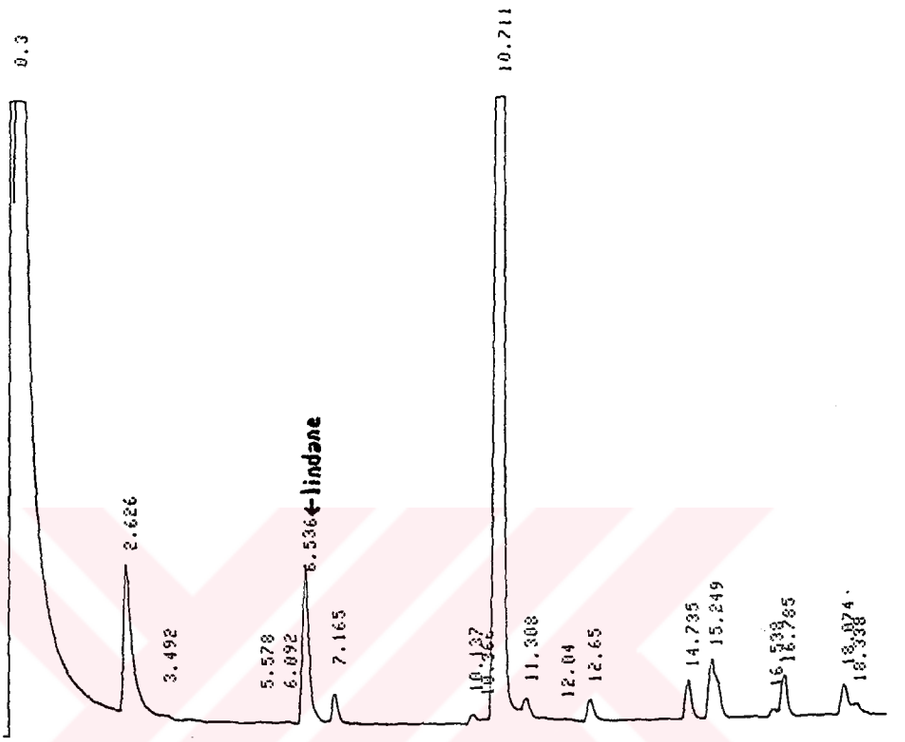
CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1
SAMPLE NO 0
REPORT NO 9

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.275	9469090	S E		98.584	
2	1.212	336	T		0.0035	
3	2.648	2545	T		0.0265	
4	6.544	1868			0.0194	
5	7.195	8092			0.0842	
6	10.171	1419			0.0148	
7	10.434	5479	V		0.057	
8	10.7	68743	V		0.7157	
9	11.299	4062	V		0.0423	
10	12.039	458			0.0048	
11	12.656	3207			0.0334	
12	14.741	5925			0.0617	
13	15.229	13220	V		0.1376	
14	16.525	1250			0.013	
15	16.787	6339	V		0.066	
16	18.095	5189			0.054	
17	18.339	7885	V		0.0821	
	TOTAL	9605100			100	

Figure 3.4.4. The GC analysis for the flask no. 4, in the fourth cycle experiments. Flask contains 115 ml Saboraud broth + 35 ml lindane solution + 2 ml T. versicolor suspension, static at room temperature, involved in fourth cycle experiments.

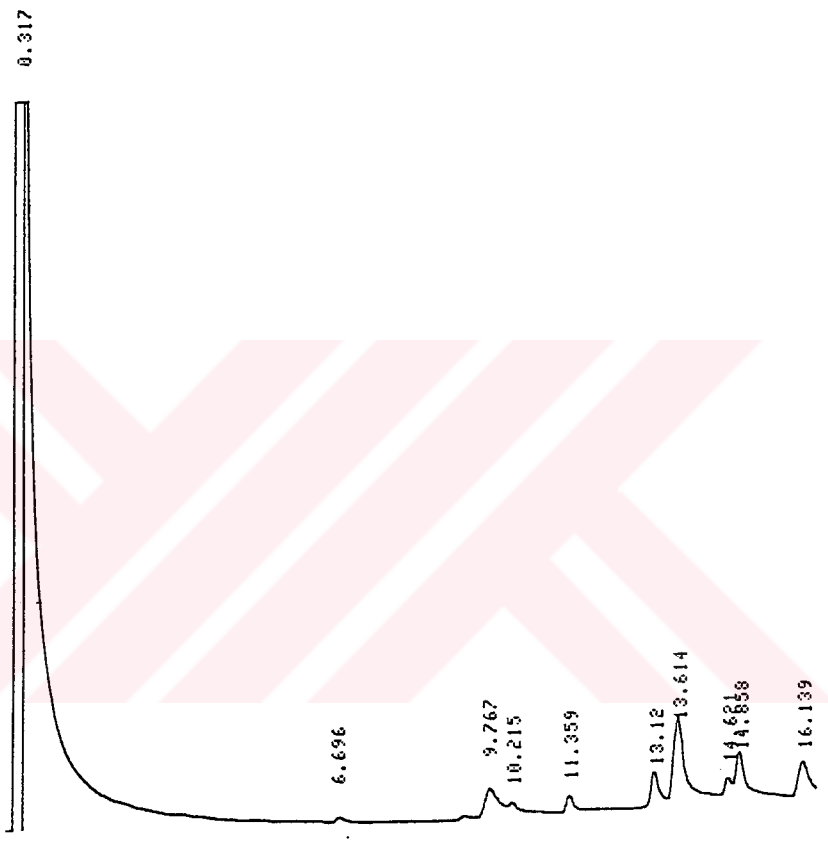


CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 7

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.3	11026411	S E		93.3977	
2	2.626	48884	T		0.4141	
3	3.492	658	TV		0.0056	
4	5.578	247			0.0021	
5	6.092	443			0.0038	
6	6.536	39372	V		0.3335	
7	7.165	6883	V		0.0583	
8	10.137	2180			0.0185	
9	10.366	990	V		0.0084	
10	10.711	613914	V		5.2001	
11	11.308	6117	V		0.0518	
12	12.04	269			0.0023	
13	12.65	5510			0.0467	
14	14.735	8941			0.0757	
15	15.249	20644	V		0.1749	
16	16.538	2248			0.019	
17	16.785	10002	V		0.0847	
18	18.074	8958			0.0759	
19	18.338	3213	V		0.0272	
	TOTAL	11805874			100	

Figure 3.4.5. The GC analysis for control flask no. 1, including 115 ml Saboraud broth + 35 ml lindane, involved in fourth cycle experiments.

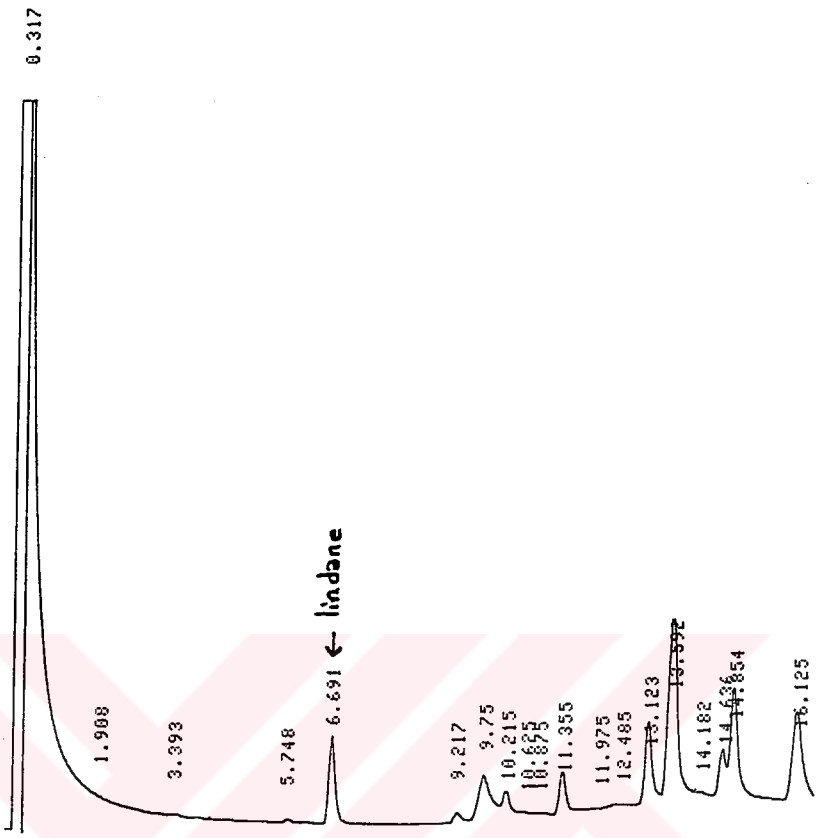


CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 4

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.317	11843194	E		99.2783	
2	6.696	1427			0.012	
3	9.767	12186			0.1022	
4	10.215	3774	V		0.0316	
5	11.359	4363			0.0366	
6	13.12	8636			0.0724	
7	13.614	28321	V		0.2374	
8	14.621	4138			0.0347	
9	14.858	14755	V		0.1237	
10	16.139	8498			0.0712	
	TOTAL	11929290			100	

Figure 3.4.6. The GC analysis for control flask no. 2, containing 500 ml Saboraud broth, involved in fourth cycle experiments.



CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 3

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.317	11892432	S E		97.9534	
2	1.908	46	T		0.0004	
3	3.393	579			0.0048	
4	5.748	791			0.0065	
5	6.691	19616			0.1616	
6	9.217	1984			0.0163	
7	9.75	19010	V		0.1566	
8	10.215	8829	V		0.0727	
9	10.625	1204	V		0.0099	
10	10.875	1231	V		0.0101	
11	11.355	10830	V		0.0892	
12	11.975	2622	V		0.0216	
13	12.485	5113	V		0.0421	
14	13.123	25877	V		0.2131	
15	13.592	68069	V		0.5607	
16	14.182	5570	V		0.0459	
17	14.636	13896	V		0.1145	
18	14.854	34363	V		0.2831	
19	16.125	28241			0.2326	
	TOTAL	12140294			100	

Figure 3.4.7. The GC analysis for control flask no. 3, containing 500 ml lindane solution, involved in fourth cycle experiments.

Table 3.3. Results of the lindane degradation for the fourth cycle experiments

Flask no	Flask Composition	Static/ Shake	For 150 ml Lindane İnitial	Lindane Last (for 150 ml)	%Removal 0 (day)	%Removal 9 (day)
1	Saboraud Broth, 40 mg/L Lindane, 2 ml P. chrysosporium	Shake	6 mg	0,054 mg	0	99,1
2	Saboraud Broth, 40 mg/L Lindane, 2 ml T. versicolor	Shake	6 mg	0,615 mg	0	89,75
3	Saboraud Broth, 40 mg/L Lindane, 2 ml P. chrysosporium	Static	6 mg	0 mg	0	100
4	Saboraud Broth, 40 mg/L Lindane, 2 ml T. versicolor	Static	6 mg	0,285 mg	0	95,25
Control 1	Saboraud Broth, 40 mg/L Lindane	Shake	6 mg	6 mg	0	0
Control 2	Saboraud Broth	Shake	0	0	0	0
Control 3	40 mg/L Lindane	Shake	6 mg	6 mg	0	0

CHAPTER 4

DISCUSSION

4.1. THE FIRST CYCLE EXPERIMENTS

According to Leatham (1986) and Ulmer et al., two genera of white-rot fungi, P. chrysosporium and Lentinula edodes, metabolize various lignin preparations only when an alternate carbon/energy source is present. Polysaccharides are required for white-rot fungi, in order to maintain not only its carbon source, but also, to enhance its secondary (idiophasic) metabolism, which will be involved in veratryl alcohol synthesis essential for enzyme activity to provide the lignin degradation.

The results of the first cycle experiments were tabulated in Table 3.1. Glucose limitation and nitrogen limitation were provided subsequently. For both glucose and nitrogen (limited cultures) the one which contained alkaline BPE and lindane showed approximately a 32 % loss of lindane. The flask No.1, which contained glucose and nitrogen (delimited culture), exhibited 29 % loss of lindane when 0 and 9 days' results were compared.

The glucose content analysis of all the test flasks at the end of the experiment indicated complete consumption by P. chrysosporium. On the 4^{th} day of experimentation, glucose content in all flasks were around zero. On the other hand, for the sec-

ond and third flasks, the protein curves indicated a sharp increase on the 4th day, followed by a subsequent decrease. These results imply that when glucose is completely consumed, extracellular enzymes, which are proteins in nature, initially appeared and then disappeared from the medium for some unknown reason.

The addition of surfactant, Tween 80, must afford the ligninase production via lignin degradation; but the mechanism through which this surfactant stimulates ligninase production in mixed cultures is not clearly understood.

UV-absorbance measurements were carried out at two wavelengths, at 254 nms, for benzene ring, and at 280 nm, for phenolic substances. After the fourth day, the UV absorbance values gradually decreased and dropped to a constant low value. These results were common to all the flasks involved in the first cycle experiments and indicated that , from beginning of the 1st cycle, treatment with P. chrysosporium decreased both the 254 and 280-nm absorbing substances, which gave initially high absorbances in the BPE and lindane containing flasks. At the end of the experimental cycle, the dark brown color of the BPE was turned to a pale yellow, indicating decolorization activity. The decolorization activity of P.chrysosporium should be attributed to its ligninase activity mainly. This should also affect dehalogenation as colour compounds in BPE, are mainly chlorinated phenols in nature.

The decreasing UV-absorbances in the first and second flasks are supporting this view. Consequently a correlation between ligninase activity and the decolorization and dehalogenation by P. chrysosporium could be assumed.

The growth measurements indicated an increase in the mycelia concentration between the zero and 4th day.

Except for the second flask, approximately, 30% degradation was achieved in all the test flasks, for lindane. For the second flask, 1% by approximation, degradation was observed. This might be due to the combination of nutrient limitation and excluding alkaline BPE in the second flask. The other peaks appeared in GC chrom-

atograms might also be the degradation by-products of lindane, as, during treatment with P. chrysosporium, compounds with fewer chlorine atoms, was compared with the hexachlorinated lindane molecule, could have been formed.

Two flasks, No. 2 and No.3., in first cycle experiments were run in parallel with the sole difference that flask no. 3 contained alkaline BPE in addition, displayed differing results. The 30% removal achieved in flask no. 3 as compared to 1% in flask no. 2 was clearly due to the effect of alkaline BPE present in this flask.

4.2. THE SECOND CYCLE EXPERIMENTS

Acetate was the replacement carbon source, used in the second cycle experiments, together with glucose. Glucose is an expensive carbon source, when compared with acetate. This replacement was thought to decrease the cost in actual size bioreactors. Glucose was used in limiting mode (2 g/l) and alkaline BPE was used to supply a source of veratryl alcohol to the fungus. The static flasks didn't contain Tween 80.

Elbistan and Beypazarı lignite coals were used in flasks 5, 6, 7, 9 and 10, to act as both solid substrate for the mycelia to adhere to, and also to induce ligninases in fungi, should lindane concentration be too low to do this. It is reported that (Gökçay et al.) P.chrysosporium switches to a different mode of metabolism when immobilized on a solid substrate. On this new mode, the fungus becomes truely non N-limited and glucose non-specific. Meaning, ligninase activity is detectable in N- sufficient media and on carbon source other than glucose, such as cheap acetate.

However, no conclusive results could be obtained in these experiments, and no discussions were made on these.

The glucose- containing cultures, especially the second and the third flasks, showed 27 and 11% loss of lindane, with nitrogen delimitation and limitation modes, respective-

ly, indicating N- sufficient mode requirement for lindane removal by this microorganism.

4.3. THE THIRD CYCLE EXPERIMENTS

Solid-phase culture studies were discussed under the third cycle experiments, which were undertaken by using both solid lindane, and lindane-impregnated Elbistan lignite coal. A 10-day incubation was employed and as a result lindane solid crystals were observed not to have disappeared within 5 and 6 days of incubation, and in the end of the ten day.

In the second phase of the third cycle experimentation, 10-day old solid cultures, showing growth in the vicinity of lindane particles were used. The proceeding incubations were made onto lindane-containing malt agar plates. With P. chrysosporium and T. versicolor a common procedure was employed. For preparing inoculum, a smear was taken. From the vicinity of a lindane particle, showing very weak growth compared to other parts of the lawn culture. The 'weak' growth appeared around lindane particles after 5-6 days of incubation. This zone was initially containing no growth but subsequently covered by this 'weak' growth suggesting a mutant being selected in due process. Also mycelia grown on lindane-impregnated coal particles were taken and an inoculum mixture was prepared by combining these with the smears. This mixed inoculum was used to inoculate lindane containing malt-agar media.

Mycelial growth on lindane-malt agars were used as source of inoculum for the subsequent fourth cycle experiments. The same procedure was applicable to both T. versicolor and P. chrysosporium.

4.4. THE FOURTH CYCLE EXPERIMENTS

The growth measurements, carried out in the fourth cycle experiments indicated much richer growth in the static cultures, when compared to the agitated (35°C, 180 rpm) cultures.

The lindane removals shown in Table 3.3 for P. chrysosporium indicate that selected mycelia indeed proved to be effective in degrading this compound. Lindane removals obtained in both shake flask and static cultures were close to 100% with this fungus.

Comparison of these results with those presented in Table 3.1. for the first-cycle experiments indicate that strain selection procedure described in third cycle experiments was successful and complete degradation of lindane was thus possible using P. chrysosporium.

Likewise, results for T. versicolor shown in Table 3.3., flasks 2 and 4 indicate that this fungus, which is also a white-rot fungus by definition, is able to affect over 90% of lindane degradation, following a strain selection procedure as described in third cycle experiments. However, no previous studies with the un-selected T. versicolor have been performed and net effect of strain selection on this out-come could not be satisfactorily demonstrated.

In this cycle of experiments the lindane concentration was almost double the concentration in the first cycle experiments but this increment is highly unlikely to cause of additional induction of the enzymes. Nevertheless enzymes were already induced in the first cycle as there were noticable removals. Normally enzyme induction is a go/no go kind of process and no in-between effects are expected.

CHAPTER 5

CONCLUSION

The white-rot fungi, P. chrysosporium and T. versicolor are known to degrade and mineralize lignin , as well as several organochlorine compounds. The nutrients provided during the course of first cycle experiments, indicate that the extracellular enzyme system of P. chrysosporium is inducable when lindane is present in the medium, and approximately 30 % degradation was achieved by common strains. Addition of alkaline BPE seems to improve lindane removal.

The solid-state cultivation experiments were carried out using P. chrysosporium and T. versicolor. During the course of experiments, lindane particles were found not to effect growth of mycelia. However no solid crystal removal could be detected.

Experiments with malt-agar containing lindane and using T. versicolor and P. chrysosporium indicated that these microorganisms can withstand high dissolved lindane concentrations. However placement of lindane crystals on lawn-growth of these fungi initially produced no-growth zones on solid cultures. However a subsequent weak growth was later observed in these no -growth zones.

A liquid culture,inoculated by an inoculum prepared from the weak growth areas on no-growth zones indicated that almost complete removal of lindane is possible by white-rot fungi, upon a careful strain selection procedure.

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APPENDIX A-1

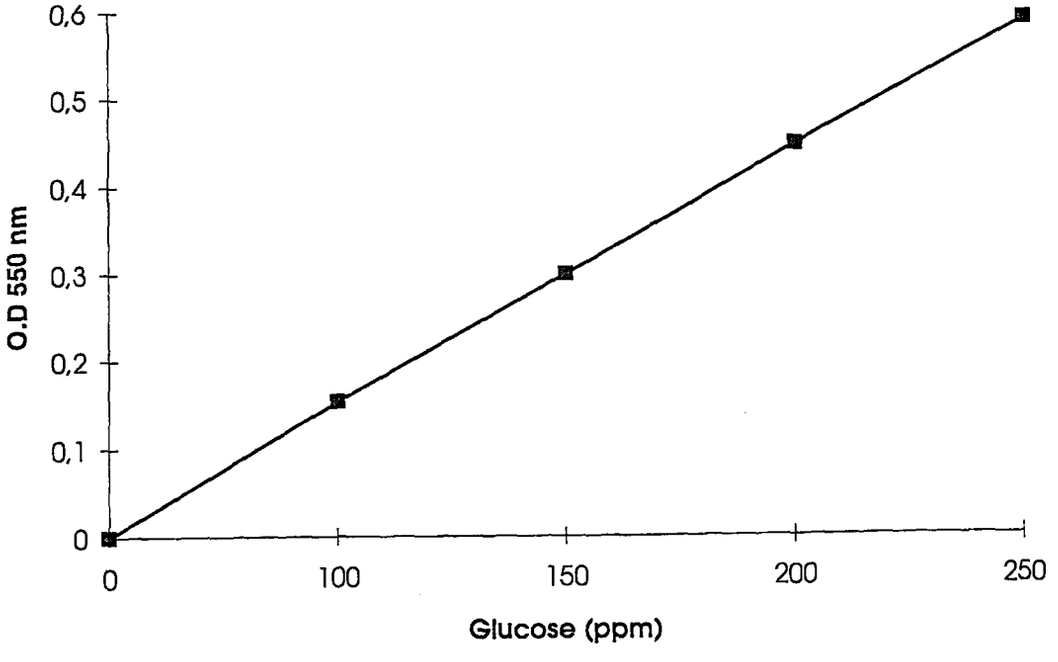
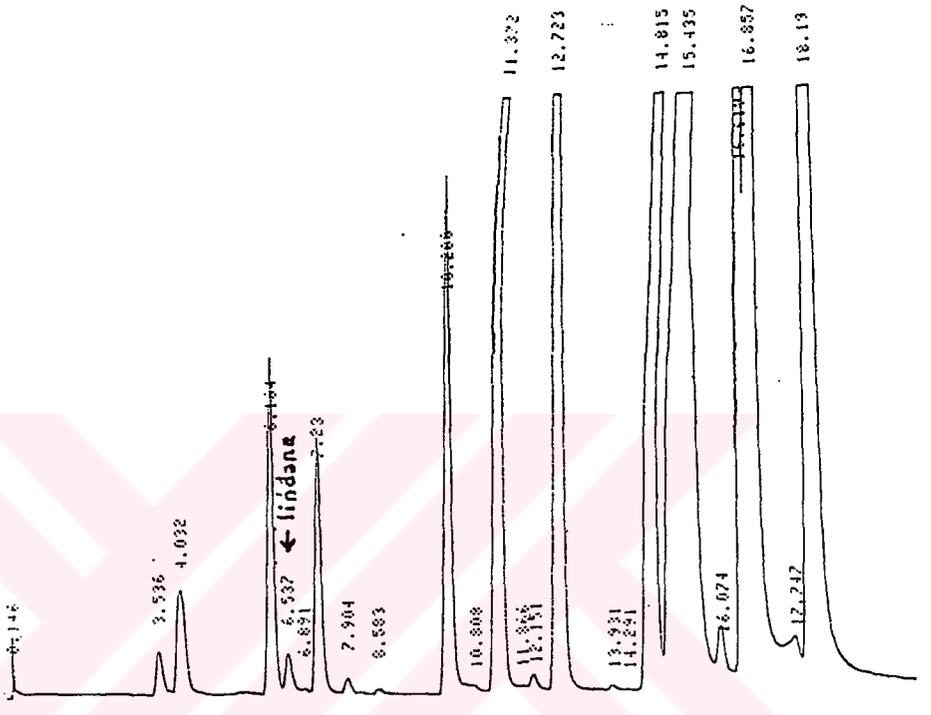


Figure A. 1. Calibration Curve for Glucose Determination

APPENDIX A-2

Figure A.2. Calibration Curve for BSA

APPENDIX A-3



CHROMATOGRAM 1 MEMORIZED

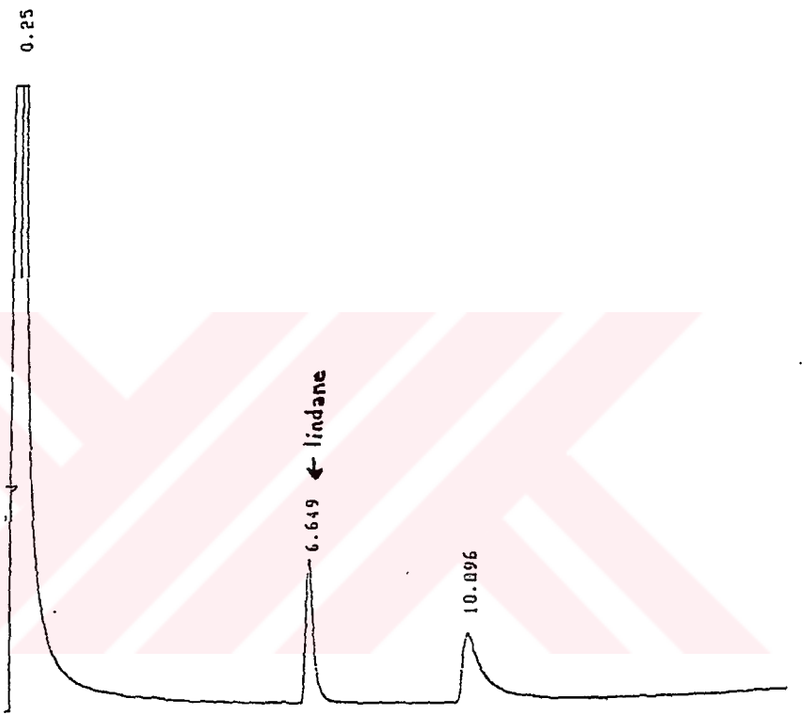
C-R5A CHROMATOPAC
CHANNEL NO 1
SAMPLE NO 0
REPORT NO 1

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.146	3119			0.0654	
2	3.536	12811			0.2685	
3	4.032	39257	V		0.8227	
4	6.164	85328			1.7882	
5	6.537	13140	V		0.2754	
6	6.891	1879	VI		0.0394	
7	7.23	67131	V		1.4068	
8	7.904	3888			0.0815	
9	8.583	1739			0.0365	
10	10.808	145631	S		3.0519	
11	10.808	468	T		0.0098	
12	11.372	333389	SV		6.9866	
13	11.966	214	T		0.0045	
14	12.151	3884	T		0.0814	
15	12.723	354947	V		7.4384	
16	13.931	1648	V		0.0344	
17	14.291	543	V		0.0114	
18	14.815	540453			11.3259	
19	15.435	1340864	V		28.0994	
20	16.074	24042	V		1.5038	
21	16.619	267551	V		5.6063	
22	16.857	728228	V		15.2611	
23	17.747	32225	V		0.6753	
24	18.19	769479	V		16.1252	
	TOTAL	4771354			100	

Figure A. 3. The GC Analysis for Several Pesticides

APPENDIX A-4



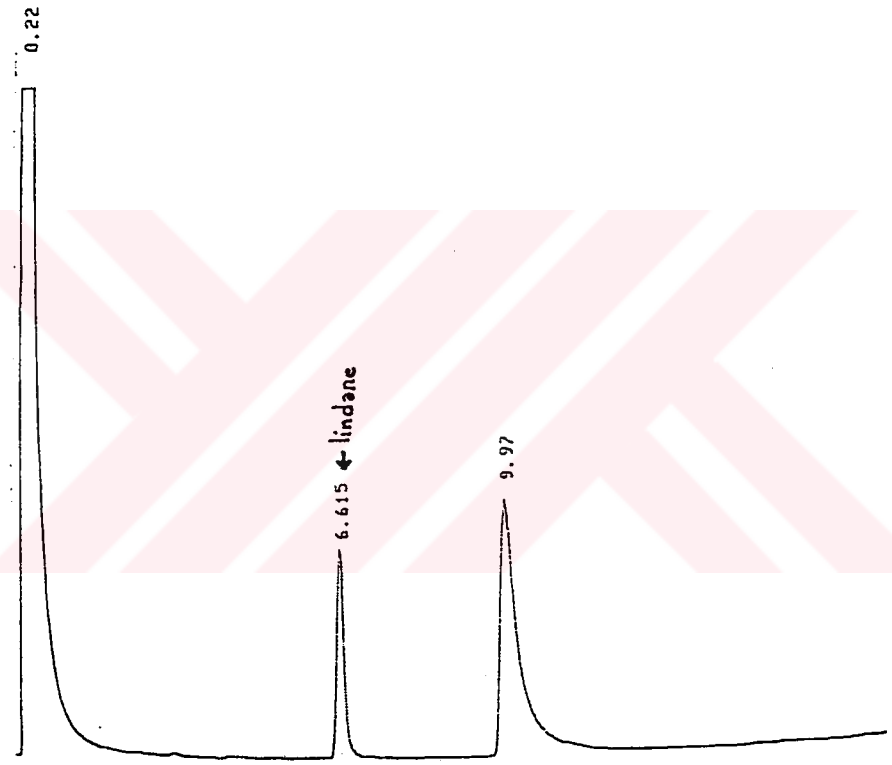
CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 3

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.25	9142042	E		99.5332	
2	6.649	43143			0.4676	
3	10.096	41445			0.4491	
	TOTAL	9226634			100	

Figure A. 4. The GC Analysis for the 1st flask, on 9th day of the First Cycle Experiments, containing 10 g/l Glucose + 100 N % + 20 mg/l lindane solution + 2 ml P. chrysosporium suspension, incubated at 35°C

APPENDIX A-5



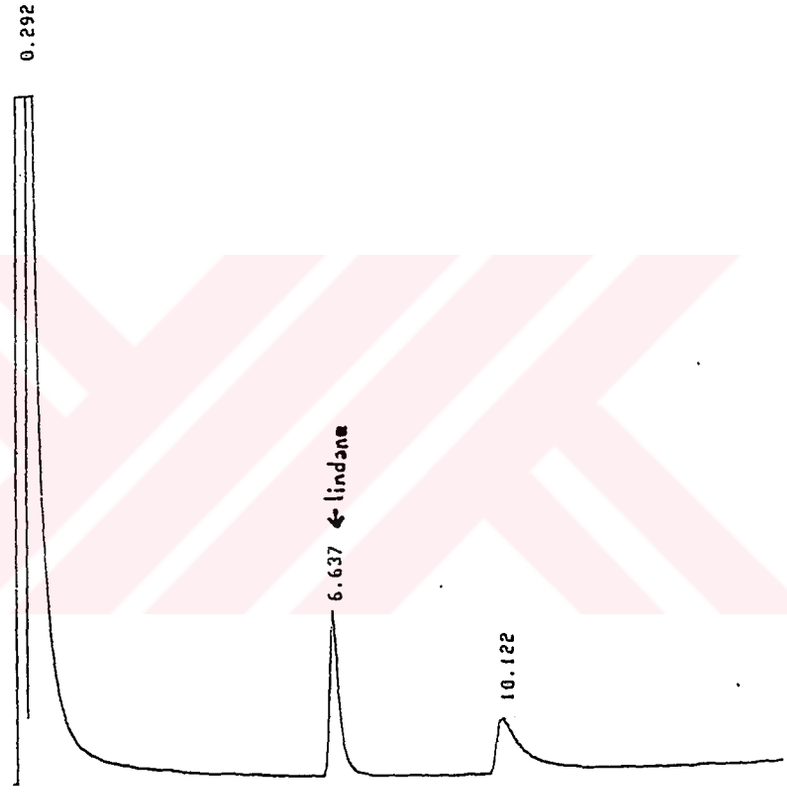
CHROMATOGRAM 1 MEMORIZED

C-ESA CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 1

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.22	7781698	E		97.9673	
2	6.615	51872			0.6554	
3	9.97	141737			1.7771	
TOTAL		7975306			100	

Figure A. 5. The GC Analysis for Flask no. 2, on 1st day of the First Cycle Experiments, containing 2 g/l Glucose + 10 N % + 20 mg/l lindane solution + 2 ml P. chrysosporium suspension, incubated at 35°C

APPENDIX A-6



CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 MET+03 41
REPORT NO 3

P.NO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.292	12485126	E		99.2573	
2	6.637	51640			0.4125	
3	10.122	41333			0.3302	
	TOTAL	12578099			100	

Figure A. 6. The GC Analysis for Flask no. 2, on 9th day of the First Cycle Experiments, containing 2 g/l Glucose + 10 N % + 20 mg/l lindane solution + 2 ml P. chrysosporium suspension, incubated at 35°C

APPENDIX A-7

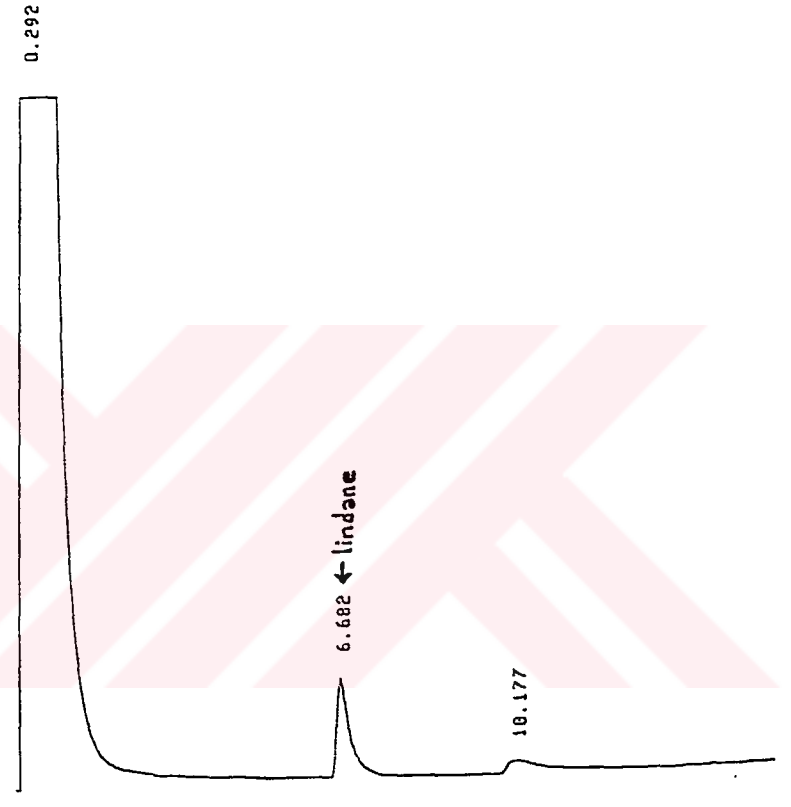
CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 4

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.203	11234599	E		99.3407	
2	6.659	60418			0.5343	
3	10.153	11866			0.1049	
	TOTAL	11306883			100	

Figure A. 7. The GC Analysis for Flask no. 3, on 1st day of the First Cycle Experiments, containing 2 g/l Glucose + 10 N % + 40 ml Alkaline BPE + 20 mg/l lindane + 2 ml P. chrysosporium suspension, incubated at 35°C

APPENDIX A-8



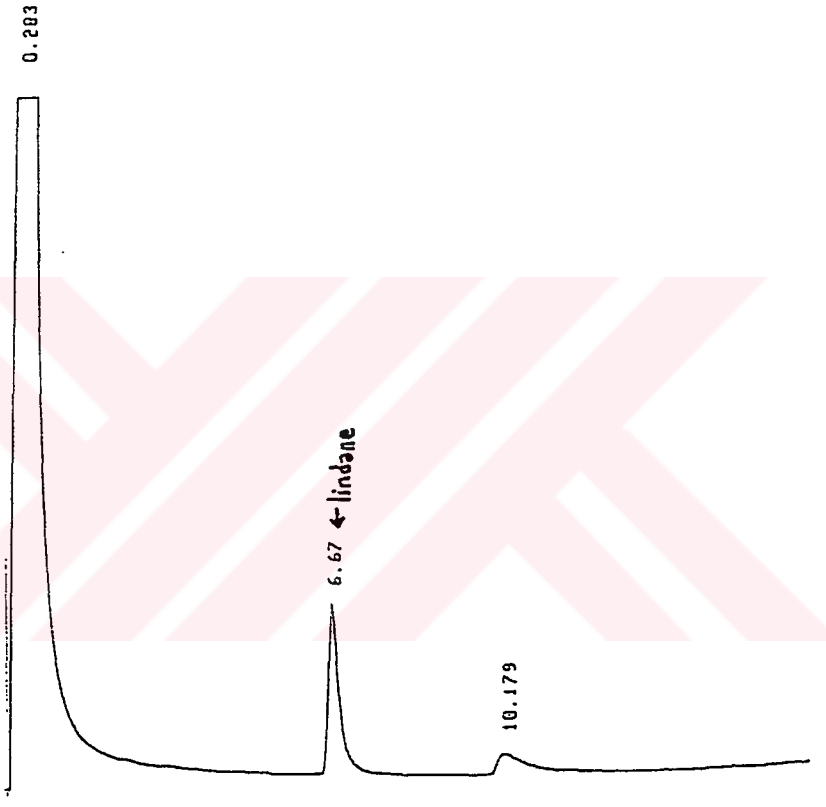
CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 5

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	0.292	14354793	E		99.7021	
2	6.682	40959			0.2845	
3	10.177	1937			0.0134	
		---------			----------	
TOTAL	14397678				100	

Figure A. 8. The GC Analysis for Flask no. 3, on 9th day of the First Cycle Experiments, containing 2 g/l Glucose + 10 N % + 40 ml Alkaline BPE + 20 mg/l lindane + 2 ml P. chrysosporium suspension, incubated at 35°C

APPENDIX A-9



CHROMATOGRAM 1 MEMORIZED

C-R5A CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 6

PKNO	TIME	AREA	MK	ISNO	CONC	NAME
1	0.283	11529976	E		99.4083	
2	6.67	68761			0.5838	
3	10.179	8570			0.0739	
	TOTAL	11599307			100	

Figure A. 9. The GC Analysis for the Control flask, containing 20 mg/l lindane + distilled water

APPENDIX A-10

Tablo A.10. $O.D_{550}$ (nm) Values for Glucose Utilization, detected in the First Cycle Experiments

Flask no	Day	$O.D_{550}$	Concentration (mg)
1	0	1,895	1553,9
1	2	0,002	1,64
1	4	0,001	0,82
2	0	0,006	43,4
2	2	0,000	0
2	4	0,000	0
3	0	0,199	130,2
3	2	0,006	3,93
3	4	0,004	2,62

APPENDIX A-11

Tablo A.11. $O.D_{550}$ (nm) Values for Protein Determination, detected in the First Cycle Experiments

Flask no	Day	$O.D_{550}$	Concentration (mg)
1	0	0,080	11,79
1	2	0,011	2,03
1	4	0,000	0,0
1	7	0,000	0,0
1	9	0,000	0,0
2	0	0,070	10,38
2	2	0,030	4,72
2	4	0,124	18,02
2	7	0,026	4,15
2	9	0,017	2,88
3	0	0,042	6,41
3	2	0,004	1,04
3	4	0,102	14,90
3	7	0.014	2,45
3	9	0,013	2,31

APPENDIX A-12

Tablo A.12. Data for UV-absorbances detected at 254 and 280 nm by Secomam S-750, in the First Cycle Experiments

Flask no	Day	$O.D_{550}$	Concentration (mg)
1	0	0,415	0,390
1	2	0,189	0,127
1	4	0,123	0,113
1	7	0,097	0,110
1	9	0,086	0,098
2	0	0,437	0,340
2	2	0,189	0,216
2	4	0,156	0,169
2	7	0,152	0,141
2	9	0,140	0,096
3	0	0,880	1,650
3	2	0,400	1,208
3	4	1,329	1,148
3	7	1,322	1,136
3	9	1,319	1,127

APPENDIX A-13

Tablo A.13. Growth Curve Measurement for P. chrysosporium, detected in the First Cycle Experiments

Day	Biomass (μg/150 ml)
0	1,70
2	3,19
4	5,73

APPENDIX B-1

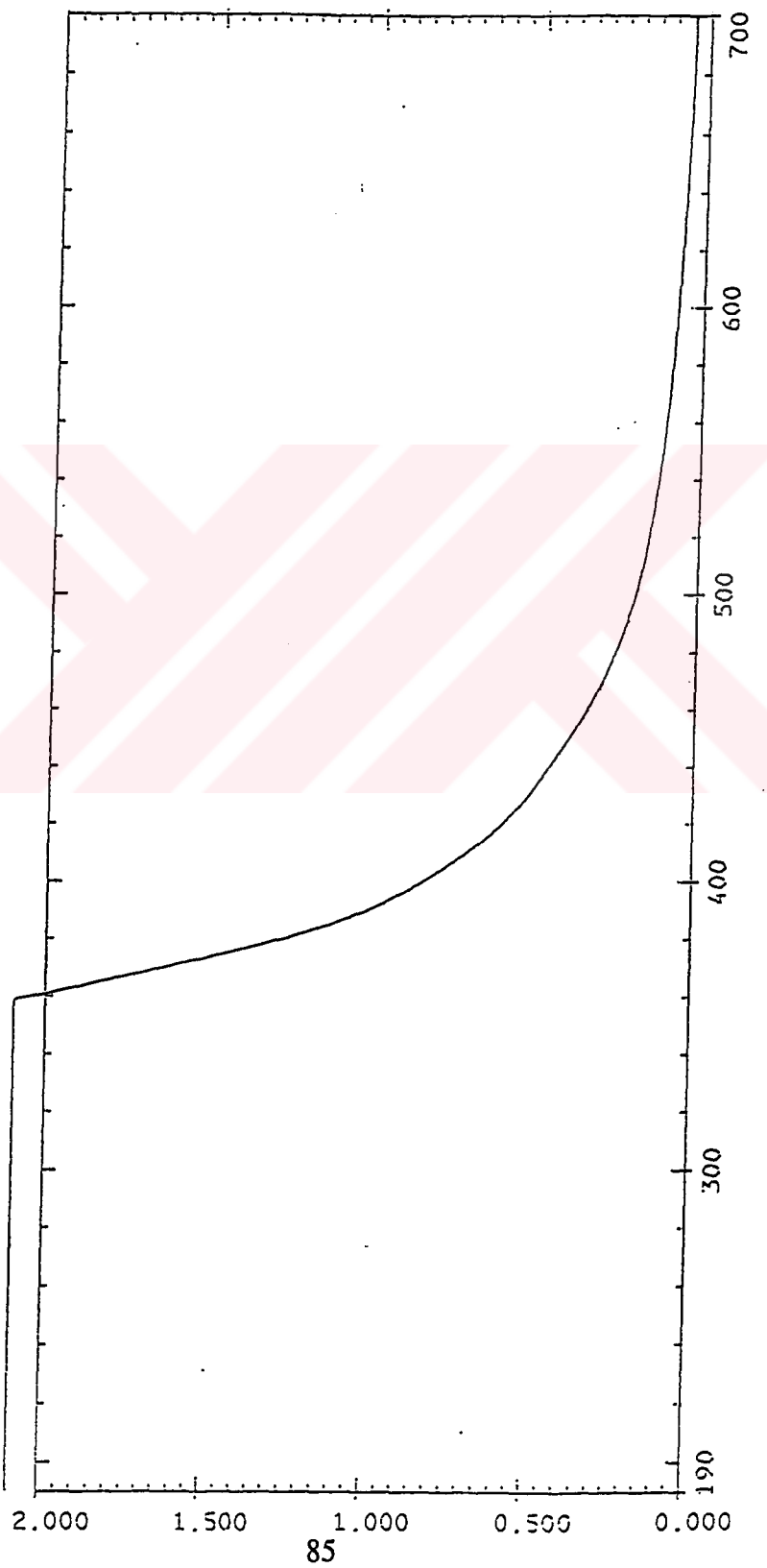


Figure B.1. UndilutedUV-Scanning Analysis of Static Grown T. versicolor involved in fourth cycle experiments

APPENDIX B-2

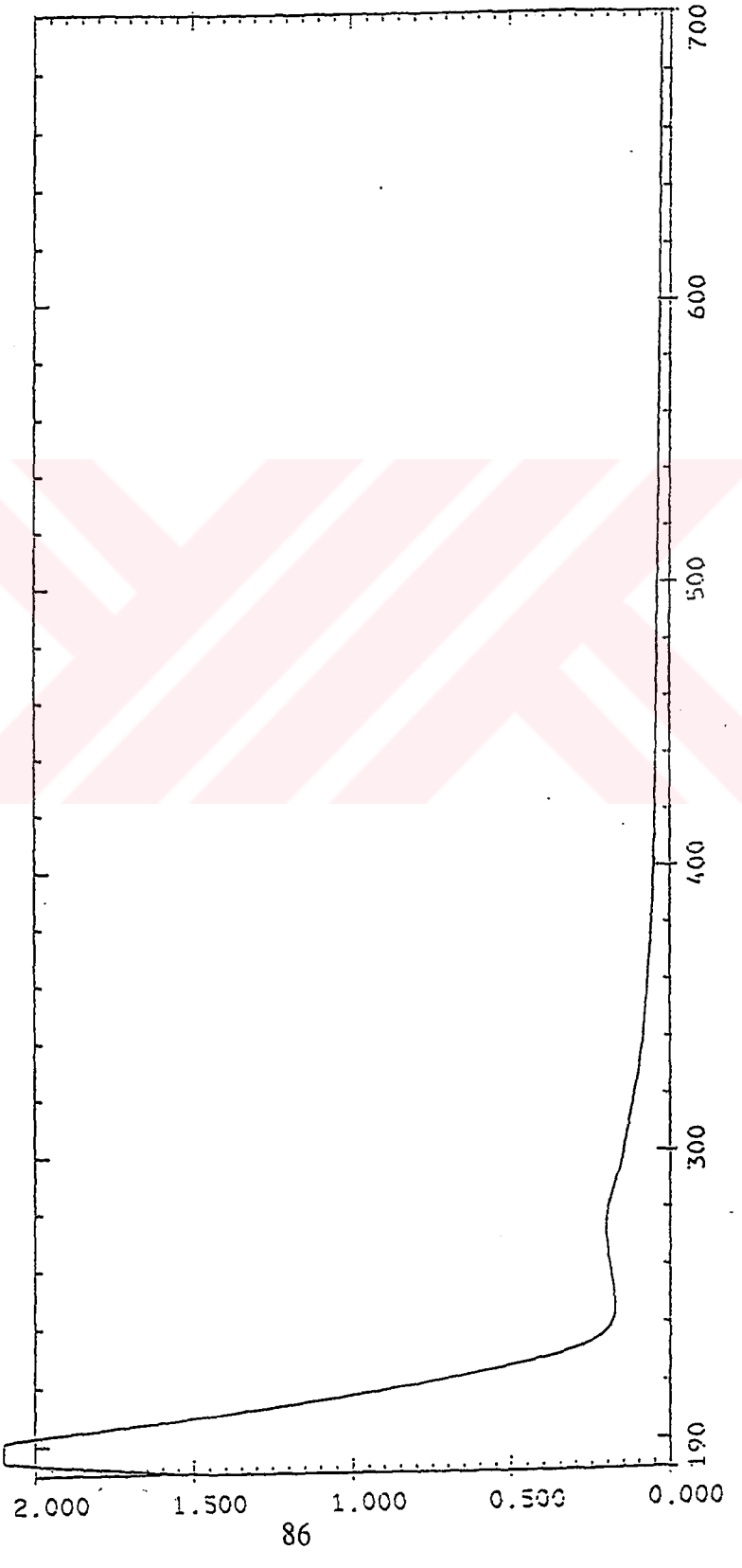


Figure B.2. UV-Scanned aliquot of the control flask, containing 115 ml Saboraud Broth and 35 ml lindane, involved in fourth cycle experiments

APPENDIX B-3

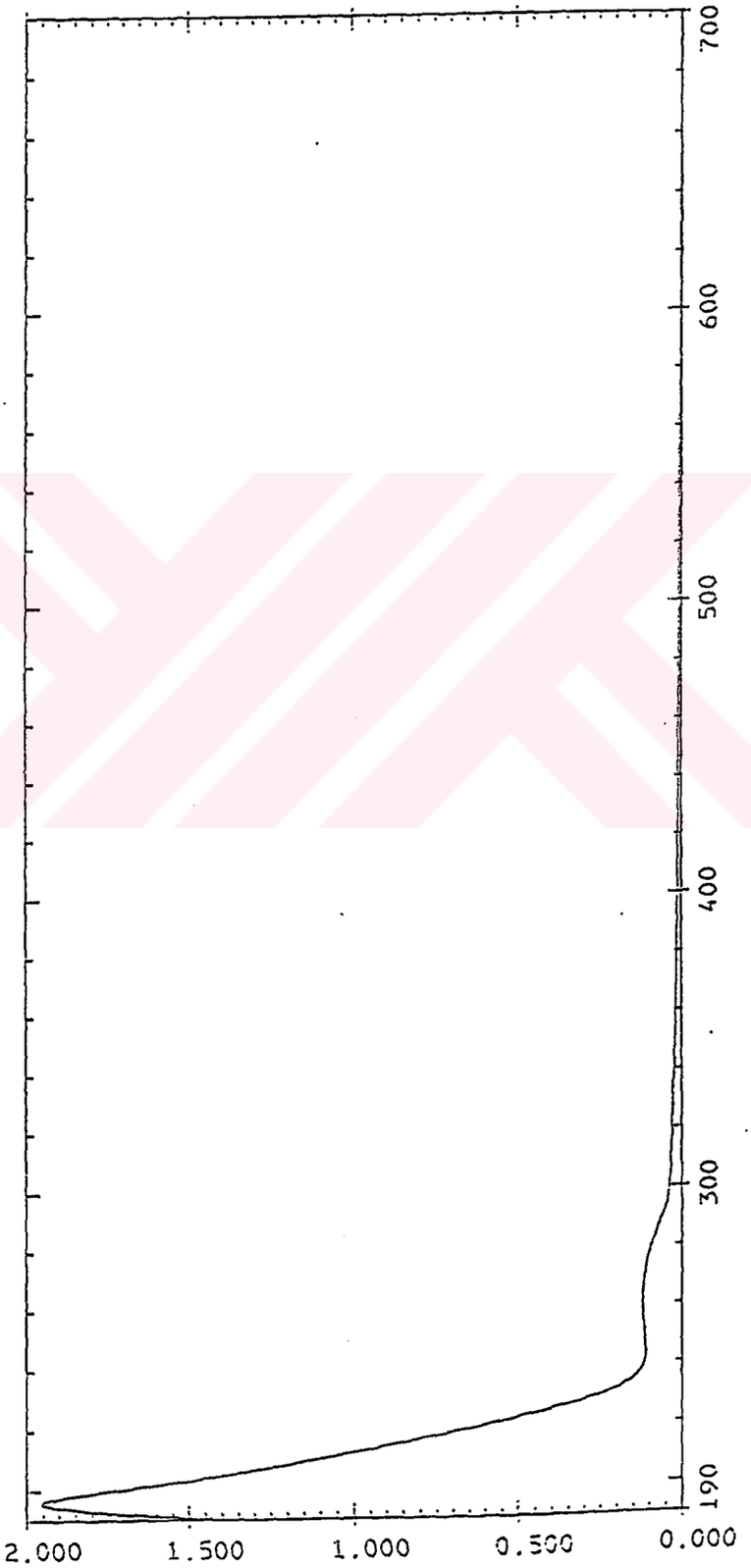


Figure B.3. UV-Scanned aliquot of the control flask, containing 500 ml Saboraud Broth, involved in fourth cycle experiments

APPENDIX B-4

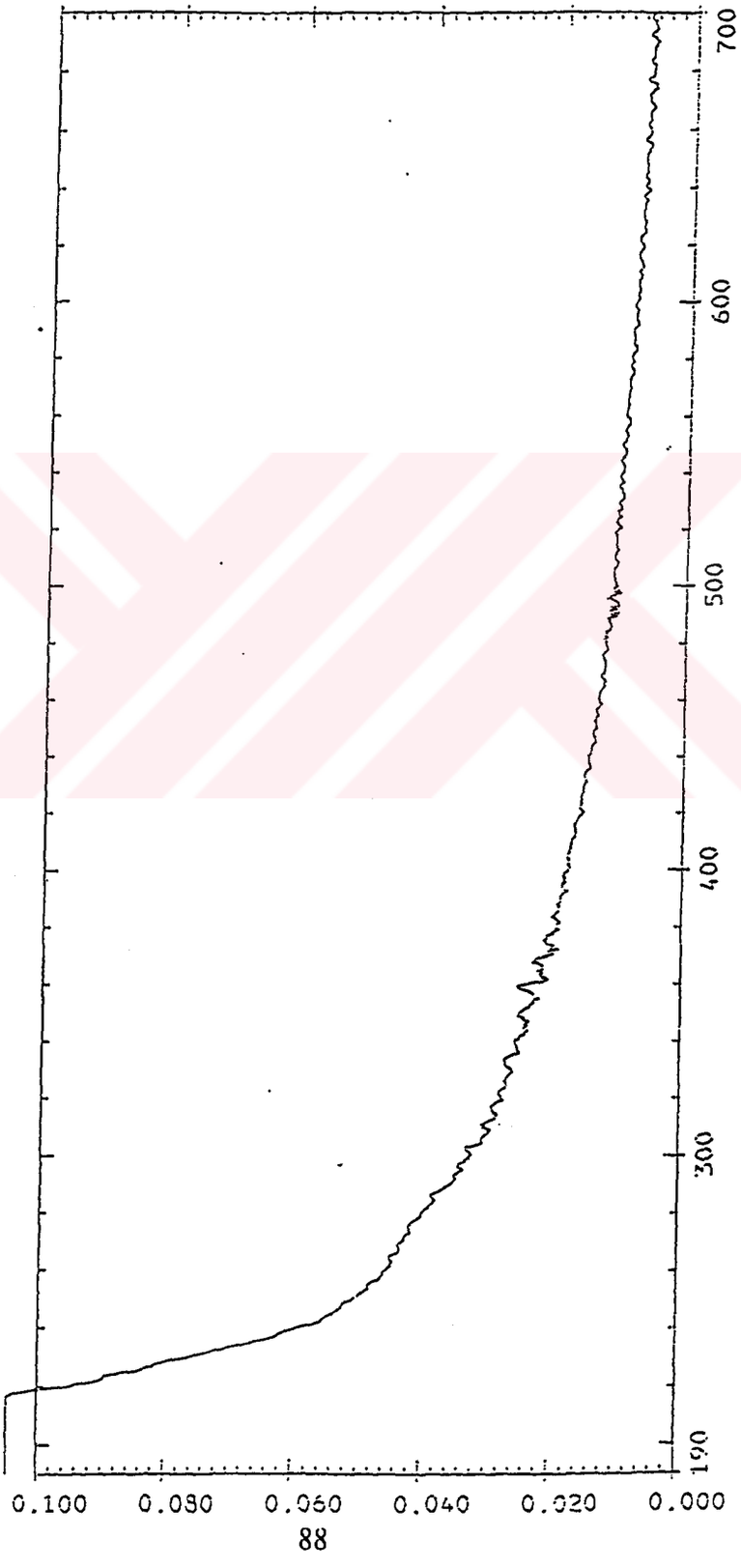


Figure B.4. UV-Scanned aliquot of the control flask, containing 500 ml Lindane, involved in fourth cycle experiments

APPENDIX B-5

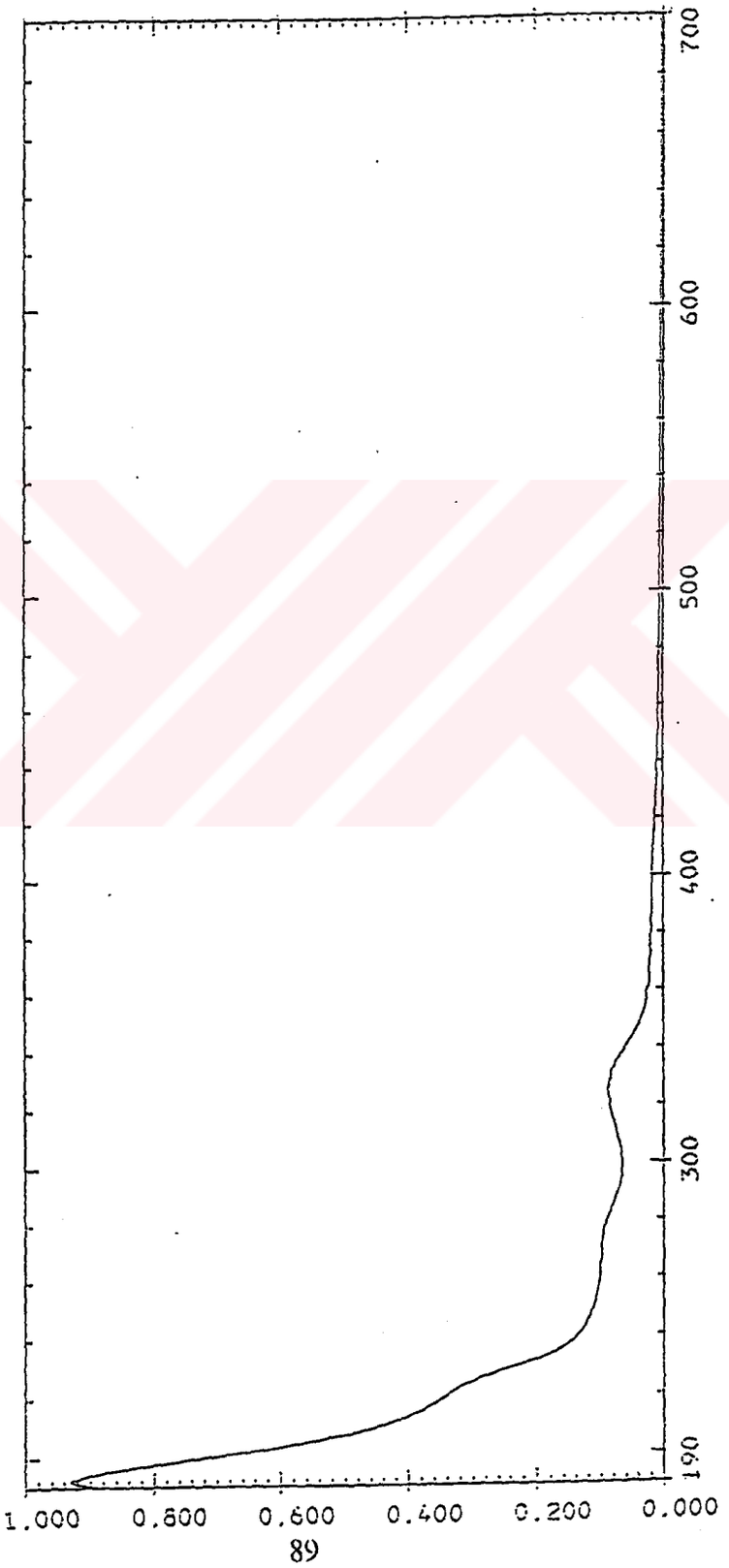


Figure B.5. UV-Scanned aliquot of the control flask containing incubated 2 ml P. chrysosporium + 115 ml Saboraud broth + 35 ml lindane, involved in fourth cycle experiments

APPENDIX B-6

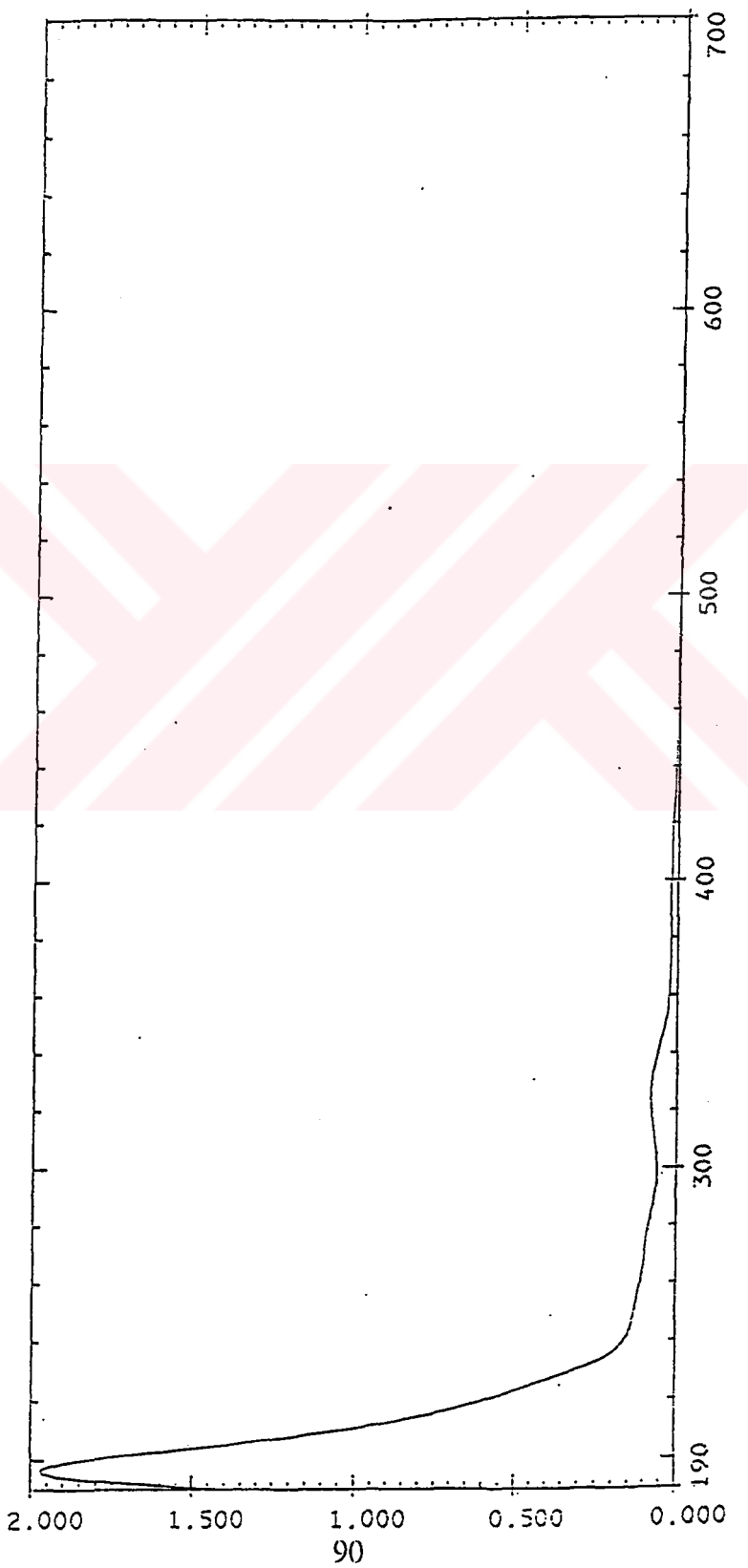


Figure B.6. UV-Scanned aliquot of the control flask containing incubated 2 ml T. versicolor + 115 ml Saboraud broth + 35 ml lindane, involved in fourth cycle experiments

APPENDIX B-7

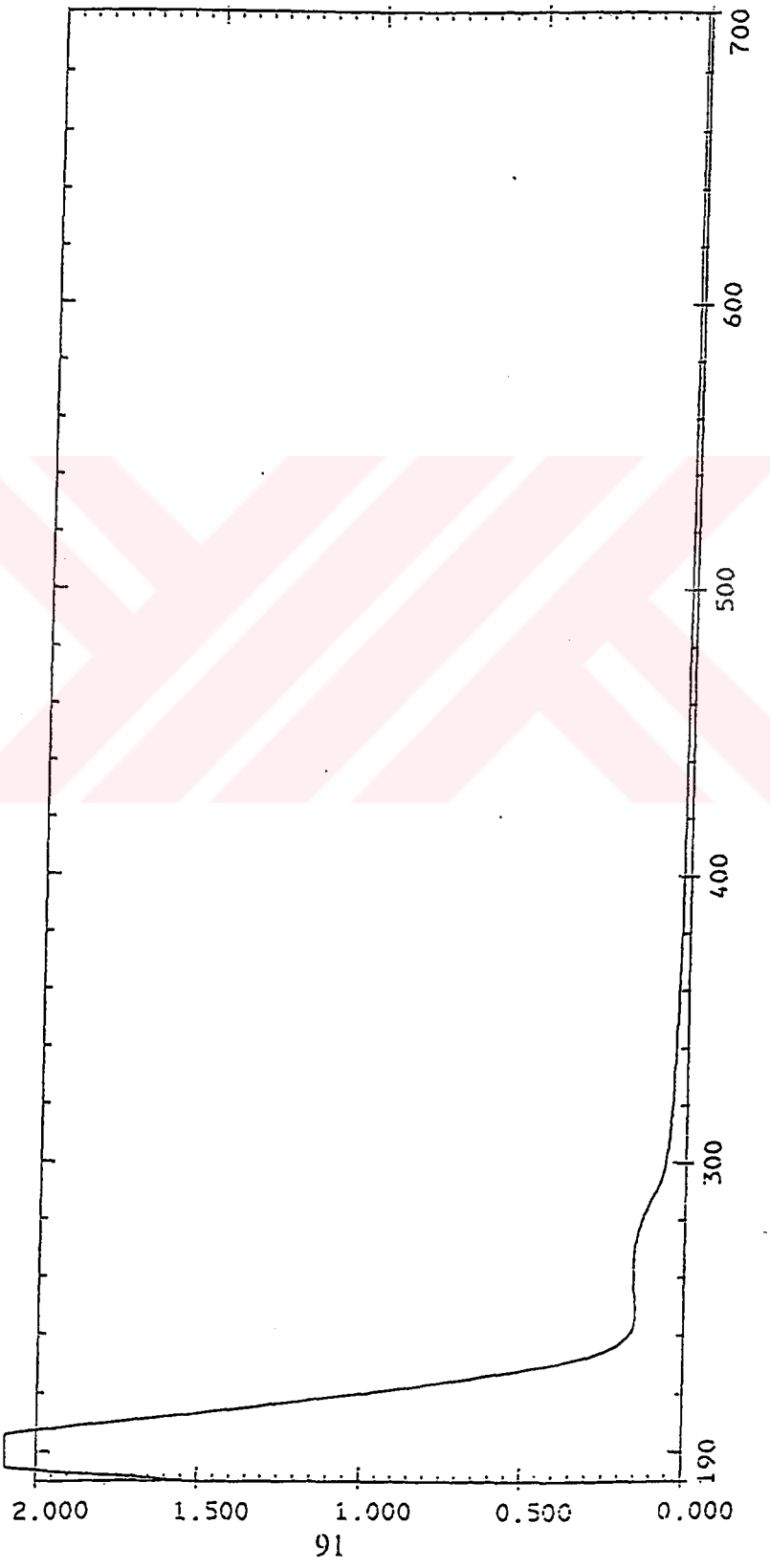


Figure B.7. UV-Scanned aliquot of the control flask containing static grown P. chrysosporium (2 ml) + 115 ml Saboraud broth + 35 ml lindane, involved in fourth cycle experiments

APPENDIX B-8

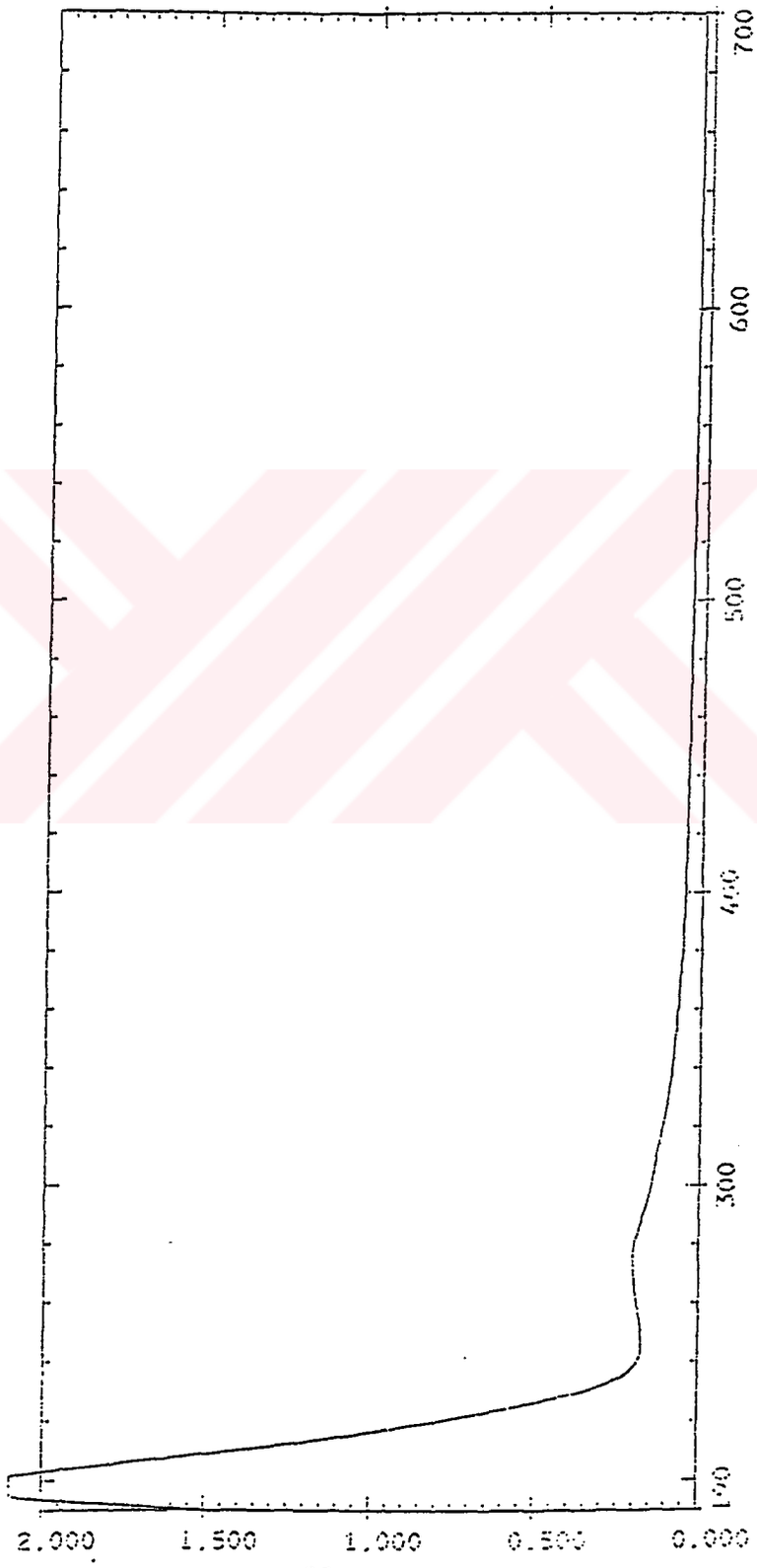


Figure B.8. UV-Scanned aliquot of the control flask containing static grown T. versicolor (2 ml) + 115 ml Saboraud broth + 35 ml lindane, involved in fourth cycle experiments

APPENDIX B-9

Tablo B.9. Absorbances Detected at UV-Scanning Analysis

Sample	(nm)	Absorbance
Saboraud + Lindane	198,5 274,0	2,238 0,129
Lindane	Undefined	Undefined
Saboraud Broth	201,0 263,5	2,394 0,158
Incubated P. chrysosporium	192,5 269,0 324,5	0,039 0,097 0,086
Static P. chrysosporium	196,0 258,0	1,952 0,118
Incubated T. versicolor	196,5 322,5	1,967 0,078
Static T. versicolor	198,5 273,5	2,237 0,201
Undiluted Static T. versicolor	Undefined	Undefined

APPENDIX B-10

Tablo B.10. Growth Measurement for P. chrysosoporium and T. versicolor detected in the Fourth Cycle Experiments

Day	Organism	Biomass (µg/150 ml)
1	P. chrysosporium	1,97
1	T. versicolor	1,33
10	Static P. chrysosporium	111,8
10	Incubated P. chrysosporium	65,7
10	Static T. versicolor	125
10	Incubated T. versicolor	85,5