Copyright Warning & Restrictions

The copyright law of the United States (Title 17, United States Code) governs the making of photocopies or other reproductions of copyrighted material.

Under certain conditions specified in the law, libraries and archives are authorized to furnish a photocopy or other reproduction. One of these specified conditions is that the photocopy or reproduction is not to be "used for any purpose other than private study, scholarship, or research." If a, user makes a request for, or later uses, a photocopy or reproduction for purposes in excess of "fair use" that user may be liable for copyright infringement,

This institution reserves the right to refuse to accept a copying order if, in its judgment, fulfillment of the order would involve violation of copyright law.

Please Note: The author retains the copyright while the New Jersey Institute of Technology reserves the right to distribute this thesis or dissertation

Printing note: If you do not wish to print this page, then select "Pages from: first page # to: last page #" on the print dialog screen

Order Number 9322431

Investigation of reactor design parameters towards optimizing biodegradation of chlorophenols by *Phanerochaete chrysosporium*

Pal, Nirupam, Ph.D.

New Jersey Institute of Technology, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Investigation of Reactor Design Parameters Towards Optimizing Biodegradation of Chlorophenols by *Phanerochaete chrysosporium*

by
Nirupam Pal

A Dissertation
Submitted to the Faculty
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

Department of Chemical Engineering, Chemistry,
and Environmental Science

January, 1993

APPROVAL PAGE

Investigation of Reactor Design Parameters Towards Optimizing Biodegradation of Chlorophenols by *Phanerochaete chrysosporium*

Nirupam Pal

Dr. Piero M. Armenante, Associate Professor
Department of Chemical Engineering, Chemistry and Environmental Science
New Jersey Institute of Technology

Dr. Gordon Lewandowski, Professor and Chairman
Department of Chemical Engineering, Chemistry and Environmental Science
New Jersey Institute of Technology

Dr. Basil C. Baltzis, Associate Professor
Department of Chemical Engineering, Chemistry and Environmental Science
New Jersey Institute of Technology

Dr. Dimitri Petrides, Assistant Professor
Department of Chemical Engineering, Chemistry and Environmental Science
New Jersey Institute of Technology

Dr. David Kafkewitz, Associate Professor
Department of Biological Sciences
Rutgers University

ABSTRACT

Investigation of Reactor Design Parameters Towards Optimizing Biodegradation of Chlorophenols by *Phanerochaete chrysosporium*

by
Nirupam Pal

The biodegradation of 2,4,6-trichlorophenol (246-TCP) and 2,4,5-trichlorophenol (245-TCP) by *Phanerochaete chrysosporium* was studied in batch and in continuous systems. Contrary to most of the previous reports from the literature, this study shows that degradation of both TCPs can occur in the absence of any measurable ligninase activity. The microorganism did not retain its degradative ability for more than about two weeks.

In order to better understand the degradation process, the individual contributions of both the biomass and the extracellular proteins were studied separately. The results show that neither the biomass nor the extracellular proteins alone can completely degrade 246-TCP, but both are required for complete degradation to occur. In addition, it was found that the rate of degradation is directly proportional to the concentration of the total extracellular protein produced by the fungus. The extracellular enzyme system (other than ligninase) responsible for degradation has a life time of 32 to 45 hours (depending upon the pH of the system). On the basis of these observations, a reaction scheme for the degradation process is proposed in which 246-TCP is first attacked by an extracellular protein (enzyme) secreted by the fungus, described by a Michaelis-Menten kinetic expression, and then finally degraded

by the cell bound protein (enzyme). The kinetic parameters were determined in continuous reactor experiments and successfully tested for other configurations.

Optimal operating parameters were determined for a packed-bed continuous reactor. Degradation of phenol and pentachlorophenol were also studied for comparative purposes.

BIOGRAPHICAL SKETCH

Author: Nirupam Pal

Degree: Doctor of Philosophy

Date: January, 1993

Undergraduate and Graduate Education:

- Doctor of Philosophy in Chemical Engineering, New Jersey Institute of Technology, Newark, NJ, 1993
- Master of Science in Chemical Engineering, University College of Science and Technology, Calcutta University, India, 1986
- Bachelor of Science in Chemical Engineering, University College of Science and Technology, Calcutta University, Calcutta, India. 1985
- Bachelor of Science with honors in chemistry, Krishnagar Government College, Calcutta University, Krishnagar, Nadia, India, 1981

Presentation and Publications:

- Biodegradation of Trichlorophenols by *Phanerochaete chrysosporium*- Minitech Competition, April 1992, Newark, NJ, jointly organized by American Institute of Chemical Engineers (AICHE),Society of Plastics Engineers (SPE) and Society for Advancement of Materials and Process Engineering (SAMPE).
- Reactor Modeling for Biodegradation of Hazardous Wastes Utilizing a White Rot Fungus - October 1992, North-Eastern State Conference of Waste Water Treatment, Atlantic City
- Poisoning of Hollow Catalyst Pellets by Parallel Poison Forming Reactions-Indian Institute of Chemical Engineers, Annual Conference, 1986.

Major: Chemical Engineering

This thesis is dedicated to my beloved sister
the late Chanda Pal

ACKNOWLEDGMENT

The author wishes to express his sincere gratitude to his advisors, Professor Gordon A. Lewandowski and Professor Piero A. Armenante, for their guidance, friendship, and moral support throughout this research.

Special thanks to Professors Dimitri Petrides, David Kafkewitz and Basil C. Baltzis for serving as members of the committee.

The author appreciates the timely help and suggestions from fellow graduate students S. Dikshitulu, Z. Shareefdeen, K.Wang, S. Ioannidis A. Gami, T. Poncet, J.Wang; members of Hazardous Substance Management Research Center (HSMRC), C. Brockway and G. San Augustin; and Y. Gandhi of the chemistry stock room.

And finally, the author would like to acknowledge the immense moral support and help from his wife Susmita and parents throughout this research without which it would never been possible to continue his studies.

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **PAGE**

LIST OF CALIBRATION CURVES

NOMENCLATURE

C =toxic compound

E= enzyme

E_B=cell bound enzyme

P=products of degradation

D=intermediates produced by breaking the enzyme-substrate complex

C_{in} = concentration of toxic compound at the reactor inlet or the initial concentration for a batch reactor (ppm)

C_{out} = concentration of toxic compound at the reactor outlet (ppm).

E_1= concentration of the particular enzyme responsible for the degradation (ppm)

E_1C = concentration of the enzyme substrate complex (ppm)

E_1^* = total concentration of the enzyme responsible for the degradation.

E_T= total measured protein concentration (ppm)

V_{max}^* = kinetic constant based on the particular enzyme responsible for degradation

V_{max} = kinetic constant based on total enzyme concentration (1/h)

K_m = kinetic constant (ppm)

τ = residence time in a CSTR (h)

t= any instant of time starting from initial time 0 (h)

NOMENCLATURE USED IN TABLES

Glu= glucose concentration (ppm)

Prot= protein concentration (ppm)

Cl = chloride Ion concentration (ppm)

Lig= ligninase activity measured by increase in absorbence in 5 minutes (AU)

Cont = concentration of the toxics in control experiment(ppm)

N = $NaNO_3$ measured as nitrogen (ppm)

R.T.= retention time in packed-bed reactor (h)

R.R. = recirculation rate in packed-bed reactor (ml/min)

BM = biomass Concentration (ppm)

D.O. = dissolved oxygen concentration (ppm)

246 = 2,4,6-trichlorophenol (ppm)

245 = 2,4,5- trichlorophenol (ppm)

PCP = pentachlorophenol concentration (ppm)

Prod = model predicted data

Exp = experimental data

CHAPTER 1

INTRODUCTION

Bioremediation is the use of organisms to improve environmental quality by taking advantage of their ability to treat toxic, hazardous or merely offensive compounds at contaminated sites or at the source of the contamination. This natural process has been used for decades to treat wastes such as municipal sewage and effluents from industrial processes such as oil refining and chemical manufacture. It is emerging as an extremely attractive alternate technology for the economical treatment of a wide range of environmental contaminants. Biodegradation can decompose waste products and hazardous chemicals into water, carbon dioxide, biomass or other innocuous products, rather than simply moving the contaminants from one site or medium to another.

Among xenobiotic compounds, chlorophenols and their derivatives are extensively used as insecticides, fungicides and herbicides for industrial and agricultural purposes throughout the world. All these chlorinated aromatics are listed hazards according to the Environmental Protection Agency (EPA), and many of them are proven or suspected carcinogens (62). The toxicity of these chemicals increases with increase in degree of chlorination. Because of its potent nature, study of chlorophenols is given immense importance. Furthermore, many chloroaromatics are biodegraded via a chlorophenol route (62). So, by studying the biodegradation of chlorophenols, a number of chloroaromatics can also be considered indirectly for biodegradation. In this study, 2,4,6-trichlorophenol (246-TCP) and 2,4,5-trichlorophenol (245-TCP) are the selected model compounds.

Due to the presence of a stable benzene nucleus, the chlorinated phenols have proven to be environmentally persistent. Numerous bioremediation strategies have been developed which use both aerobic and anaerobic microorganisms to treat this group of chloroaromatics. Activated sludge processes, anaerobic digestion, aerated lagoons, trickling filters, rotary biological contactors, land farming and aerobic composts are examples of such systems (25,49). However, only partial success has been achieved. In many cases only a few of the isomers of a particular compound have been degraded. Furthermore, with increasing degree of chlorination biodegradation becomes more and more difficult.

Phanerochaete chrysosporium, can degrade halogenated compounds in a somewhat non specific manner. *P. chrysosporium* is a distant relative of edible mushrooms. But unlike mushrooms, this type of basidiomycete can be seen by the naked eye only when cultured in huge numbers and coalesced into white, paper-like mats. As a result, the organism, a voracious devourer of dead wood, is known as a white rot fungus.

P. chrysosporium was isolated in the sixties by a group of mycologists in the former Soviet Union. To access the cellulose in dead wood, this fungus secretes a system of enzymes to disintegrate the lignin. In the last decade, studies showed that the fungus is capable of mineralizing different aliphatic and aromatic compounds and could be of immense use for biodegradation purposes of different xenobiotic compounds.

There are some definite advantages of using this fungus over other microorganisms. The fungus can work over a wide range of temperature and pH, compared to other studied bacterial processes where the microorganisms are very much pH and temperature dependent (14). The fungus is not very compound specific and could be used for a wide variety of pure and mixed

substrates. Since the degradation process is an enzymatic process, a very low concentration of compound can be treated. However there are some particular drawbacks in the use of *P. chrysosporium*. The specific growth rate of this fungus is slower than many bacterial species (49). The pollutants can not be used as the primary carbon source for this fungus. Another carbon source must be used. Moreover, this fungus is sensitive to shear stress and can not be used in a conventional CSTR type reactor. However, all the above problems can be overcome by proper selection of reactor configuration and operating parameters, as shown in this study.

The purpose of this research was to explore and optimize the reactor configuration and design which is best suited for biodegradation of two recalcitrant model compounds (2,4,6-trichlorophenol and 2,4,5-trichlorophenol) using *P. chrysosporium*. This study also aimed at revealing the degradation scheme, followed by kinetic modeling. Degradation of phenol and pentachlorophenol were also studied for comparative purposes considering an industrial effluent that may contain a mixture of many chlorophenols and phenol.

CHAPTER 2

LITERATURE REVIEW

The white rot fungus *Phanerochaete chrysosporium*, a wood decaying basidiomycete, is a voracious devourer of dead wood. The hyphe of this White Rot Fungus are able to penetrate wood by virtue of their ability to degrade lignocellulosic material. The resulting decay causes the wood to become a light colored spongy mass containing white pockets or streaks (white rot) separated by thin areas of firm wood.

The fungus was originally noted for its lignin degrading ability. Lignin is a complex, three dimensional structure consisting of methoxylated and non-methoxylated aromatic rings joined by carbon-carbon and carbon-oxygen bonds, having a molecular weight ranging from 600 to 1000 kilo daltons. The lignin structure can also contain chlorinated molecules, excreted by the plant body and accumulated in plant bark (18). Although the subject of intense research for many years, details of the mechanism of lignin biodegradation have only recently been established. It was found that the fungus was able to release a system of extracellular enzymes that are effective lignin degraders. By the late 60's and 70's work in a number of laboratories had shown that lignin degradation is a non specific, extracellular, oxidative process initiated by nutrient nitrogen, carbohydrate or sulfur starvation (39). Studies utilizing the fungus to degrade aromatic compounds were initiated because of their similarity to lignin (18). In 1980, Crawford (12), published an extensive survey of compounds related to lignin.

In 1983, Leatham and co-workers (41) showed that this fungus was able to degrade phenolic compounds in a non-specific manner. They reported that a

mutant strain of *Phanerochaete chrysosporium* (POL 88) could degrade a variety of phenolic compounds. Out of 36 chemicals tested, 16 were degraded 50% and the other 28 compounds were degraded about 20% in less than three days. They reported that all these phenolic compounds were degraded via a muconic acid route.

Phanerochaete chrysosporium was used by Huynh et al (31) to degrade chlorinated organics in a wastewater treatment system. Although the concentration they treated was lower than 10 mg/l, most of the chlorinated phenols and low molecular weight components were removed. They also noticed that veratryl alcohol was the major end product, and concluded that the mechanism involved methylation, oxidation, and reduction in successive stages.

Bumpus et al. (7,8,10) reported the ability of *Phanerochaete chrysosporium* to mineralize recalcitrant organohalides, namely lindane (hexachlorocyclohexane), polychlorinated dibenzo(p)dioxins, DDT (1,1-bis 4-chlorophenyl) 2,2,2-trichloroethane and polychlorinated biphenyls. They observed that degradation persisted for more than 50 days, while its rate decreased in exponential fashion with time. They found that degradation is most favorable in nitrogen-limited conditions.

Bumpus et al. (9) showed that *Phanerochaete chrysosporium* was able to mineralize pentachlorophenol (PCP). This fungus was able to degrade 12 ppm of PCP to below detectable limit within 35 hours. Moreover, they found that the fungus was able to survive at 500 ppm of PCP. However, it must be mentioned here that the solubility of PCP in neutral water is around 30 ppm, and no mention was made how a 500 ppm solution was made. No control experiments were cited in this study.

A number of studies were conducted to determine the particular enzyme primarily responsible for these degradation processes. The studies concluded

that lignin peroxidase (or ligninase) was the key enzyme, and that degradation occurs via a free radical mechanism (1 - 4, 8,15 to 17, 19,20,22 - 25,30,34,36,39,41,44 - 55,58 - 60).

In 1985, Sanglad and his co-workers (63) reported rapid degradation of benzo(a)pyrene to carbon dioxide by the fungus. They attributed this degradation to ligninase only, although they used whole cells in their experiments. However, they also reported that this type of degradation occurred in the absence of ligninase with a lag phase of about 10 to 12 hours. Thus they themselves contradicted their opinion on the validity of ligninase in these degradation processes. They observed three intermediates, and concluded that a C_α-C_β cleavage occurred.

2.1 Studies of Different Strains of *Phanerochaete chrysosporium*

In the early 80's the enzymatic activity of the fungus attracted many workers to find the best suitable strain for biodegradation purposes, by separating from the wild type as well as by genetic mutation. By this time it was strongly believed that ligninase or lignin peroxidase was the sole responsible enzyme for degradation of both lignin as well as the toxic compounds. In 1986, Kirk and Tien (38) did a comparative study on three wild types, namely ME-446, K-3 and BKM-F-1767. Three cellulose negative mutants were derived from K-3 (called 3113, 13132-176, 85118-22), and an other mutant was derived from BKM-F-1767 (namely SC-26). They showed that SC-26 has the ability to produce the maximum amount of ligninase. They also found that all three cellulose negative strains also degraded lignin to CO_2 when no ligninase activity was detected. They suggested that either ligninase activity was not obligatory for lignin degradation or ligninase concentration is so low that it could not be detected. This was the first time that the effectiveness of ligninase was questioned,

however reports continued to be published proclaiming that ligninase is the solely responsible enzyme.

In 1990, Munheim et al.(53) studied another species of white rot fungus *Bjerkandera adusta* along with *Phanerochaete chrysosporium*. They detected aryl alcohol oxidase from both species which can oxidize veratryl alcohol to veratryl aldehyde, and they found that the optimum pH is around 5.7. They showed that aryl oxidase can not work in the absence of oxygen, while ligninase can.

Although the effectiveness of ligninase in the degradation purpose was highly questionable, investigators continued to mutate the fungus to overproduce the lignolytic enzymes. Orth et al. (54) in 1991 reported that a mutant of *Phanerochaete chrysosporium* (PSBL-1) can produce 4 to 10 times more lignolytic enzymes than the wild type. However, enhancement in enzyme production was never tested for any degradation purpose and the effectiveness of such mutants was never tested.

Ulmer et al.(68) found the lignin degradation rate by ^{14}C labeled lignin for the case of *Pleurotus ostreatus and Pycnoporus cinnabains 115* are the same and quite comparable to that of *Phanerochaete chrysosporium,* although further studies with these species were not reported.

In 1988, Burgos et al. (6) studied another strain of soft rot fungus named *Lecythophora hoffmannii* for degradation of nine phenolic compounds and found that this fungus can degrade low molecular weight aromatic compounds via the protocathechuic acid route at a rate compared to that of *Phanerochaete chrysosporium.* Moreover initial studies showed that the aromatic compounds are metabolized by the fungus via the β-ketoadipate pathway. An advantage of this fungus is that the activity is not affected by concentration of nitrogen source (unlike *Phanerochaete chrysosporium).*

2.2 Parameters Affecting Degradation

For *Phanerochaete chrysosporium* the media requirement for growth is different from that of the degradation phase. Although questions were raised regarding the singular importance of ligninase in the degradation process, most studies sought to maximize ligninase production by affecting the concentration of the primary carbon source (generally glucose), nitrogen, oxygen, trace nutrients and surfactants. The effect of shear-stress was also investigated.

2.2.1 Effect of Nitrogen

The concentration of nitrogen was found to be the most important parameter for lignolytic activity. Kirk and his co-workers (39) studied the effect of various parameters during lignin degradation in batch culture. They observed that the source of nitrogen had little effect on lignin degradation. The fungus could utilize ammonium salts, nitrates or amino acids as the nitrogen source. However, the concentration of nitrogen is very important. At higher nitrogen concentrations, the degradation rate decreased. At 24 mM nitrogen concentration, the degradation rate was 30% of that observed at 2.4 mM. They also observed that to degrade each 5 mg of lignin, 100 mg of glucose was metabolized. This observation also indicates that lignin degradation is an energy consuming process.

Reid (58,59) extensively studied the effect of nitrogen on degradation of Aspen wood lignin. He found that simple nitrogen sources like NH_4Cl, urea and aspergine inhibited lignin degradation, while low concentrations of NH_4NO_3 increased lignin degradation (although according to Kirk (39) the source of nitrogen is immaterial). This is another contradiction between the different reports. Reid also observed that secretion of secondary metabolites, like

veratryl alcohol, occurred at higher rates when the nitrogen concentration was low.

Buswell et al. (11) obtained very high levels of lignin-degrading enzymes from the mutant *Phanerochaete chrysosporium* INA-12, under non-limiting conditions of nitrogen, (although nitrogen limitation was prerequisite for the onset of significant lignin degradation). This again contradicts the reports by both Kirk (37,39) and Reid (58,59), as they support that production of ligninase requires nitrogen limited condition. Buswell et al. (11) concluded that degradation was inhibited by high concentrations of nitrogen, but the production of secondary metabolites were not affected. They reported that use of glycerol instead of glucose induced this secondary metabolism as the glycerol metabolization rate was very low. Other secondary metabolites were also produced by this mutant fungus. However, how this affected the degradation rate of lignin or any other compound was not reported.

From the observations and conclusions of Kirk (39), Reid (58,59) and Buswell (11) it looks like as though the effectiveness of ligninase has not been throughly studied. If the above observations are correct, the other explanation of this inhibitory effect may be that the nitrogen source works as an competitive inhibitor for the enzyme and binding could be irreversible. Thus the concentration of nitrogen or most likely the type of nitrogenous compound is related to the mechanism of enzymatic degradation.

Faison and his co-workers (21) reported higher degradation rates with both nitrogen and carbohydrate limited sources. They observed maximum ligninase activity 24 to 36 hours after the depletion of nitrogen source. Moreover they also observed that the concentration of lignocellulosic material induced the production of ligninase, suggesting that lignin degradation is inducible by lignocellulosic material. They also reported that mycelium growth ceases 36 to

48 hours after inoculation, and after an additional 36 hours, the ligninase activity and lignin degradation begin. Addition of glutamate or NH_4Cl strongly suppressed the ligninase system as well as lignin degradation. This contradicts the previous reports (39,58,59,11) and may be an indication that the nitrogen source may actually bind with the enzymes and inhibit enzymatic reactions.

Jager et al.(32) studied the effect of nitrogen concentration in agitated cultures during lignin degradation. They observed that higher nitrogen concentration delayed or completely suppressed the ligninase activity. They concluded that degradation of lignin and appearance of ligninase activity were both associated with growth limitation. Depletion of nutrient nitrogen triggered the onset of iodophase, when secondary metabolism starts. This contradicts the findings by Buswell et al. (11), who reported that the nitrogen source did not suppress lignolytic activity.

As described above, many workers focused on ligninase activity as the key factor in degradation of lignin and other toxic compounds. As a result they also focused on the importance of low nitrogen concentration to increase ligninase activity. However, recently the effect of nitrogen on degradation has become a debatable issue. In 1988, Mileski (51) and his co-workers found that considerable degradation of pentachlorophenol (PCP) occurs in a nitrogen rich cultures. Moreover the degradation due to excretion of ligninase was not enough to explain the phenomena. They concluded that the presence of a mechanism other than ligninase may exist.

More recently (1991), Orth et al.(54) found high production of ligninase from a mutant of *Phanerochaete chrysosporium* -PSBL-1 in nitrogen rich medium. According to this study, the fungus secretes a family of enzymes while in the log phase of growth. The activity of the enzymes were 4 to 10 fold higher than that of the wild type *Phanerochaete chrysosporium.* However, the

degradation rate with the mutant fungus was not reported. This report contradicts the conclusion by Buswell et al.(11).

Janshekar et al.(33) studied bioalteration of Kraft lignin, and used chemostats for the purpose of their study. They concluded that lignolytic activity can not be attributed to nitrogen limitation, as considerable degradation was observed in the presence of nitrogen. According to Fenn et al. (22), ligninase suppression occurs above 0.7 mM of nitrogen, which again contradicts many previous reports about the effectiveness of ligninase and its relation to nitrogen concentration.

Yang et al.(71) showed that ammonium nitrate plus aspergine at low concentrations stimulated lignin degradation by *P. chrysosporium* in red alder thermochemical pulp, but high doses inhibited lignin degradation. However, there are no quantitative estimations of the nitrogen concentration at which inhibition occurred.

2.2.2 Effect of Carbohydrates

Kirk et al.(37,38,39) demonstrated that carbohydrates are necessary growth substrates for decomposition of lignin by white rot fungus. Carbohydrates such as glucose, cellulose, cellobiolose and zylos can serve as primary carbon sources. Kirk reported (39) that low glucose concentration enhances ligninase activity. However, a glucose concentration below 0.1% suppressed the ligninase activity. Reid et al.(59) showed that the culture stopped mineralizing when the carbon/energy source was depleted. A similar observation was made by Leisola and his coworkers (44) who reported that enzyme production ceases in a carbohydrate depleted environment. Janshekar et al. (33) reported that limitation of carbohydrate and sulfur triggered ligninase activity by the fungus.

Faison et al.(21) observed a strange phenomenon during degradation of lignin by *Phanerochaete chrysosporium*. They observed that the lignin degrading system was induced by lignocellulosic material although the lignolytic system was considered non-inducible by lignin. However, they observed a marked increase of ligninase activity and H_2O_2 production when pre-incubated with birch lignin. Nevertheless, they concluded that it is unlikely that lignin could act as an inducer; its size and insolubility precludes its crossing the cytoplasmic membrane to interact with DNA. Thus, if true induction is involved, the actual effect would most plausibly be production of a soluble, low-molecular weight compound derived from or related to lignin. This observation indicates lignin as a possible secondary carbon source for the fungus.

Miranda and his co-workers (50) made a similar observation to Faison et al (21). They used ^{14}C labeled lignin to study the mycelium binding and depolymerization. They observed higher degradation rates to a concentration of 1 mg of lignin/ mg of protein. They concluded from their result that lignin was not bound, depolymerized or oxidized to CO_2. They also concluded that the entire lignolytic system is a secondary metabolite.

2.2.3 Effect of Oxygen

Kirk et al.(39) showed that the rate of degradation of lignin was enhanced by two to three fold when pure oxygen (100%) was used insted of air (21% oxygen). The degradation rate became practically zero when 5% oxygen was used.

Dorosetz et al.(17) studied the effect of oxygenation conditions on submerged cultures for degradation of lignin by *Phanerochaete chrysosporium*. They used periodic flushing, continuous flushing, and continuous bubbling with both oxygen and air; and measured glucose metabolization rate as the indicator for the oxygen utilization rate. They concluded that continuous flushing and

bubbling with pure oxygen resulted in the maximum glucose consumption rate in the iodophase. However, no significant effect was observed during the growth phase for the first two days. Moreover, in all cases use of 100% oxygen gave higher metabolization rate compared to that with air. They also concluded that the oxygenation condition has a direct regulatory effect on the production and decay of lignolytic enzymes. Although an increase in oxygen tension increased the level and rate of formation of ligninase and Mn-peroxidase, it also enhanced their decay, associated with faster substrate depletion and temporal increase of iodophasic protease activity. They also observed that submerged cultures with continuous flushing with air resulted in a completely ligninase negative system, but considerable degradation of lignin was observed.

Explicit studies of mass transfer limitation during high fungal biomass concentration could not be found in the literature. However, Ulmer et al.(68) proposed that oxygen transport may be affected in a concentrated slurry. In line with these observations, Leisola et al. (43) observed the degradation rate in slightly agitated cultures to be twice as effective as in non-agitated cultures.

2.2.4 Effect of Trace Nutrients

Kirk et al. (38,39) suggested that enzyme production could be increased by addition of excess trace metals such as manganese, iron and zinc to nitrogen starved cultures. The reason for enhancement may be attributed to the mechanism of this degradation process, where a single electron mechanism (via a reduced oxygen species) has been proposed via H_2O_2, and cytochrome plays a part in that mechanism. They also proposed that copper may increase the enzyme activity without any supporting evidence.

2.2.5 Effect of pH

The pH of the system was found to be an important factor for optimizing both growth and degradation rate. As found by Kirk et al.(37), the optimal pH for lignin degradation was approximately 4.5, with substantial suppression of decomposition below 3.5 and above 5.5. However, the optimal pH for growth is somewhat higher than for lignin degradation. Aitken et al.(1) studied the degradation of 2-chlorophenol, o-cresol, 2-nitrophenol and pentachlorophenol at different pH. Qualitatively they observed a dramatic difference in removal rates at pH values 3.0 and 4.75. Higher initial rates of transformation were observed at pH 3.0 for the case of o-cresol. Initial rates of oxidation of veratryl alcohol were reported to increase as the pH decreases. For the case of ligninase, they reported a pK of 3.1. Thus, they concluded that ligninase is more stable at higher pH. While the initial transformation rate of toxics may be higher at pH 3.0, the overall removal rate would depend upon the life time of the ligninase.

Almost all of the degradation studies used a buffer solution, so very limited information regarding the effect of pH is available (2,6,37,38,39)

2.2.6 Effect of Agitation

The inhibition of microbial activity in high turbulence is a common phenomenon. Recently Toma et al. (67) presented an excellent study on inhibition of microbial growth and metabolism by excess turbulence. They termed this phenomenon *turbohypobiosis*. They studied four different bacteria and found a pronounced decrease in growth and biosynthesis at higher rpm for all species. However, the cutoff rpm varies from species to species. The main reason for this inhibition was shear effects causing decreased adenosine triphosphate (ATP) generation, lower O_2 uptake, and lower specific growth rate of bacteria.

As *Phanerochaete chrysosporium* is a filamentous microorganism the effect of *turbohypobiosis* is very pronounced for the fungus (30,55). The effect of

agitation on ligninase activity was also studied (30,35,39,44,55). Most of the studies were carried out in suspended cultures and showed that moderate to high agitation suppressed the ligninase activity (39) but a very gentle agitation enhanced the ligninase activity. Reid et al. (58,59) showed that cultures agitated on a gyratory shaker degraded lignin to carbon dioxide as effectively as static cultures. Similar results were obtained by Pak (55), who was able to obtain a substantial amount of degradation of 2-chlorophenol in cultures which were agitated on gyratory shaker. However, he was unable to obtain any significant degradation activity in stirred tank reactors.

Leisola et al. (43) reported that the extracellular H_2O_2-dependent ligninase activity of *Phanerochaete chrysosporium* was observed in agitated culture conditions when veratryl alcohol or veratryl aldehyde was added to the culture. However, no explanation was given by those investigators. Moreover veratryl alcohol itself is a secondary metabolite of this fungus. Thus induction by the alcohol did not seem to be a plausible explanation of their findings. Leisola and his co-workers (43) also reported that ligninase activity was completely suppressed at a high agitation speed.

In 1985 Faison and Kirk (21) studied the effect of different operating parameters on ligninase production. They found that ligninase activity was completely eliminated by agitating the growing cultures, which resulted in formation of mycelial pellets. Activity was also absent when a mat was allowed to grow before agitation.

In order to overcome the sensitivity of the ligninase production to agitation and shear stress, utilization of immobilized *Phanerochaete chrysosporium* spores both in agrarose and agar gel beads was studied by Linko et al.(47). They observed a 30% increase in ligninase activity in the beads. This contradicts the findings by Ulmer (68) who observed no ligninase production in a

culture continuously bubbled with air (whereas Linko and his co-workers observed increased ligninase production in continuously bubbled cultures).

Haq (30) found that *Phanerochaete chrysosporium* was not active when grown in suspension in a well agitated reactor. Although the fungus grows well, it exhibited no degradation activity. Although both Haq and Pak (30,55) claimed an increase in ligninase activity by immobilization, the enzyme activity measurement technique was incorrect and can not be used for any conclusive evidence. Moreover their results for the packed-bed reactor could not be duplicated with the same experimental techniques. The total mass balance and reactor characteristics used by Haq (30) were also incorrect.

2.2.7 Effect of Additives

Research to maximize degradation by addition of additives started in the late 80's. Different additives were used, namely veratryl alcohol, Tween 80, and other surfactants. However, the irony is that all the efforts were diverted to increase the ligninase production, which was believed to be the solely responsible enzyme, but these developments with additive additions were never tested for any real degradation activity.

2.2.8 Effect of Veratryl Alcohol

Harvey et al. (27) reported that the role of veratryl alcohol in lignin degradation was as an enzyme mediator. Veratryl alcohol was oxidized to a radical cation which was not rapidly degraded. Therefore it could act as a single electron oxidant. Faison et al.(21) extensively studied the role of veratryl alcohol on ligninase activity in *Phanerochaete chrysosporium*. They showed that out of six ligninase proteins from a strain of BKMF 1767, ligninase H2 had a several fold higher activity than ligninase H8, and the increase in ligninase activity by addition of veratryl alcohol was due to an increase in ligninase H2 production.

They observed a two-fold increase in ligninase activity by addition of 0.4 mM veratryl alcohol. Based on protein assay, they concluded that veratryl alcohol increases the ligninase activity by increasing the production of certain ligninase enzymes. As addition of both birch lignin and veratryl alcohol increased the ligninase activity, they suggested that lignin is converted to low molecular weight compounds, perhaps in part to veratryl alcohol itself, which stimulate enzyme secretion. However, according to Kirk et al.(37,38,39) the recognized relationship between veratryl alcohol biosynthesis and lignin degradation does not encompass the entire lignolytic system. Moreover, Liwicki et al. (48) have demonstrated that there is no relationship between the secondary metabolite produced and lignin degradation for a mutant strain of *Phanerochaete chrysosporium.*

Leisola et al. (43,44) isolated two oxidation products of veratryl alcohol from lignolytic cultures of *Phanerochaete chrysosporium.* IR and H-NMR spectra of the purified compounds showed the absence of benzene rings. However, it was not clear whether this ring cleavage was carried out by ligninase protein or some other unidentified protein. Thus if veratryl alcohol itself was degraded, how it helps in the over-production of ligninase was not explained.

2.2.9 Effect of Surfactants

So far all efforts have been directed either to increase the ligninase production or to increase the activity of ligninase, since almost all workers concluded that ligninase is the solely responsible enzyme. In recent years, efforts have been made to increase ligninase production by the addition of additives such as oils and surfactants. In 1990, Lestan and co-workers (42) studied the effect of additives on ligninase production. They observed that an emulsion of oleic acid enhanced ligninase production by *Phanerochaete chrysosporium*, while addition

of linseed oil and rape seed oil decreased the specific activity by a factor of 2.9 to 6.6. They concluded that peroxidases are located mainly inside the fungal biomass and must pass through the plasma membrane to be extracellularly active. A changed environment, such as the impact of oleic acid on the plasma membrane lipids, can influence and presumably promote peroxidase transport. However, they did not study the degradation of any compound or the effect on degradation by the enhanced activity.

Jager et al.(32) found that Tween 80 (Polyoxyethylene-sorbitan dilaureate) enhanced the ligninase activity. Tween 80 at a concentration of 0.2% gave maximum activity on the sixth day. They also found that both Tween 20 and CHAPS (3-dimethyl 1-propane sulphonate) gave comparable results, and the maximum activity was found on the fifth and sixth day, one day before Tween 80. They concluded that Tween 80 and Tween 20 supply fatty acids to the culture. However they did not come up with any plausible explanation for this effect, and moreover, no study was made as to how this enhancement effected the degradation rates.

2.3 Time Decay of Fungal Activity

Ulmer et al (68). studied the degradation of lignin by *Phanerochaete chrysosporium* in batch culture. They observed during the first 2 to 3 days that 1.0 gm/l of lignin was 90 to 100% degraded. However, with time, the degradation rate went down. After 7 days, the degradation rate was only 65% of its original rate, and the glucose or extracellular energy source was completely depleted during the next 7 days. After that degradation ceased completely. Reid (58,59) also had similar findings during lignin degradation.

Ander et al. (2) concluded that the enzyme production of lignolytic enzymes depends on the availability of both carbohydrates and nitrogen source, and stops as they get exhausted.

A very nice paper was presented by Asada and Miybe (4). They found from a basic study that without supply of continual energy, the fungus would not produce lignolytic enzymes. Peroxidase produces hydrogen peroxide by oxidizing NADH, and thus is an energy requiring process. Thus as soon as the supply of the energy yielding molecule ceases, degradation also stops. This also indicates that no energy is derived from lignin degradation.

According to Merril and Cowling (49), wood rotting fungi can recycle nitrogen. When glucose is present, lysis of the biomass occurs until the carbon source is consumed. In the absence of nitrogen, but presence of glucose, some activity of the fungus would be possible (although not for an extended period).

It was found that the fungus can retain its degradative ability for a definite time in a substrate exhausted condition, and ligninase has a definite time of activity (11,19,21,34,39). The activity period is maximum around 10 days, considering two burst phases as suggested by Dutta et al(16). In spite of the limited lifetime of this enzyme, Bumpus (8-10) during the study of a few toxins observed a continued degradation for least 25 days. Janshekar et al. (33) found lignin degradation for around 40 days. Similarly degradation of PCP was observed by Lin et al (45-46) for more than 20 days and by Lamar et al (40) till 50 days in soil. The prolonged degradation cannot be explained by ligninase activity alone.

2.4 Enzymology and Enzymatic Mechanism

In the last decade, except for a few reports (5,17,68) all workers reported that degradation by the fungus *Phanerochaete chrysosporium* is a completely

enzymatic process, and ligninase or lignin peroxidase is the only enzyme responsible for this enzymatic degradation.

In 1971, Dagley (15) proposed that degradation of low molecular weight aromatics follows a mechanism similar to that of lignin, and he envisioned a scheme as follows: lactonization, delactonization, isomerization, hydrolysis, dehydrogenation and decarboxylation, with each reaction requiring a separate enzyme. He also found that monooxygenase, dioxygenase and phenol oxygenase are the lignin and lignin-related degrading enzymes.

However in 1975, Chang and his co-workers (13) found no evidence of such type of mechanism for lignin degradation. They concluded that in the catabolism of aromatic compounds, the preparation of ring cleavage may be the rate limiting step. Wood et al.(70) showed that the enzymes responsible for the ring cleavage by hydroxylation and/or demethylation are monooxygenases. The monooxygenases require a reducing coenzyme such as NADH or NADPH for their activity. Thus, this is an energy requiring process. Wood also showed that aerobic cleavage of aromatic rings by microorganisms is catalyzed by deoxygenate type enzymes. These enzymes are non-heme iron containing proteins, and require no co-enzyme for their activity. Both atoms of molecular oxygen are incorporated into the aromatic ring to yield an aliphatic acid. A similar observation was also made by Kirk et al.(39) during degradation of lignin.

In the same year (1975), Ander et al (2) detected phenol peroxidase in lignin degrading broth. The enzymes lactase oxidoreductase, tyrosine oxidoreductase, and peroxidase are all copper containing proteins having the property of catalyzing the direct oxidation of their respective substrates by atmospheric oxygen.

The importance of hydrogen peroxide in lignin degradation has become increasingly apparent. Kersten and Kirk (36) reported that under lignolytic

conditions, *P.chrysosporium* produces extracellular hydrogen peroxide. A number of potential substrate such as simple aldehyde, hydroxyl carbonyl and dicarbonyl compounds were tested for degradation. The highest activity was suggested at pH 6.0 with methylglyoxal and glyoxal as the secondary product. No H_2O_2 producing oxidase activity was observed in carbohydrate depleted conditions.

To explain the importance of hydrogen peroxide, Asada et al. (4) suggested that one of the possible physiological roles of NADH peroxidases was to supply H_2O_2 to lignin peroxidase by oxidizing NADH. Manganese stimulated the reaction by 3 to 4 fold. Green and Gold (23) suggested that intracellular fatty acyl-coenzyme-A oxidase, may be an important source of extracellular H_2O_2 They observed an increase in H_2O_2 production in the presence of steryl coenzyme-A with mycelia premobilized with Triton X-100 as the detergent. The possible involvement of intracellular enzymes in H_2O_2 production was studied by Kelly and Reddy (34,35). They reported the isolation of intracellular glucose 1-oxidase, and suggested that this enzyme was the primary source of peroxidase enzyme in the lignolytic cultures. They also suggested that intracellular glucose oxidase produces the extracellular H_2O_2 . Leisola et al. (43) showed that in the liquid culture of *Phanerochaete chrysosporium,* 21 hemoproteins were found, all of which had peroxidative activity. Fifteen of these enzymes oxidized veratryl alcohol in the presence of H_2O_2. Six enzymes were Mn-dependent peroxidases, which reached their activity earlier than lignin peroxidase in the culture. They suggested that the many extracellular enzymes of *Phanerochaete chrysosporium* can be divided into two basic groups: Mn-dependent peroxidases and lignin peroxidases. However it was not clear why the fungus produced two types of extracellular peroxidases. They appear and reach their maximal activity at

different times and have different function in lignin or other toxic compound degradation.

Glenn et al. (24) reported that the hydrogen peroxide requiring enzymes are responsible for the degradation of a lignin model compound (2-keto-4-thiomethyl butyric acid) and formation of an intermediate ethylene. However, the explanations were more intuitive than experimental.

Hyunth et al. (31) observed the fungus to produce an extracellular aromatic methyl ester and identified an aromatic methoxyl-demethylase. They concluded that both esterase and demethylase were components of the lignolytic enzyme complex.

Aitken et al. (1) studied the degradation of 2-chlorophenol, 0-cresol, 2-nitrophenol and pentachlorophenol utilizing the fungus. They observed an increased degradation rate in the presence of Mn and concluded that Mn(II) could serve as an electron donor to phenoxy radicals generated enzymatically, thereby reducing the radicals back to the parent phenol. Addition of both Mn(II) and veratryl alcohol increased the degradation rate. They attributed the total removal to ligninase only.

In 1991, Datta et al. (16) identified and separated six different kinds of enzymes from this wood rotting fungus *Phanerochaete chrysosporium*. They detected manganese peroxidase (MnP), lignin-peroxidase (LiP) and glyoxal oxidase (GLOX). Interestingly the major peroxidase protein was MnP. A partial amino acid sequence provided evidence that it differs from the dominant MnP found in optimized liquid cultures. The second most dominant enzyme was GLOX, and in the solid wood culture GLOX was the most abundant enzyme. During the study of the time course of the peroxidase activity, they observed two bursts phases The first burst phase on the third day and the other one on the 7th day, with the activity dying out after ten days. Moreover, they detected

protease which inhibit the peroxidases and GLOX. While a typical protease inhibitor like phenylmethyl sulphonyl fluoride was not able to inhibit the protease activity, macroglobulin is effective for protease inhibition.

Kelly and Reddy (35) reported that glucose oxidase is an important source of hydrogen peroxide in lignin degrading cultures of *Phanerochaete chrysosporium* They purified the protein and found that the apparent native molecular weight is 180,000, whereas that of the denatured protein is only 80,000. The optimum pH for the enzyme is between 4 to 5.0. It was inhibited by Ag++, but not by Cu++ or NaF. They found the Michaelis-Menten constants for glucose D and found the enzyme to be very specific. However, this enzyme can disintegrate xylose, so they suspected that this enzyme may utilize other lignocellulosic materials for metabolization. In another study, Kersten and Kirk reported (36) that the activity of glucose oxidase is very much dependent on both glucose and nitrogen concentration in the medium. They also concluded that glucose oxidase plays an important role in H_2O_2 production. However, they didn't show any specific examples as to how this is related to the lignolytic system.

Paszczynski et al.(56) studied the enzymatic activities of an extracellular manganese dependent peroxidase from *Phanerochaete chrysosporium* They reported that the enzyme oxidizes various phenols and amines in the presence of Mn^{++} . This enzyme never requires H_2O_2. Moreover, hydrogen peroxide is a product of the reaction between the enzyme and reduced glutathione or NADPH. They concluded that Mn-dependent peroxidase plays a central role in lignin degradation. Thus, the simultaneous role of both LiP and MnP was considered in this study. This was the first time that simultaneous action of multiple enzymes was studied.

Sanglad and his co-workers (63) studied the role of extracellular ligninase in biodegradation of benzo(a) pyrene by *P. chrysosporium*. They concluded that ligninase is the only responsible enzyme for degradation of aromatic compounds, and this is fairly non-specific and non-stereo selective. However,since they did not study any stereo isomers or homologues, it is not clear how they reached this conclusion.

Schoemaker et al. (64) studied the enzymatic lignin breakdown mechanism in great detail. They proposed that lignin degradation occurs via C_α-C_β cleavage They proposed a single electron transfer model. This mechanism is a single electron transfer from the methoxylated aromatic ring to a high redox potential center yielding a radical cation in the substrate. The aromatic radical cations may also act as electron transfer agents. They proposed that lignin degradation products in the form of radical cations, produced either enzymatically or by direct interaction with hydroxyl radical, may act as electron transfer agents to induce the formation of free radicals in the remote lignin structure, thus causing degradation in polymers not accessible to the large enzymes located in the hyphal surface.

Tien and Kirk (65,66) also supported the same view of Schoemaker (64) and proposed that the enzyme catalyzes non-stereospecifically the alkyl side chain for the lignin. Moreover they found that the ligninase contained iron but not Cu, Zn, Mn and Mo or Co. They found the optimal pH for veratryl oxidation is around 3.0.

Umezawa and Higuchi (69) also studied the degradation of lignin model dimers and found that the model compounds are degraded by lignin peroxidase and H_2O_2, and the intermediates are carbonate, oxalate, formate, etc.

Although a good number of studies were conducted on the reaction scheme of lignin degradation, no reaction mechanism or scheme was proposed for degradation of pollutants,

2.5 Stereospecificity of Degradation

It was reported by all workers that degradation by *Phanerochaete chrysosporium* is non-specific in nature. Because of unavailability of any comparative scale, it was not possible to quantify the stereospecificity. Only Zitelsberger et al. (72) studied the stereospecificity of veratrylglycerol β-2,4-dichlorophenyl ether. They reported that for three stereoisomers, no stereo-specificity could be detected, and concluded that the degradation process in not stereospecific in nature. However, it must be noted that this is a qualitative conclusion. Leatham et al.(41), when studying different positional isomers, obtained different degradation rates.

2.6 Reactor Design and Modeling

Very little information is available regarding reactor design and modeling. In 1989, D.Pak (55) used packed bed and fluidized bed reactors for degradation of 2-chlorophenol. Pak found that *Phanerochaete chrysosporium* can effectively degrade 2-chlorophenol. In his experiments with shaker flasks, he was able to degrade 20 ppm of 2-chlorophenol in 4 hours. He was able to show fungal degradation in packed bed as well as in fluidized bed reactor experiments. However, he reported that the degradation rate became insignificant when a stirred tank batch reactor was used. He analyzed various types of reactor configurations and came to the conclusion that a packed-bed with porous silica beads as inert support was the optimal reactor configuration. He showed that the fungus can mineralize 500 ppm of chlorophenol in 20 hours in a packed bed.

He also showed that the fungus can survive at 2-chlorophenol concentrations as high as 1400 ppm. Later Haq (30) also supported similar findings.

In 1990, Lin and his co-workers (45) studied degradation of PCP. According to their scheme, both biomass and extracellular protein individualy can degrade PCP. They found that some intermidiates were produced, then the rate of decay of these intermediates were the rate limiting steps. Thus they studied extensively different biomass and protein concentrations and came up with a second order model that added two effects: one involving biomass coupled with extracellular protein concentration, and another involving biomass coupled with pollutant concentration. They showed good agreement with predicted and experimental data. However, the maximum concentration they studied was only 12 μM. Such a low concentration may only show an artifact of the true enzyme kinetics. Moreover, as the intermediates vary from experiment to experiment for the same compound (40,45,) a model based on intermediates becomes useless when the feed contains a mixture of compounds and it become very difficult to find the rate limiting compound in the presence of so many others. In 1991, Lin et al. also described a coimmobilized system that can enhance the degradation rate by this fungus (46). However, they did not quantify the improvement, and moreover, the system concentration was so low that it is very difficult to perceive the increase.

CHAPTER 3

OBJECTIVE

The objectives of the present work were to test the biodegradability of 2,4,6-trichlorophenol and 2,4,5-trichlorophenol by *Phanerochaete chrysosporium*, to maximize the degradation rate by optimizing the operating parameters and reactor configuration. To elucidate the degradation reaction scheme, individual effects of biomass and extracellular protein concentrations on degradation of 2,4,6-trichlorophenol were studied, and a kinetic model of the process was developed. To better understand the packed-bed behavior, the growth parameters were studied in detail and correlated with the packed-bed characteristics. Finally to further test the fungal activity, degradation of phenol and pentachlorophenol were also studied.

CHAPTER 4

ANALYTICAL METHODS

In this chapter different analytical techniques used for measurement of various parameters are described in detail.

4.1 Nitrogen Assay

$NaNO_3$ was used as the nitrogen source for fungal growth in this study. Hence, nitrate concentration was measured. However, during the study of growth parameters in long standing cultures, NH_4^+ ion (the possible product of lysed biomass) was also measured in order to have an indication of lysis of fungal biomass. Both nitrate and ammonium were measured in a similar fashion by using ion sensitive electrodes. An Orion nitrate electrode from Orion Research Inc. Boston, MA (Model 93-07) was used to measure nitrogen as nitrate. Similarly, an Orion electrode (Model 95-12) was used to measure ammonium as nitrogen. Before measuring the concentration of either nitrate or ammonium ion, calibration curves were prepared as described in the next two sections.

4.1.1 Determination of Nitrogen Concentration as Nitrate

The procedure from the instruction manual for this electrode was followed. A double junction reference electrode (Orion Model No. 90-02-00) was also used. The outer chamber of the reference electrode was filled with 2% ionic adjuster solution (ISA). The ISA solution was prepared by dissolving 25.60 g of $(NH_4)_2SO_4$ in 100 ml of deionized water. A nitrogen standard was prepared by adding 0.3035 g of sodium nitrate in 1 liter of deionized water, which resulted in a nitrogen concentration of 50 ppm of nitrogen as atomic nitrogen (N). Since the

concentration of nitrogen in the cultures never went above 30 ppm, the upper limit of 30 ppm was enough for all measurements. From the 50 ppm solution, standards of 40, 30, 20, 15, 10, 5, 2.5 and 1.25 ppm were made by dilution. To determine the nitrate concentration 0.05 ml of ISA were added to 5 ml of standard in a small 10 ml beaker, and mixed by swirling the solution. The electrodes were placed in the solution. Then the electrode voltage in millivolts corresponding to each concentration was measured using an Orion pH/ISE meter (Model No: 720). A calibration curve was prepared by plotting the nitrogen concentration vs. to the millivolts readings. Contrary to the suggested method in the electrode manual of plotting ln (mV) vs. ppm, this calibration curve was prepared by plotting mV on the X axis and ppm of nitrogen concentration on the Y axis, to have better clarity and accuracy in reading the calibration curves. A typical calibration curve is shown in Figure 1 in Appendix III. However, the calibration curve changed from time to time because of the loss of sensitivity of the membrane. Moreover, different electrodes for same solution also show slightly different electrode characteristics. During the measurement of nitrate concentration in samples the identical method used with the standards was followed. 5 ml of sample were collected from either the continuous reactors or the batch reactor or shaker flasks, and 0.05 ml of Ionic Strength Adjuster (ISA) were added. The millivolts corresponding to the sample concentration were recorded and then converted to ppm nitrogen using the calibration curve. A linear regression over a portion of the calibration curve was also used for an accurate measurement of nitrogen concentration, since the total calibration curve could not be approximated as a straight line. The nitrate concentration was always reported as elemental nitrogen (N).

It must be noted that magnetic stirrers should not be used during these measurements, since the nitrate sensitive membrane at the bottom of the

electrode is very sensitive to any mechanical abrasion. Any slight mechanical friction with solid surfaces damages the membrane, and would give erratic readings. For instance, use of polishing papers (Orion Inc.), normally used to clean the electrode surface, also alters the electrode characteristics. However, mixing by magnetic stirrers was accomplished before dipping the electrodes in the samples or standard. To maintain a longer life of the membrane, the electrode was always stored in 100 ppm nitrogen solution. Electrode slope and calibration were checked twice a week. The calibration check was done by measuring the voltage of a standard solution. To check the electrode slope, the voltage corresponding to 1 ppm and 10 ppm of nitrogen (N) was measured. The difference in these two readings is known as the slope for the electrode. This value is to be set on the meter dial labeled slope. If at any time this slope is beyond a range of 52 mV to 58 mV, the membrane is no-more usable. Normally, the membrane was normally replaced after every 12 to 14 weeks, depending upon wear and tear. Before every measurement, the inner and outer liquid level in the reference electrode was checked, as level changes affect the measurements.

4.1.2 Determination of Nitrogen Concentration as Ammonium

In a similar way, ammonium concentration was measured using a Orion ion selective electrode (Model No: 95-12). Here analytical grade ammonium chloride was used as the standard. To prepare the standard, 0.38215 g of NH_4Cl were dissolved in 1000 ml of water to give a standard concentration of 100 ppm of nitrogen as N. Then standards of 50, 40, 30, 25, 20, 25, 10, 5, 2.5 and 1.25 ppm were prepared by dilution of the 100 ppm solution. A calibration curve was drawn with mV vs. ppm in a similar way to that of nitrate as shown in Figure 2 in Appendix. The samples were tested in a similar fashion and the

concentration of nitrogen was found by using the calibration curve. The ammonium ion concentration was always reported as elemental nitrogen (N). The ammonium ion sensitive electrode took 2 to 3 minutes time to reach a steady reading, compared to 1 minute in case of nitrate sensitive electrode. This is due to nature of the specific ion electrodes. Here a magnetic stirrer was used for both standards and samples, since the membrane component allows their use, as recommended by the manufacturer.

4.2 Chloride Ion Assay

Measurement of chloride ion was used as an indicator for dechlorination of chlorophenols. An ion sensitive combination chloride electrode from Orion Research Inc., Boston, MA, (Model No: 96-17B) was used. To make standards, 0.1648 g of analytical grade NaCl were dissolved in 1000 ml of deionized water resulting in a 100 ppm standard chloride solution (as Cl^-). Then standards of 25, 20, 15, 10, 5 2, 1 and 0.1 ppm were prepared by dilution of the 100 ppm chloride solution. According to the electrode instruction manual, the use of any magnetic stirrer was not recommended with combination chloride electrodes. However, mixing by a glass rod or by swirling was conducted prior to placing the electrode in the sample or standard solution. An Orion pH/ISE meter (Model No. 720) was used. A calibration curve was prepared as described for nitrate ion. A typical calibration curve is presented in Figure 3, in Appendix. The samples from the reactors or the shaker flasks were measured in an identical fashion as the standards and converted to ppm chloride by using the calibration curve. The voltage reading is inversely proportional to the concentration. Thus, at higher Cl^- ion concentrations the millivolts readings are low. If the reading for a particular sample was lower than 100 mV, then the samples were diluted with deionized water to have a higher millivolts reading (typically greater than 150

mV), since at higher concentrations of chloride, the change in millivolts due a slight change in chloride concentration could not be detected by the electrode. However, by dilution, the difference could be magnified and the concentration could be measured with greater accuracy. Complete mineralization of 1 ppm of 246-TCP or 245-TCP would liberate 0.5392 ppm of chloride. Thus measuring the chloride concentration at the inlet and the outlet of the reactor (or at initial time and at some other time in the shaker) would give the corresponding amount of TCP mineralized.

4.3 Determination of Dissolved Oxygen Concentration

To monitor the dissolved oxygen (DO), an Ingold oxygen measurement system (Ingold Electrode Inc.; Wilmington, MA) was used in conjunction with a dissolved oxygen meter (New Brunswick Scientific, NJ; Model No. DO-50). To calibrate the DO meter, the probe was immersed in deionized water contained in a 14 liter Microferm fermenter (working volume 10 liters). Air was bubbled for 4 hours through the water at a rate of 3 liters/min, with continuous stirring at 200 rpm. The temperature was maintained at 32.2° C. The DO meter was then set at 100%. The probe could not be used for continuous monitoring, since biomass grew on the DO membrane if the probe was left in the reactor for long periods of time. Therefore, to measure DO in a continuous reactor, the probe was periodically inserted in the uppermost liquid part of the reactor and DO was recorded. Caution was taken to avoid contact of the membrane with the packing material as this could damage the membrane. According to the DO instruction manual, at 32.2° C, 100% saturation corresponds to 7 ppm of dissolved oxygen. Other measurements were made by considering a linear relationship over the DO meter dial gage as specified by the manufacturer (New Brunswick Scientific,

NJ; Model No: DO-50). The DO electrode was always stored in 1% KCl solution as recommended by the manufacturer.

4.4 pH Measurement

The pH was measured directly using an Orion pH electrode (Model N0: 95-56) connected to an Orion Expanded ion-Analyzer meter (Model No: EA 920). Standard buffers of 4.0 and 7.0 from Orion research were used for calibration. The pH was measured in samples collected from shaker flasks or from the reactors. The measurements were done by dipping the tip of the electrode in the sample liquid, and noting the reading when the meter gave a constant value and the 'ready' signal appeared on the display.

During experiments to study the effect of nitrogen concentration at constant pH (section 8.4), the pH was continuously monitored in the packed-bed reactor. For this purpose a long-stem pH probe (Ingold, MA) connected to a pH meter (New Brunswick Scientific, Model # pH 450)) was used. This pH probe was calibrated at room temperature (22° C) but used at the reactor operating temperature (32° C) by inserting it through the PET bed. Since the pH is a strong function of temperature, a difference in reading was observed for a same liquid at 22° C and at 32.2° C. However, the electrode is accurate enough for continuous monitoring of the reactor pH. Since for all the experiments the pH was measured at 22° C, to maintain uniformity, a small portion of liquid was drawn out of the reactor, allowed to cool down to room temperature and the pH was measured with another probe. However, the pH shown at 32.2° C, by the inserted pH meter was used as the guide line to trace the change in pH during the experiment.

4.5 Glucose Assay

Glucose was assayed using the ortho-toluidine method (73). At high temperature (around 100° C), o-toludine reacts with glucose in the presence of an acid to form a blue-green colored complex. The intensity of the color is proportional to the glucose concentration. To eliminate interference from other compounds, 0.05 ml of 1.0 mM EDTA (sodium salt) solution were mixed with 2 ml of sample. The liquid was centrifuged at 13,000 rpm for 10 minutes in an ultracentrifuge (IEC Ultracentrifuge, Model No: Centra-M). Then, 0.1 ml sample, 0.1 ml of standard glucose, and 0.2 ml of distilled water were placed in three different test tubes (10 ml, 10 ml and 20 ml sizes respectively) and labeled respectively as sample, standard and reference. Then 5 ml of o-toludine mixed with 3% trichloroacetic acid (w/v) were added to each sample and standard, and 10 ml to the reference test tube. (Since a dual beam spectrophotometer was used for all the spectroscopic measurements, twice as much volume of reference fluid were required to that of sample volume to fill two cuvets for blanking purposes). The three test tubes were placed in a boiling water bath and allowed to boil for ten minutes. The test tubes were placed in another beaker containing tap water at room temperature and allowed to cool for 3 minutes. Then absorbence of both the standard and sample were measured at 635 nm wavelength using a Varian (DMS-200) spectrophotometer. The test tube containing distilled water was used as a blank. The concentration of glucose in the standard was 1 g/l. Since the absorbence is directly proportional to the concentration, a direct measurement of the sample was done knowing the absorbence for the standard. If the glucose concentration was greater than 2.5 g/l, it was diluted with deionized water, since above that concentration (2.5 g/l) the method is not very accurate. In addition, the accuracy of the method is about ± 5 mg/l. However,

in the present study, it was not necessary to accurately determine the glucose concentration below that lower range.

4.6 Assay for 246-TCP, 245-TCP, Phenol, And PCP

An HPLC (Waters, Inc., Model 600E) provided with a tunable detector (Waters Inc., Model 484) and auto-sampler (715 Ultra-WISP) was used in conjunction with a C18 bonded phase column (Alltech Associates Inc.) to measure the concentration of chlorophenols or phenol. A mixture of methanol with 1% acetic acid, and deionized water with 1% acetic acid, were used as the mobile phases in a 60:40 (v/v) ratio. Absorbence was detected at 280 nm wavelength. Calibration curves were prepared for the trichlorophenols, phenol and pentachlorophenol by using standards. To prepare standards for 246-TCP, 0.108 g of 246-TCP were added to 1 liter of a 0.1 (N) KOH solution and mixed thoroughly for two hours using a magnetic stirrer. This resulted an 246-TCP concentration of 108 ppm. Then, dilutions of 54, 39, 27, 19.50, 13.50, 6.75, 3.38, 1.69 ppm were made by successive dilution with deionized water. It should be noted here that due to convenience of manipulation for dilution, fractional concentrations of standards were prepared. This reduced instrumental and manual error, as measurements of fractional volume are not very accurate. Similarly, standards for phenol, 245-TCP and pentachlorophenol (PCP) were prepared. Since the sample pH was always in the acidic range, the pH of the standards was adjusted between 4.0 and 5.0. The calibration curves for 246-TCP, 245-TCP, phenol and PCP are presented in Figures 4 to Figure 7, in Appendix. During analysis of any sample aqueous samples from the reactors were spun for 10 minutes at 13,000 rpm in an ultracentrifuge (IEC Centra-M, International Equipment Co.) to separate the biomass. Then, 25 µl of each sample were injected for analysis via auto-sampler. Nelson software was used

for data acquisition through an on-line computer. The standard deviation of the measurements were within ± 0.29 ppm.

4.7 Lignolytic Enzyme Assay

A lignolytic enzyme assay was conducted on the supernatant of centrifuged samples by measuring the rate of oxidation of veratryl alcohol to veratryl aldehyde, as described by Tien et al.(65). A 2 ml sample was collected from shaker flasks or from the continuous reactor and the biomass was separated by centrifuging at 13,000 rpm for 15 minutes. Each sample (1.375 ml of supernatant) was mixed with 2 cc of a 5 mM veratryl alcohol solution and 0.625 ml of the 0.8 mM sodium tartarate buffer. A blank was prepared by adding 4 ml of veratryl alcohol, 1.25 ml of sodium tartarate buffer and deionized water to produce a final volume of 9 ml. The absorbence of the sample was measured at 308 nm using a Varian DMS 200 spectrophotometer. Initially the spectrophotometer was zeroed by using the blank. Then 1.0 ml of H_2O_2 solution was added to the sample to make a final volume equal to 5.0 ml and the absorbence was immediately measured. Then the reaction mixture was incubated at 32.2° C. Since the lab spectrophotometer was not equipped with temperature controller, incubation was conducted outside the spectrophotometer chamber at 32.2° C. This water was always available from the water bath used to maintain the continuous packed-bed reactor temperature. After five minutes of incubation, the absorbence was again measured. The total increase in absorbence in five minutes was measured in Absorbence Units (AU) and recorded. After it was confirmed that ligninase was not primarily responsible for the degradation, this test was no longer conducted.

The veratryl alcohol, tartarate buffer, and hydrogen peroxide solutions were prepared as described in next three sections.

4.7.1 Preparation of Veratryl Alcohol Solution

The veratryl alcohol solution preparation was the most important step in the measurement of ligninase activity. Veratyl alcohol (3,4-Dimethoxybenzyl alcohol) 96% purity was purchased from Aldrich Chemical Company Inc. The commercial veratryl alcohol contained some ketones since veratyl alcohol is readily converted to ketones in the presence of air. These ketones are preferred by ligninase over veratryl alcohol and are converted to organic acids. Since the assay method is based on formation of veratyl aldehyde from veratyl alcohol the presence of ketones makes the assay unsuccessful and create interference. Thus the veratryl alcohol required to be purified by distillation. Approximately 10 g of veratryl alcohol were transferred to a 25 ml distillation flask, then vacuum distilled under 700-720 mm of Hg at a temperature of 76-80° C. When the veratryl alcohol in the distillation flask started turning yellowish brown, the distillation was stopped. The distillate was then transferred to a rubber-capped, air-tight, dark glass vial, to prevent photo oxidation. The vial was previously purged with nitrogen to displace any air. Approximately 4 to 5 g of purified veratryl alcohol could be obtained in this process from 10 g of raw material. The purified product was stored at 4° C in the freezer. The alcohol so prepared would maintain its purity for approximately 3-4 weeks. It was periodically checked by spectrophotometer and discarded when other peaks appeared. The purified veratryl alcohol was used to prepare a stock solution by transferring 0.841 g to 200 ml of distilled, deionized water to make a 5.0 mM veratryl alcohol solution. This was also stored in a dark volumetric glass flask at 4° C, since the alcohol is known to be light sensitive. The shelf life could be enhanced by storing the materials at -20° C.

4.7.2 Preparation of Tartarate Buffer

Sodium tartarate from Fisher Scientific, NJ was used for the preparation of the tartarate buffer. Then 0.18407 g of sodium tartarate were dissolved in 1.0 liter of water to give a buffer solution 0.8 mM tartarate concentration having a pH of 3.7. A portion (0.625 ml) of this solution was used for preparation of 5.0 ml of final reaction mixture, where the tartarate concentration was 0.1 mM.

4.7.3 Preparation of Hydrogen Peroxide Solution

Hydrogen peroxide (30% by volume H_2O_2) solution was purchased from Sigma Chemical Company and stored in the freezer. To prepare a 4.0 mM H_2O_2 solution, 0.408 ml of the 30% solution was transferred to a 1 liter of deionized water. Then 1.0 ml oh this solution was subsequently used for ligninase assay. To maintain good activity of hydrogen peroxide, fresh solutions were prepared every day before the measurement of ligninase activity.

4.8 Protein Assay

The protein content of the liquid part of the samples was determined via biuret reaction using a BCA (Bicichoninic Acid) Protein Assay reagent (Pierce Chemicals Co; Rockford; Illinois). The purple reaction product formed by interaction of two molecules of BCA with one Cu^+ ion, is water soluble and exhibits a strong absorbence at 562 nm. This allows spectrophotometric quantification of protein in aqueous solutions.

The protein measuring reagent was prepared by mixing 50 ml of reagent-A with 1.0 ml of reagent-B. After centrifuging the sample at 13,000 rpm for 15 minutes 0.2 ml of the supernatant were mixed with 4 ml of reagent and incubated for 30 minutes at 37^o C. When the protein concentration was lower than 20 mg/l, the samples were incubated at 60^o C for 30 minutes. In presence of protein, the initially colorless solution turned pink after incubation. Absorbance

was measured at 562 nm. Bovine albumen (Pierce Chemicals Co.) was used for calibration purposes. The concentration of protein in the standard vials were 2.0 g of protein/ml, which was subsequently diluted to concentration of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g of protein/l by mixing with deionized water. Absorbence for each sample was measured and recorded. Then a calibration curve of protein concentration (y axis) vs. absorbence as AU (X axis) was generated. A typical calibration curve is shown in Figure 8, Appendix 1. The calibration curve was checked for each new bottle of reagent. The protein concentration of each sample was directly read from the graph by measuring the absorbence. The accuracy of the method was within 2% of the measured values when measured within 10 minutes.

4.9 Determination of Biomass Concentration

In order to minimize the experimental error, the biomass concentration was determined using a significant amount of slurry (100 ml) taken from either the batch fermenter, the shaker flasks, or the continuous reactors. In the case of the shaker flasks, this volume was collected from all the flasks used in a given experiment (conducted in quadruplicate or sometimes in duplicate).

Each 100-ml sample was centrifuged at 6,000 rpm for 20 minutes. Then, about 80 ml of the supernatant was pipetted out and replaced with deionized water. The biomass was resuspended, and the sample was centrifuged again. This washing procedure was repeated three times. The biomass suspension was transferred to a pre-weighed aluminum foil dish, dried at 90 ^{o}C for 24 hours, cooled to room temperature in a desiccator, and weighed. One aluminum foil dish was always used as the control. A slight difference in tare weight was observed before and after heating. This was most likely due to absorbed surface moisture.

CHAPTER 5

MATERIALS AND METHODS

5.1 Organism and Innoculum

Phanerochaete chrysosporium BKM-1767 (ATCC 24725) was obtained from the American Type Culture Collection (ATCC). The fungus was maintained on yeast malt agar media. The yeast malt agar was prepared by dissolving 0.3 g of yeast extract, 0.3 g of malt extract, 0.5 g of peptone, 2.0 g of agar and 1.0 g of glucose in 100 ml of deionized water. The medium was autoclaved for 20 minutes at 121° C and then cooled to 38-40° C. The medium was then poured into 6 to 8 petri dishes and allowed to cool to room temperature. The plates were ready for streaking with the fungus. The fungus was inoculated under sterile conditions and the plates were incubated at 37° C for three days. At the end of the incubation period, a thin white layer of fungus appeared on the agar plates. The plates were sealed with parafilm and stored at 4° C in refrigerator. New plates were made every four to six weeks. The fungus from these plates was used as inoculum in all experiments

5.2 Culture Medium

Media and solutions were prepared with deionized water. Different media were used for growth phase (Growth Medium) and during degradation (Induction Medium). Another solution named 'wash solution' was used to study the degradation by separated biomass. The basic compositions of both growth and induction media were taken from the thesis of Haq (30) and Pak (55). However, the compositions of both media were changed after performing some

experiments, as mentioned in subsequent section 6.2.1. The composition of each medium is described below.

5.2.1 Growth Medium

The growth medium used to grow *Phanerochaete chrysosporium* in all types of experiments had the following composition

Compound	**Amount**
Glucose	6.0 g
KH_2PO_4	2.0 g
$NaNO_3$	0.2 g
$MgSO_4$	0.5 g
$CaSO_4$	0.1 g
Mineral Salt Solution	5 ml
Thiamin hydrochloride	5 mg
Deionized Water	1.0 liter

The final pH of the solution was between 4.4 to 4.45.

5.2.2 Induction Medium

The induction medium is a substrate deficient medium, used to induce secretion of lignolytic enzymes. The composition is as follows

Compound	Amount
Glucose	0.5 -0.9* g
KH_2PO_4	2.0 g
$NaNO_3$	0.02-0.04* g.
$MgSO_4$	0.5 g
Mineral Salt Solution	5 ml
Thiamin hydrochloride	5 mg
Deionized Water	1.0 liter

*The concentration was varied as mentioned specifically in the description of each experiment.

The final pH of the solution was adjusted between 3.4 to 5.8 by adding 0.1 (N) KOH or 0.1 (N) tartaric acid as required by the experiment. The range of pH varied since experiments were conducted at pH of 3.6, 4.6 and 5.6.

5.2.3 Wash Solution

In the experiments with separated biomass, wash solution was used to prevent any fungal growth and any abrupt change in pH. The wash solution had the following composition:

Compound	Amount
KH_2PO_4	2.0 g
$MgSO_4$	0.5 g
Mineral Salt Solution	5 ml
Deionized Water	1.0 liter

The final pH of this solution was 4.4 - 4.5. The pH was then changed by adding 0.1 (N) KOH or 0.1 (N) tartaric acid as required during experiments. The final pH was adjusted to 3.6, 4.6 or 5.6, depending on the experiment.

5.2.4 Mineral Salt Solution (MSS)

A mineral salt solution was used to provide the fungus with trace elements. The composition of the mineral salt solution is given in the table below.

Compound	Amount
$MgSO_4 ,7H_2O$	3 g
$MnSO_4 H_2O$	0.5 g
NaCl	1 g
$FeSO_4 ,7H_2O$	100mg
$CoSO_4$	100 mg
$CaCl_2$	2 mg
$ZnSO_4$	100 mg
$CuSO_4 ,5H_2O$	10 mg
H_3BO_3	10 mg
$NaMO_4$	10 mg
$AlK(SO_4)$	10 mg
Deionized water	1 liter.

5.3 Preparation of Different Chemical Solutions

The procedure for preparation of different reagents and solutions are described below.

5.3.1 246-TCP and 245-TCP Solution

A stock solution of 200 ± 10 mg/l for both 246-TCP and 245-TCP (98% purity, Sigma Chemical Co.) was prepared in a 0.1N KOH solution. Since the solubility of both TCP in water is high, no special technique is required for preparation of TCP solution.

5.3.2 Phenol and Pentachlorophenol (PCP) Solutions

A 1000 ppm solution of phenol (Sigma Chemical Co) was prepared in distilled water. An aliquot was added to the induction medium as required. Like the TCPs, the solubility of phenol in water is very high so preparation of aqueous solution was pretty straight forward.

The preparation of pentachlorophenol (Aldrich Chemical Company) solution was very difficult. The reported solubility of PCP is around 14 ppm at neutral pH and room temperature (25). Due to extremely low solubility, a stock solution could not be made as in the case of the TCPs and phenol. In the case of PCP, the whole induction medium was used to dissolve the PCP. Twenty liters of induction medium were prepared and then 500 mg of PCP were added to the liquid. This solution was stirred for 24 hours with a magnetic stirrer and filtered through a glass fiber filter paper. This resulted in a PCP concentration of 23.4 ppm at pH of 8.5, and at room temperature. This solution was directly used as the induction medium.

5.4 Selection of Temperature for the Study

In this study the temperature was selected on the basis of literature reports and no experiments were performed to determine the optimal temperature. As reported by previous workers (35,36,67,72), the optimum temperature for growth is between 39° C to 40° C. However, Glasier (23) reported that the growth rate stabilizes above 30° C and then starts falling often above 40° C. Since the

present study was not concerned with growth of the fungus, the lower optimum (30° C) could have been selected. A lower operating temperature would minimize enzyme deactivation and is more energy efficient. To further simulate an industrial sludge tank condition, initially room temperature was used in this study. However, room temperature fluctuates over a range of 13° C to 26° C from day to day and over different seasons. Thus, it was not possible to use room temperature for the experiments. This necessitated the use of a water bath to maintain a constant temperature. The lab hot water bath setting required a minimum set temperature 6° C above room temperature for good control, i.e. at 32° C. Since the lab thermometers are graduated in ° F, 90° F was the actual temperature in all experiments.

5.5 Apparatus

Different types of reactors were used in this study for different types of experiments as described below.

5.5.1 Batch Reactor for Growing the Fungus

A Microferm 14-liter batch fermenter (New Brunswick Scientific Co., NJ) with built-in aeration, heating, and stirring systems was used for the sole purpose of growing the fungus. The fermenter was charged with 10 liters of growth medium and inoculated with the fungus from an agar plate culture. The aeration rate was set at 50 ml/ (min. × liter of slurry), the temperature was maintained at 32.2 °C, and the agitation speed was 80 rpm. Glucose, nitrogen, and pH were monitored twice a day. On the fifth day the glucose and nitrogen concentrations were about 60 mg/l and 1 mg/l, respectively, and a thick biomass slurry was observed. At this time, a fraction of this fungal slurry (typically about 1 liter out of the 10 liters of slurry in the fermenter) was removed from the fermenter to conduct the

shaker flask experiments or for further processing, as described below in greater detail.

5.5.2 Batch Reactor for Studying the Effect of Substrate Concentrations on Growth

To study different parameters during the growth of *Phanerochaete chrysosporium* a 3.0 liter batch reactor (Bioflow II, New Brunswick Scientific Co., NJ) with a 2.5 liter working volume was used. The reactor was equipped with built-in pH, dissolved oxygen and temperature monitoring and control systems. Two and one half liters of growth medium were prepared, sterilized and placed in the reactor. The temperature was allowed to reach 32.2° C using the hot water heating system. Then the content was aerated for 4 hours to allow it to saturate with CO_2 and eliminate any pH effects due to dissolution of atmospheric CO_2. Since the reactor is constantly aerated, initial saturation with CO_2 ensured that any further pH effects would be due to fungal activity only. Then inoculum from the plate culture was added, and the concentrations of glucose, nitrogen as nitrate, nitrogen as ammonium, protein, pH, and biomass were monitored and recorded for about three weeks.

5.5.3 Shaker Flasks

All shaker flask experiments were conducted in 250 ml Erlenmeyer flasks with a liquid content of 100 ml. The mouths of the flasks were plugged with cotton and no additional aeration was used. A water bath gyratory shaker (New Brunswick Scientific Co.; NJ. Model No. G-76 was) used for shaking the flasks at 45 rpm. The temperature of the water in the shaker was maintained at 32.2° C.

5.5.4 Packed-Bed Reactor (for Supernatant Production)

A continuous packed-bed reactor was used to produce fresh fungal extracellular protein after the fungus had been immobilized in it. This protein (in the form of separated supernetant) was used in some of the batch experiments in shaker flasks, as described below.

The reactor consisted of a jacketed vessel (length 72.5; I.D: 5.0 cm) made of acrylic polymer. Clear polyethylene terephthalate (PET) flakes, irregular in shape and size (cross-sectional area = 2-15 mm^2; thickness $\cong$ 0.5 mm) were obtained from the Polymer Recycling Plant, Rutgers University, New Brunswick, NJ; and used as the random packing material. The polyethylene terephthalate (PET) flakes were first washed with a 0.1 (N) H_2SO_4 solution and then several times with distilled water. During washing, dusts or finer flakes settled at the bottom and they were discarded. The PET flakes were autoclaved for 1 hour at 100^0 C. The void volume in this particular experiment was 63%, measured as described in the previous chapter.

After growing the fungus in the fermenter, about 1 liter of fungal slurry was transferred to the reactor with the simultaneous addition of the PET flakes. The fungus was allowed to attach itself to the packing for 12 hours. Aeration was maintained at 50 ml of air/(liter of slurry $\times$ min.) throughout the entire procedure. The reactor temperature was maintained at 32 °C by circulating hot water through the jacket. After this waiting period growth medium was continuously fed to the bottom of the reactor and removed from the top at a flow rate of 1.5 ml/min. The retention time was 9.97 hours. In order to establish good internal mixing the reactor was also provided with an external top-to-bottom recirculation loop (flow rate = 15 ml/min). The fungus was allowed to grow for five more days in the reactor, and then the feed was switched from growth medium to induction medium. The induction medium is a substrate-deficient medium used here to

induce the fungal production of the extracellular enzymes responsible for the degradation process. After four days of induction medium regime some 50 ml of fungal supernatant were removed from the reactor and used for separate batch experiments in shaker flasks.

5.5.5 Packed-Bed Reactors (for Continuous Experiments)

During the study of continuous systems, jacketed vessels (used as packed bed reactors) made of glass or acrylic polymer were used. The reactors are identical to those described in the previous section for the production of fresh supernant. The same PET flakes were used as packing. However the void volume varied from experiment to experiment. The void volume of each individual experiment is reported in individual results section. Five different reactors were used. The height of the jacketed vessels varied from 56 to 72.5 cm, and diameter from 4.0 to 5.0 cm. Each of the reactor was equipped with five sampling ports. A schematic of reactor #1 (R1 in the table below) is shown in Figure 5.5.6 with all its dimensions.

The dimensions of each reactor are given in the table below

Reactor	Height (cm)	Diameter (cm)
R1	72.5	5.0
R2	60	5.0
R3	60	5.0
R4	60	5.0
R5	56	4.0

5.5.6 Enzyme Reactor

During the experiments with the packed-bed continuous reactor, a Continuous Stirred Tank Reactor (CSTR), named enzyme reactor was often placed at the outlet of the packed-bed continuous reactor. The enzyme reactor was an acrylic cylindrical vessel, with a working volume varied from 2.0 to 4.0 liters. The reactor was not jacketed and was left open to the air. There was no device to aerate or agitate the liquid in the enzyme reactor. The reactor was maintained at room temperature. A schematic of the combined packed-bed and enzyme reactor is shown in Figure 5.5.6.

5.6 To Find a Stable Nitrogen Source to be Used in this Study

NH_4Cl has commonly been used as the nitrogen source in many published studies (2,6,33,47,53). Pak and Haq (55,30) in this group (worked in this lab on white rot fungus) also used ammonium chloride as the nitrogen source. However, to check for the stability of the NH_4Cl a blank study was done. NH_4Cl and $NaNO_3$ were separately dissolved in 100 ml of deionized water in 250 ml Erlenmeyer flasks. The air purging rate was maintained at 50 ml/min x liter. The nitrogen concentration (as N) was approximately 30 ppm in each of the flasks. The flasks were kept at room temperature in the hood. The nitrogen concentration was measured periodically in both flasks.

5.7 Experimental Procedure For Studies in Batch System

In the following subsections, the change of different parameters during the growth and subsequent phases, namely iodo phase has been monitored to gain a insight in fungal growth cycle. Later it can be used to explain different observations in packed-bed reactor studies.

5.7.1 The Effects of Growth Parameters During Growth and Subsequent Phases

In these experiments the growth parameters were monitored in the absence of TCP or any other toxic compounds.

5.7.1.1 To Determine the Optimal Ratio of Glucose to Nitrogen in Growth Medium

It was necessary to optimize the concentration of glucose to nitrogen to grow the fungus. Previously Pak (55) and Haq (30) in this group and others (4,13, 36, 37,39) used 10.0 grams of glucose and 0.12 grams NH_4Cl for preparation of the growth medium. This gives a carbon to nitrogen ratio of 127.3 by weight. However, the average chemical formula for dry biomass of microbes is $C_{86}H_{160}O_{45}N_7P_{0.3}K_{0.25}Mg_{0.17}Fe_{0.008}S_{0.003}$ as given by Tamaya (61), where the ratio of carbon to nitrogen is 10.53 by weight. Although this may not be a true expression for the composition of *Phanerochaete chrysosporium* and it may be taken as a starting point. To check the validity of the proportion and to minimize unnecessary use of substrates, experiments were done to find the actual usage of glucose and nitrogen by the fungus during growth. This was conducted by starting with an initial glucose concentration of 7.0 grams with the concentrations of all other components equal to those used by previous workers. Then the progressive decrease of both glucose and nitrogen, along with the increase in biomass concentration were monitored from the time of inoculation.

5.7.1.2. To Determine the Effect of Glucose and Nitrogen Concentrations on the Change of pH

Nearly all experiments reported in the literature (with the exception of those conducted by our group) were performed in buffer solution to maintain constant pH. However, the pH is one of the most important factors concerning enzymatic reactions. Since this whole work was performed without buffering the systems, it

was essential to monitor the pH changes during growth and degradation phases. To study how the pH changes, to find the pattern of pH change, experiments were conducted. Attention was also given to find the effects of glucose, nitrogen on the phenomenon of pH change. The parameters were monitored for 20 days.

5.7.1.3 To Determine the Effect of Biomass Concentration on Change of pH

Initial experiments showed that pH changes over a wide range during growth and in the subsequent phases. During the packed-bed experiments, it was observed that if the growth medium or induction medium fed to the reactor was suddenly stopped, the pH of the system would increase abruptly. Since the concentration of biomass in the packed-bed was much higher than in a four or five days old batch cultures in microferm, it was suspected that the biomass concentration could be a major factor the change in pH for a substrate depleted medium. To verify this hypothesis, the fungus was grown in the batch fermenter as described above. On the fourth day, the glucose and nitrogen were mostly depleted. Then another 15 grams of additional glucose, 0.5 g of $NaNO_3$, 1.5 g $MgSO_4$, $7H_2O$, 5 grams of KH_2PO_4, 20 mg of thiamine and 12.5 ml of mineral salt solution were added and the fungus was allowed to grow for two days. At the end of this period the carbon and nitrogen sources were completely depleted and the pH had gone down from 4.4 to 2.58. Stirring was stopped and the biomass was allowed to settle for 4 hours. About 2 liters of supernatant were decanted and replaced with 2 liters of fresh growth medium containing 2.5 liters equivalent of all substrates (e.g. 15 grams of glucose, 0.5 g $NaNO_3$ etc.) and allowed to grow again. All the parameters were routinely monitored throughout the process.

5.7.2 Determination of the Degradation Scheme

The experiments were conducted in shaker flasks. In all cases the shaker flasks were incubated at 32 ^{o}C on a gyratory shaker gyrating at 45 rpm.

A stock solution of 200±10 mg/l 246-TCP (98% purity, Sigma Chemical Co.) was prepared in a 0.1N KOH solution as specified above. Aliquots of the stock solution were added to the shaker flasks, as required. The pH in all shaker flasks was then adjusted to the desired value of 3.6 or 4.6 or 5.6 by adding 0.1N KOH or 0.1N tartaric acid.

Samples of different volumes (2 or 5 ml) were taken from the shaker flasks to measure the lignolytic enzyme activity and the concentrations of protein, chloride ion, 246-TCP and glucose. Flasks containing only deionized water, 246-TCP and acid/base as pH adjusters were used as controls in all experiments.

Five different sets of experiments were conducted. The first three (with whole fungal slurry, with separated supernatant, and with separated biomass) were carried out in parallel using the same source of fungal slurry. The other two (with additional biomass, and additional supernatant) were conducted separately.

5.7.2.1 Experiments with Whole Fungal Slurry

These experiments were performed to establish the overall degradation ability of the fungus. About 500 ml of fungal slurry from the batch fermenter were transferred to a 1-liter beaker and spiked with 246-TCP from the stock solution to make the final concentration equal to about 45 mg/l. After pH adjustment the content of the beaker was transferred to four 250-ml Erlenmeyer flasks (100 ml/flask) in order to perform the same experiment in quadruplicate. The degradation process was followed by monitoring the concentration of 246-TCP

as a function of time. The concentration of total protein, chloride ion, nitrogen, and glucose, as well as the ligninase activity were also periodically measured.

Experiments in which fungal slurry was placed under nitrogen blanket were also conducted. The objective was to test for the presence of ligninase since previous studies had indicated that lignin peroxidase is effective under oxygen-negative conditions.

5.7.2.2 Experiments with Separated Supernatant

This set of experiments was conducted to determine the individual degradation capability of the extracellular fungal protein alone. Some 500 ml of the fungal slurry from the fermenter were transferred to screw-cap centrifuge bottles (approximately 50 ml/bottle). After centrifugation at 6,000 rpm for 20 minutes some 90% of the clear supernatant from all bottles was transferred to a single container, spiked with 246-TCP, and pH adjusted. The liquid was finally transferred to four 250-ml Erlenmeyer flasks (100 ml/flask). The degradation rate and other parameters were monitored as before.

5.7.2.3 Experiments with Separated Biomass

The biomass left in the bottles after separating the supernatant (as described in the previous section) was washed, resuspended, and centrifuged three times with a wash solution having a composition similar to that of the growth medium, but containing no carbon and nitrogen sources to prevent any further fungal growth. The biomass in the bottles was resuspended, and transferred to a single container to which an aliquot of the 246-TCP stock solution was added. After pH adjustment, the suspension was brought up to 1000 ml of final volume by adding the wash solution, and transferred to four 250-ml Erlenmeyer flasks

(100 ml/flask). These experiments were performed to determine the role of biomass alone in the overall degradation process.

5.7.2.4 Experiments with Additional Biomass

An initial biomass separation procedure was conducted as described in the previous section. As a result, four 250-ml shaker flasks, each containing 100 ml of biomass suspension, were prepared and incubated at 32 ^{o}C.

From preliminary experiments, it had been observed that after the washing procedure associated with the biomass separation the concentration of the extracellular proteins in the wash solution fell below the detectable limit. However, after about five to six hours of incubation, the separated biomass started to release extracellular proteins.

After 8 hours of incubation, when the majority of the extracellular protein had been liberated by the biomass in the flasks, a new biomass separation was conducted on fresh fungal slurry from the batch fermenter. Then, approximately 2 grams each of the newly separated, wet biomass were added to two of the four shaker flasks containing the initially separated biomass suspension. This approach was followed in order to establish whether the biomass concentration was the limiting factor in the degradation process.

5.7.2.5 Experiments with Additional Supernatant

In this set of experiments the effect of the extracellular protein concentration on the degradation rate was studied. Initially, biomass separation was conducted on the slurry from the fermenter, as described before, and four 250-ml flasks containing suspended biomass were incubated. No protein was detected in the associated liquid during the first five hours. Then, proteins began to be released. After three additional hours, during which time the majority of the

proteins had already been released, 10 ml of the supernatant from the continuous packed-bed reactor were added to each of two of the four flasks. This produced a significant increase (nearly double) in the protein concentration since the protein content of the added supernatant (25 mg/l) was much higher than that originally in the flasks (1.9 mg/l). The biomass concentration in the supernatant from the reactor was only 12.0 mg/l. Therefore, no separation was performed since the total amount of biomass added to the flasks in this way was negligible.

5.8 Experimental Procedures For Packed-Bed Continuous Reactor Studies

A major part of the experimental studies were conducted in packed-bed continuous reactor system. Before conducting any experiment, the reactor characteristics were studied in detail. These procedure is described below in the following subsections.

5.8.1 Determination of the Residence Time

Before beginning any experiments with the fungus, the residence time distribution (RTD) of the packed-bed reactor was determined.

A typical RTD experiment for the reactor (R1) is described below. The reactor was filled with the PET packing and water. Copper Sulfate ($CuSO_4$) solution was used as the tracer and a positive step input was chosen to conduct the RTD study. The absorbence of the inlet solution was 0.97 AU. The RTD was obtained from a reactor with a void volume of 760 ml, inlet flow rate of 1.65 ml/min with a internal recirculation rate of 8 ml/min and air flow rate of 40 ml/min. The residence time was 7.7 hours. Samples were taken from three different ports of the reactor and the absorbence was checked at 278 nM wavelength by using a Varian DMS-200 spectrophotometer. The resulted Residence Time

Distribution curve (RTD) is shown in Figure 5.8.1. Below a recirculation rate of 6.0 ml/min, the reactor showed a RTD in between a CSTR and plug flow reactor.

The minimum recirculation rates to give a CSTR characteristics for R2, R3 and R4 was , 5.0 ml/min. For the smaller reactor (R5), a recirculation rate of 4.0 ml/min was enough to force the reactor to behave as a CSTR.

5.8.2 Determination of Void Volume in Packed-bed

The void volume changed from time to time when packing was changed and varied from 60 to 68%. To determine the void volume without aeration, a dry column was packed with PET flakes up the mark (a little below the outlet nozzle) and then a measured volume of liquid was slowly poured in the reactor till it reached the level of the outlet nozzle. This gave an approximate volume of liquid in the reactor, and enabled the air flow to be set at 50 ml/min/lit of liquid volume.

In order to determine the void volume with aeration, the water was drained out and the packing removed. The reactor was thoroughly cleaned and dried. Then dried PET flakes were filled the same previous level. The air flow rate was fixed at the rate of 50 ml/min/lit as previously determined. While the air was passing through the column, water was slowly poured till it reached the level of the outlet nozzle. The amount of liquid required this time to fill the reactor equaled the void volume under aerated condition.

5.8.3 Measurement of Flow Rate of Induction Medium

The flow rate of the induction medium was measured by collecting the outlet flow over 36 hours to minimize error normally encountered when the flow rate is measured over short period of time.

5.8.4 Growth And Immobilization of the Fungus in the Packed-bed Reactors

Four days old slurry from the Microferm fermenter was poured in to the reactor along with simultaneous addition of PET flakes. Simultaneous addition of PET and fungal slurry gives a uniform distribution of fungal biomass over the flakes and later a uniform fungal mat was observed over the flakes. The fungus was allowed to settle for 12 hours, or normally overnight. The liquid became clear as the filamentous fungus adhered on the flake surfaces. Then introduction of growth medium was started from the bottom of the reactor and taken out from the top. This was continued for 2 to 10 days, the time depended on how thick biofilm we wanted to grow.

5.8.5 Degradation of 246-TCP and 245-TCP

All five reactors (R 1 to R 5) described earlier were used in these experiments. The different reactor parameters used in each experiment are reported in result section. After growing the fungus in the packed bed as described in the previous section, the growth medium was replaced by induction medium mixed with 246-TCP solution, and was introduced in the bottom of the packed reactor to induce the release of the lignolytic enzyme system. Samples from different sampling ports up the length of the reactor were analyzed for pH, nitrogen, glucose, chloride, 246-TCP, total protein concentration and ligninase assay. After 7 to 8 times the residence time, the system reached a steady state. Since the recirculation rate (4 to 20 ml per minute) is much larger than the feed rate (0.5 to 3.0 ml/min) the packed bed reactor always behaved as a well mixed reactor and could be modeled as a CSTR. The CSTR behavior was confirmed by analyzing the samples from different ports of the reactor which gave the same value of a particular parameter irrespective of the port. The experiment was continued for about three weeks. The total feed was prepared at one time to

have constant properties and concentration of the substrates. Here it is to be noted that the feed in the inlet reactor vessel gets contaminated (observed by fungal growth in the container itself) when kept for more than three days. Thus the feed solution was sub-divided in six 1 gallon bottles, kept air tight, and autoclaved every alternate day. The inlet tank should be cleaned daily with hot water, at which time the inlet feed bottle was changed. Then approximately one and half days feed was transferred from the stored 1 gallon bottle to inlet tank.

5.8.6 Determination of the Effects of Operating Parameters on Degradation

Different experiments were conducted with continuous reactors to determine the effect of operating parameters on degradation of chlorophenols.

The objective of this set of experiment was to study the effect of glucose concentration on the degradation rate of 246-TCP. Five experiments in this set were conducted using the reactor (R 3) with a void volume of 700 ml and a flow rate of 1.689 ml/min. Approximately identical inlet 246-TCP concentration was maintained in all these experiments. Since the unsteady state results had no significance, hence, initial readings were not recorded. Readings were recorded after the third day, when steady state had reached, and the recording of results continued for two more days to confirm that the system reached steady state.

To study the effect of nitrogen concentration on degradation rate, similar experiments as in the case of glucose, were tried by varying the outlet nitrogen concentration. In one set of experiments, an pH controller was also used to control the pH. A tartaric acid solution (0.1 N) was used to lower the pH of the system when required. In these three experiments in this set, reactor R2 was used.

The literature review revels that the fungus is very sensitive to the shear stress (section 2.2.6). Thus, it was very necessary to see the effect of shear

stress on the degradation rate and to determine the critical mass flux at which the fungus becomes ineffective. To study this phenomena, the fungus was grown in the immobilized packed-bed reactor. The reactor void volume was 760 ml (R 1). The total reactor volume was 1430 ml. Then 245 TCP mixed with induction medium was started from the bottom in the usual fashion. The inlet flow rate was kept constant at 1.5 ml/min. The system was allowed to reach a steady state then all the parameters were recorded. Then the internal recirculation rate was changed, keeping all other parameters constant. The system was again allowed to reach steady state. These operations were repeated four times and four different steady state results were obtained for four different recirculation rates. The feed rate and inlet feed concentration for the substrates, the target compound 246-TCP were kept constant within experimental range.

5.8.7 Determination of the Model Constants for 246-and 245-TCP

These experiments were a major part of the experimental work. The fungus was grown as described earlier. Then an induction medium mixed with either 246-TCP or 245-TCP solution was introduced to the packed bed reactor. System parameters were monitored until steady state was reached. After the system reached steady state, samples from different ports up the length of the reactor were analyzed for pH, nitrogen, glucose, chloride, 246-TCP or 245-TCP and total protein concentrations. The CSTR behavior was always checked by collecting samples from three ports of the reactor. The data were recorded once a day, and the system was allowed to run at least 2 more days after reaching steady. This was done to confirm that it had lined out. Here the most difficult part of the experiment was to control the outlet pH of the reactor. This depends completely on the experience of running the system. The following four factors

dictate the outlet pH: (a) biomass concentration in the reactor, (b) inlet glucose concentration (c) inlet nitrogen concentration (d) the residence time in the reactor. It is worth mentioning here that concentration of pollutant had no effect on the pH. The mathematics behind the determination of model parameters will be discussed in Chapter 7. This study was conducted at three different pH values (3.6, 4.6 and 5.6) for both 246-TCP and 245-TCP. For each compound at a particular pH a minimum of 6 experiments were conducted. On average each experiment took about 6 to 9 days. The main problem encountered in these experiments was line clogging by growth of fungus in flow tubing. In that case the experiment was stopped, the whole system was dismantled, autoclaved and reinstalled.

5.9 Degradation of Phenol

Degradation of phenol by Phanerochaete chrysosporium is not reported in the literature. However most chlorophenol containing streams would contain phenol. This experiment was conducted in the same manner as done for TCP's, with the only difference that phenol was mixed with induction medium in place of TCP.

5.10 Degradation of Pentachlorophenol

For comparative purposes the degradation of pentachlorophenol (PCP) was studied in the continuous reactor system. Again, these experiments were conducted in the same manner as with TCP where PCP was the target compound.

CHAPTER 6

RESULTS FROM BATCH STUDIES

The main emphasis of this thesis work is to optimize reactor configuration for degradation of chlorophenols, to model the system, and to determine the optimized operating conditions for maximizing the degradation rate of chlorophenols. However, before presenting results for the continuous reactor system, some parameters and operating conditions were extensively studied in batch reactors for better understanding of the continuous systems. At the same time the fungal behavior during different phases of growth and degradation (iodo phase) were also studied. To find the reaction scheme for the degradation process, the individual effects of the separated biomass and the separated supernatant on the degradation of 246-TCP were studied separately. The results of these batch experiments are discussed in this chapter.

6.1 Ammonium and Nitrate as the Suitable Nitrogen Source

In many studies as reported in the literature (2,3,7,21,33,55) NH_4Cl was used as the sole nitrogen source. However, due to the dissociative nature of this chemical, the stability of the ammonium chloride (NH_4Cl) in the growth medium was determined. The results of this experiment are tabulated in Table 6.1 and shown in Figure 6.1. The loss of ammonium chloride from the medium was substantial. During 24 hour period at pH a 5.0, approximately 50% of the ammonium chloride was lost to air and profuse loss continued in a decreasing fashion. The loss of nitrogen is due to the fact that in an aqueous NH_4Cl solution, a part of the NH_4^+ ion is converted to aq(NH_3), and is removed from the solution continuously by air stripping, resulting in a loss of NH_4Cl with time.

Since.the pK_a of NH_4Cl is 9.3 at 25° C, it could be presumed that at lower pH there should be minimal loss of ammonia. However, according to the electrode manual from Orion Research Inc, at a pH of just above 7, 50% of the ammonium is lost in 6 hours from 100 ml beaker under stirred condition. This information supports our observations that even at a pH below it's pK_a there could be significant loss of ammonium. Thus, use of NH_4Cl as the nitrogen source for fungal growth would result in a reduced availability of nitrogen to the fungus. This may result in a nitrogen limited condition in the early growth phase and may inhibit fungal growth.

As seen from the Figure 6.1, the loss of $NaNO_3$ is negligible as expected. Moreover, the microorganism grew well with $NaNO_3$.. Hence NH_4Cl was replaced with $NaNO_3$.. in the present study as the nitrogen source for the fungus.

6.2 Growth parameters

The effect of nutrient concentrations like glucose to nitrogen ratio, concentration of nitrogen and its relation to biomass growth, the relation between pH to biomass concentration were extensively studied. This was necessary to understand the operational behavior of the packed-bed reactor. The results of one such experiment are presented in Table 6.2. The results of this experiment are discussed in the following subsections.

6.2.1 Optimization of Glucose and Nitrogen

In almost all of the previous work (2,33,55), researchers used 10 grams of glucose and 0.12 grams of NH_4Cl per liter of growth medium. This proportion of glucose to nitrogen ratio is exceedingly high compared with the C:N (Carbon : Nitrigen) ratio in dry biomass of known microorganisms (61). Thus, experiments were carried out to find the optimum nitrogen to glucose ratio in the growth

medium. According to results in Figure 6.2.1 and Table 6.2, it is seen that for every 30 ppm nitrogen utilized, approximately 5 grams/l of glucose was consumed by the fungus out of the 7.0 grams/l initially charged. This glucose and nitrogen consumption occurred in 3 to 4 days. Approximately 2 grams of glucose were left unutilized for more than 24 hours, as observed from the figure. After this period it appeared that biomass lysis took place and the excess glucose was consumed. Similar results were observed in other experiments. From these results it was concluded that for every 0.12 grams/l of NH_4Cl (≡ 31 ppm nitrogen ≡ 0.2 grams/l of $NaNO_3$), 5.0 grams of glucose would be required. The ratio of carbon to nitrogen in this case is N : C=1:66. Although, this ratio is much higher than normally observed in bacterial species (61), this study shows that out of 10 grams/l of glucose previously used by other workers (23,5,33,55,57,48), only 50% is consumed for every 30 ppm of nitrogen. To maintain a little higher carbon source than required, 6.0 grams/l of glucose were used for each 30 ppm of nitrogen for the growth medium in all experiments in the present study.

6.2.2 Effect of High Toxic Concentration

It is worth mentioning here that the presence of toxic compounds at considerably high concentrations did not effect the growth characteristics to any extent. A 1000 ppm of phenol or 70 ppm of 246-TCP or 66 ppm of 245-TCP in separate experiments were found to have no influence on growth. These concentrations did not inhibited the growth and usual growth behavior was observed.

6.2.3 Changes in Nitrogen Concentration

Nitrogen concentration is of vital importance for the lignolytic activity as well as for growth. Thus it is very important to know the fate of nitrogen during the

growth phase of *Phanerochaete chrysosporium.* The change in nitrogen concentration with time is shown in Table 6.2. Initially the nitrogen consumption rate was much higher than that observed in later stages. However, the biomass growth rate was not proportional to that of nitrogen consumption during this growth period, as shown in Figure 6.2.3. The initial high nitrogen consumption rate could be attributed to catabolic metabolism with synthesis of different amino acids, without any appreciable increase in biomass. After this period, the logarithmic growth phase started. It is important to note that after the initial two days of growth (50 hours from inoculation) a very small amount of nitrogen was utilized compared to the initial stages. The nitrogen concentration at this stage is comparable to the concentration of nitrogen in the induction medium. This indicates that the fungus may grow to a certain extent with a small nitrogen supply. In the later stages, when the nitrogen concentration was below the detectable limit, lysis of biomass was also observed. Cell lysis was indicated by an increase in protein concentration, decrease in pH, and increase in ammonium concentration after 228 hours. Although no information or explanation is available in the literature, this phenomenon could be due to recycling of stored nitrogen in the biomass. It is interesting to notice that although a significant decrease in biomass concentration was observed, a simultaneous decrease in carbohydrate concentration (glucose) was also detected (Table 6.2). According to Merril and Cowling (49) this type of fungus can recycle nitrogen. Another possible explanation could be that the fungus stores some nitrogen in some form in the initial stages when nitrogen is abundant, and releases the nitrogen contained in protein/enzymes in later stages of the iodophase. The release of enzymes (amino acids) in a nitrogen depleted condition supports this explanation.

6.2.4 Changes in pH

The pH is a very important parameter in enzyme kinetics. Due to the enzymatic nature of this degradation process, it is important to know how the pH of the system changes with time. This information is not available in the literature. Most of the previous studies were performed in constant pH buffered medium. In the present study, pH was carefully monitored during fungal growth as shown in Tables 6.2. and 6.2.4. Figures 6.2.4.a & b show how the pH changes with time during growth and iodo phases. One can observe that initially (up to 100 hours) the pH of the solution dropped very fast. Then the rate of decrease in pH slowed down. After 252 hours, when the carbon source was completely exhausted, the pH remained steady for around 24 hours. Then the pH of the system increased and reached a steady state. The increase in pH during a substrate limited condition was also observed for mixed bacterial culture by another investigator (14).

To determine the effect of biomass concentration on the change in pH, another set of experiment was performed. The result of this experiment is presented in Table 6.2.4. It was observed that the rate of increase in pH and the total increase in pH are dependent on the biomass concentration present in the system. The biomass concentration in the previous experiment (Table 6.2) was 300 ppm and the pH increased from 2.85 to 3.11 in 48 hours. However when the biomass concentration was 5300 ppm (Table 6.2.4), the pH increased from 2.6 to 6.5 in 24 hours (Figure 6.2.4b). The pH never increased beyond 6.7 whatever might be the biomass concentration.

No specific explanation for this pH change was found in the literature. However, this could be explained as follows. Initially the fungus needed a suitable environment to metabolize the glucose, for which an acidic environment was required (7,21). It may also be possible that some of the products initially

excreted are acidic by nature and lowered the pH. Contrary to the initial phase, the pH of the system increased in the iodophase. Possibly the increase in pH is due to some basic excretory product from the fungal biomass for unknown reasons. It has also been observed that the pH always reached a maximum of around 6.7 irrespective of biomass concentration. This constant value (pH 6.7) indicates that the pH of the secreted protein could be around 6.7. The increase in pH was observed for all cases in substrate depleted conditions. However, streaking agar plates with a 7 weeks old culture under a substrate depleted condition, resulted in good growth, indicating the viability of the fungus after a long starvation period.

The above observations led us to conclude that the pH of a medium increases only in a substrate depleted condition and decreases in a growing phase. The rate of increase in pH depends on the amount of biomass present in the system.

6.2.5 Changes in Biomass and Extracellular Protein Concentrations

The biomass and protein concentrations changed significantly during the growth and subsequent phases as shown in Table 6.2 and Figure 6.2.5. In this figure we observed a direct relationship between biomass concentration and the extracellular protein concentrations. From the figure it could be noticed that a decrease in both biomass concentration and extracellular protein concentration started at the same time under the substrate limited conditions. No explanation is available for this behavior.

6.3 Degradation of 246 and 245-Trichlorophenols in shaker flasks

The biodegradative ability of *Phanerochaete chrysosporium* for 246-TCP and 245-TCP were tested in shaker flasks. The following subsections describe the results of those experiments.

6.3.1 Degradation of 246-TCP in Shaker Flasks

The degradation of 246-TCP was tested in shaker flasks. The average of the duplicate results is tabulated in Table 6.3.1 and Figure 6.3.1. Figure 6.3.1 shows that the concentration of 246-TCP decreased with time and continued to do so for about 160 hours. Then the degradation rate slowed down and reached zero for all practical purposes. Table 6.3.1 and the Figure 6.3.1 are showing an increase in the concentration of chloride ion liberated by mineralization of TCP. One can also observe that in 240 hours the 246-TCP concentration decreased from 22.40 to 15.98 ppm. By chemical stoichiometry, each ppm of TCP on mineralization would liberate 0.532 ppm of chloride ion. Thus, the decrease of 246-TCP from 22.40 to 15.98 should release 3.4 ppm of chloride ion. The actual increase in chloride concentration was 3.2 ppm, which is 91% of the theoretical amount. This result is quite satisfactory considering the use of specific ion electrodes for the measurement of chloride ion concentration. However, it is important to note that the rate of degradation is extremely low, i.e. 0.02675 ppm/hr. In another set of experiment, (Table 6.3.1b) the concentration of 246-TCP came down to 38.6 ppm from an initial 44.9 ppm in 256 hours. This corresponds to a degradation rate of 0.024687 ppm/day. Although the rates were about the same, the biomass concentration in the later case was maintained at 270 ppm, which is nearly twice that of the first case (147 ppm). However the pH in the latter case was 5.6, and 3.7 in the former case.

Nevertheless, it would appear that the concentration of biomass per se may not be a rate determining factor in the degradation of 246-TCP.

6.3.2 Degradation of 246-TCP in a Ligninase-Depleted System

One of the most important observation from these experiments (Table 6.3.1 and 6.3.1b) was that trichlorophenol degradation occurred in a ligninase depleted system. Figure 6.3.2, shows the ligninase concentration in terms of AU vs degradation of 246-TCP. In Figure 6.3.2, ligninase was detected only for the first 31 hours, after which the ligninase concentration dropped below detectable limits. However, the degradation of TCP continued uninterruptedly for at least 160 hours. This process was accompanied by stoichiometric chloride recovery indicating mineralization. These results clearly indicate that ligninase is not responsible for the degradation of 2,4,6-TCP. Lamar et al. (35) in 1991 were not able to explain the degradation of PCP by ligninase alone, and indicated that some other system might be responsible for the degradation process. In the present work degradation of 245-TCP and PCP were also observed in ligninase depleted systems (Table 6.3.5 and Table 8.9). These observations indicate that ligninase plays no role in the degradation of chlorophenols. For the case of pentachlorophenol degradation, Mileski et al. (51) suggested that another enzyme system (other than ligninase) is likely to cause degradation. Furthermore, in conjunction with the observations made by Boominathan (5) and Dosoretz (17) for the case of lignin, the effectiveness of ligninase for degradation of other compounds is highly questionable and may not be of any significance to the degradation of toxic organics. This observation could be a turning point in the research of biodegradation utilizing *Phanerochaete chrysosporium,* since to date many of the workers believe that ligninase is the responsible enzyme for this type of degradation.

We strongly believe that ligninase is just a secondary metabolite of the fungus *Phanerochaete chrysosporium* having nothing to do with the degradation of the chlorophenols or other hazardous organic compounds.

6.3.3 Time Decay of Fungal Activity

It is essential to know how long the fungus can retain its degradative ability in a substrate depleted condition. From Table 6.3.1 and Figures 6.3.1, 6.3.2, 6.3.4, and 6.3.5, it is observed that the degradation rate of 246-TCP reached zero for all practical purpose after about 150 hours (approximately one week). Similar observations were made by Ulmer et al. (67) for the case of lignin degradation. These researchers reported that after 6 to 8 days from inoculation degradation decreased to 35% of the initial rate, and in the following 7 days, the degradation ceased completely under substrate depleted conditions. According to these workers, the degradation ability of the fungus decreases with time under substrate limited condition and is completely lost after 14 days from the day of inoculation. According to our experiment, the degradation of 246-TCP stopped after 13 to 14 days, same as reported by Ulmer and his co-workers. It is possible to explain this above observation from the energy consumption point of view. According to Asada et al. (4) lignin degradation is an energy consuming process via NADH oxidation by hydrogen peroxide. When all the carbon and nitrogen sources are consumed, the fungus stops degrading. Ander et al. (2) also postulated that lignolytic enzyme production depends on the availability of carbohydrate and nitrogen sources. According to Merril and Cowling (49), in the absence of nitrogen but presence of carbohydrates, some fungal activity is possible. However, the activity does not continue for a long time. The prolonged degradative ability of this microorganism as observed by Bumpus et al (7) for 30 days and by others (36,40,41) is not well understood at this point.

6.3.4 Addition of Substrate to a Substrate-Depleted System

In the previous section we have seen that the fungus cannot retain its degradative ability for a long time after the substrates are exhausted. An experiment was conducted to determine the effect of substrate addition in a substrate depleted medium. In Table 6.3.4 and Figure 6.3.4 it can be seen that after 190 hours, the degradation practically stopped, and the 246-TCP concentration remained constant up to 240 hours. On the 242nd hour, both the glucose and nitrogen ($NaNO_3$) were added. As seen from the Table 6.3.4 and Figure 6.3.4, within a very short time after addition of substrate, degradation started again. However, the rate of degradation in this period was much less than that observed initially. Previously, the degradation continued for more than 180 hours, however after addition of glucose and nitrogen, the degradation continued for only about 100 hours, and then stopped. This phenomenon cannot be adequately explained at the moment. According to Kelly et al. (35), in-situ production of H_2O_2 and protease may inhibit the rest of the enzyme system. According to Dorosetz et al (17), protease is one of the enzymes produced by this fungus, which can cleave other enzymes and accumulate in the batch system. Moreover, if the life time of the inhibitors is more than that of the active enzymes then the inhibitors would buildup and deactivate the enzymes in the later stages of the reaction. This could be a possible explanation for the reduced degradation rate observed after the addition of substrates. The fungus cannot maintain the degradative activity for a long time in a substrate depleted condition. To maintain the prolonged enzyme activity, a continuous supply of nutrients is necessary.

6.3.5 Degradation of 245-TCP in Shaker Flasks

Degradation experiments were also conducted with 245-TCP in shaker flask. The results are shown in Table 6.3.5 and Figure 6.3.5. Similar results were obtained for 245-TCP as for 246-TCP. Degradation of 245-TCP was observed up to about 150 hours, as in the case of 246-TCP. There is no fundamental difference in the degradation process of 246-TCP and 245-TCP.

6.4 Determination of Reaction Scheme

It is clear from the results reported above that the fungus has the ability to degrade both 246-TCP and 245-TCP. However, no specific degradation scheme can be obtained from these batch experiments. Therefore new experiments were performed to determine the degradation scheme utilized by the fungus for the degradation of 246-TCP.

6.4.1 Separated Supernatant (Extracellular Protein)

Figure 6.4.1 shows the results for these experiments (run in quadruplicate and having an average standard deviation of 0.24 mg/l). Here the separated supernatant (containing 210 mg/l of extracellular fungal protein) was only able to produce a slight decrease in 246-TCP concentration. These results are directly comparable to those obtained with whole fungal slurry, since they were all conducted in parallel using the same source of slurry. However, the rate of 246-TCP disappearance was much lower in the experiments with separated supernatant than in those with whole slurry in spite of the fact that the initial protein concentration (including ligninase concentration) was nearly identical in both systems. No chloride was recovered in the supernatant experiments.

However, a new peak was detected having a higher retention time in the HPLC than that of 246-TCP. This suggests that 246-TCP was not completely degraded by the extracellular enzyme system produced by the fungus, but

formed some intermediate(s) which are quite stable in the absence of biomass. The results for a similar experiment are shown in Table 6.4.1b.

6.4.2 Separated Biomass

In these experiments (also run in parallel with the previous set) the degradation capability of the biomass alone was tested. Figure 6.4.2 shows the results of a typical experiment run in quadruplicate (standard deviation: 0.27 mg/l). During the first four hours no significant change in 246-TCP concentration was observed, and the concentration of protein in the medium surrounding the biomass remained at undetectable levels. However, during the fifth hour the biomass began releasing significant amounts of protein and continued to do so at an average rate of 0.55 mg/l hr up to the eighth hour, thus bringing the protein concentration to 2.2 mg/l. After the eighth hour the rate of protein release decreased to about 0.01 mg/l hr. This resulted in only a slight increase in the protein concentration over the next 62 hours, during which the protein concentration went from 2.2 to 2.8 mg/l.

A number of similarly repeated experiments exhibited the same phenomenon of protein appearance after the biomass had been washed, separated, and resuspended. Therefore, this rapid release of extracellular protein appears to be an intrinsic characteristic of the biomass removed from its supernatant.

During the period in which the rapid protein release occurred a marked decrease in the concentration of 246-TCP was also detected. The rate of 246-TCP disappearance during this phase was 0.79 mg/(l × hr). This rate is comparable to that observed during the initial stage of the degradation process in the presence of whole slurry. No ligninase was detected throughout the

experiment, thus confirming that ligninase does not contribute to 246-TCP degradation, as noticed before in the experiments with whole slurry.

The decrease in 246-TCP concentration following the phase of rapid protein release was also accompanied by chloride ion production (Tables 6.4.2 & 6.4.2b). However, the percentage of chloride recovery was only a fraction (typically between 32 and 79%) of the stoichiometric amount recoverable from the complete mineralization of 246-TCP. In addition, the chromatograms obtained at different times (Figure 6.4.2b) during the experiment indicated that an unidentified intermediate having a retention time higher than that of 246-TCP was being produced but not completely degraded.

Figure 6.4.2 shows that after 40 hours from the beginning or the experiment no further degradation occurred, which implies that the extracellular protein has a finite lifetime. By contrast, 246-TCP degradation continued for about 150 hours in the whole slurry experiments.

The results obtained in this set of experiments show that biomass alone cannot degrade 246-TCP unless extracellular fungal protein is present. However, the results obtained with separated supernatant indicate that the extracellular fungal protein alone is also unable to produce significant and complete degradation. *Therefore, it appears that both biomass and extracellular fungal protein are necessary for 246-TCP degradation to occur.*

In another set of experiments, 0.25 ml of a 0.18 M trichloroacetic acid solution were added 8 hours after the beginning of the experiment (i.e., after the bulk of the protein release had occurred) in order to denature the newly released protein. Following this addition, the pH was immediately adjusted to the original value. In these experiments practically no degradation of 246-TCP was observed during the next 60 hours. However, streaking agar plates with a loop immersed in this suspension produced fungal growth, thus confirming the

viability of the microorganism at the end of the experiment. These results support the idea that the presence of extracellular protein is essential to carry out the degradation process.

6.4.3 Additional Biomass

The initial procedure for this experimental set was identical to that with separated biomass discussed in the previous section. Of the four identical flasks used here (each containing 44 mg/l of biomass), two were incubated and left untouched while a new biomass addition was made to the other two on the eighth hour from the beginning of the experiment (i.e., after the first major protein release had occurred), thus bringing their biomass concentration to 60 mg/l. The results are shown in tables 6.4.3 and 6.4.3b and in Figure 6.4.3, in which each point represents the average of an experiment run in duplicate.

During the first 14 hours, both systems produced nearly identical results in terms of 246-TCP degradation. During this period the extracellular protein-time profiles for both systems were also very similar, including the rapid protein release some five hours from the start. However, beginning at time t = 14 hours, the system containing the additional biomass showed a second marked decrease in 246-TCP concentration. At the same time, a new rapid release of additional extracellular fungal protein occurred. This release occurred 6 hours after the new biomass addition was made. The lag time between such biomass addition and the following protein release was nearly the same as the lag time between the beginning of the experiment and the first protein release.This experiment not only confirms the previous conclusion that the extracellular protein and the biomass are both required for 246-TCP degradation but also shows that *the extracellular fungal protein released by the biomass is the limiting factor in the degradation process.*

6.4.4 Additional Supernatant

These experiments were similar to those discussed in the previous section with the major difference that 8 hours after the beginning of the experiments additional supernatant from the continuous reactor (instead of additional biomass) was added to two of the four flasks. The results are reported in Figure 6.4.4. Even accounting for the effect of dilution, this figure shows that the rate of 246-TCP degradation was significantly higher when additional supernatant was present at constant biomass concentration. This confirms the conclusion reached in the previous section that the extracellular fungal protein concentration is the limiting factor in 246-TCP degradation.

Figure 6.4.4 also shows that 246-TCP degradation ceased almost entirely 40 hours after the extracellular protein was released. These results are similar to those reported previously in Figure 6.4.2, indicating once again that the enzyme system contained in the extracellular protein has a finite lifetime.

The results for another experiment is also produced here in Table 6.4.4b for further confirmation of the results obtained in the previous experiments.

6.5 Oxygen Requirement

Experiments were conducted to find out whether oxygen is required in the degradation of 246-TCP. The results are shown in Table 6.5 and Figure 6.5. It is clearly seen that without the presence of oxygen no degradation of 246-TCP takes place. So presence of oxygen along with biomass and extracellular protein is necessary for complete degradation of 246-TCP.

This short experiment was very important to verify the absence of ligninase in the degradation process and was a confirmative test to our findings that the observed degradation was not due to ligninase. In 1990, Munhein and his co-workers (53) showed that ligninase can express its activity in an anaerobic

condition. However, this experimental result shows that no degradation occurred in the absence of air, which confirms beyond doubt at this point that ligninase was not present in the system, otherwise it could have degraded 246-TCP in the absence of oxygen.

CHAPTER 7

REACTION SCHEME AND DEGRADATION MODELING

The degradation of toxic compounds by *Phanerochaete chrysosporium* is an enzymatic process where the biomass or the cell-bound enzymes play an important role in breaking down the intermediates. The structural features and large size of molecules such as lignin (mw 600-1000 kilo-daltons) suggest an extracellular enzymatic reaction as the first step. Particularly in experiments with separated supernatant or separated biomass, unidentified intermediates are observed in chromatograms of the batch liquor.

Four typical chromatograms for 246-TCP are presented in Figure 7.1 to 7.4. These reveal three small peaks at 4.48, 5.07 and 5.37 minutes after injection (the 246-TCP peak occur at 3.28 minutes after injection). Similarly in Figure 7.3 and 7.4 we detected the same compounds as indicated by identical retention time in the chromatogram.

The reaction scheme proposed in Figure 7.5, TCP binds with the extracellular enzyme (s) (E_1) to form an enzyme-substrate complex (E_1C), which is then transformed to an intermediate D, releasing the enzyme (s) En. Although more than one intermediate was detected in the chromatograms, for simplicity we assume in the reaction scheme that only one intermediate is formed. In the next step, D reacts with the cell bound enzyme E_B producing the final products P.

$$E_1 + C \Leftrightarrow E_1C \tag{7.1}$$

$$E_1C \xrightarrow{k_2} E_1 + D \tag{7.2}$$

$$D + E_B \Leftrightarrow E_B D \quad (7.3)$$

$$E_B D \Leftrightarrow E_B + P \quad (7.4)$$

Where C is TCP, E_1 is the extracellular enzyme, E_1C, the enzyme-substrate complex, D the break down intermediate of the enzyme-substrate complex, E_B is the cell bound enzyme, E_BD is the second enzyme-substrate complex formed between the intermediate (D) and cell bound enzyme (E_B), and P is the final products.

As a further assumption, the enzyme activity of E_1 was assumed to follow a step function. That is, full activity for the first 32 hours to 45 hours (depending on pH) followed by no activity (see also Section 6.4.2 and 9.1).

During the experiments with separated extracellular protein containing supernatant, we observed stable unidentified intermediates as seen in Figures 7.3 and 7.4. A close look at these two chromatograms indicates that the area of the peak corresponding to the retention time 5.4 progressively increases. On the other hand although the same peaks were detected with whole slurry experiments, they are further transformed as indicated from the area of the peaks at 5.4 minutes as shown in Figure 7.1 and 7.2. Chang et al. (13), also concluded that the step leading to ring cleavage is the rate limiting step. Therefore we assumed that reaction 7.2 is the rate-limiting step.

From reaction 7.1,

$$K_1 = \frac{[E_1C]}{[E_1][C]} \quad (7.5)$$

Total concentration of enzymes (E_1^*) is given by that bound plus free enzymes:

$$[E_1^*] = [E_1] + [E_1C] \quad (7.6)$$

$$\text{or } [E_1] = [E_1^*] - [E_1C] \quad (7.7)$$

Substituting in (7.5) we obtain,

$$K_1 = \frac{[E_1C]}{[E_1^* - E_1C][C]} \tag{7.8}$$

$$\text{or, } [E_1C] = \frac{K_1[C][E_1^*]}{1+K_1[C]} \tag{7.9}$$

Now from reaction step (7.2), we can write the rate of reaction

$$-r_s = K_2[E_1C] \tag{7.10}$$

Substituting [EC] from (7.9) in (7.10) we obtain,

$$-r_s = \frac{K_1K_2[C][E_1^*]}{1+K_1[C]} \tag{7.11}$$

$$\text{or } -r_s = \frac{K_2[C][E_1^*]}{\frac{1}{K_1}+[C]} \tag{7.12}$$

We now assume that at any instant, $[E_1^*] \equiv [E_1]$ since $[E_1C] << [E_1^*]$

Therefore, by dropping the parenthesis as the symbol for concentration one obtains

$$-r_s = \frac{V_{max}^* C E_1}{K_m + C} \tag{7.13}$$

Where $V_{max}^* = K_2$ and $K_m = 1/K_1$ (7.14)

Since it is not possible to measure the particular enzyme involved in the degradation, the total protein concentration which can be measured easily was taken to be proportional to E_1:

$$E_1 = K^* E_T, \tag{7.15}$$

where K^* is the proportionality constant and E_T is the total enzyme concentration.

The proportionality constant now can be absorbed in V^*_{max}, Then,

$V_{max} = K^* V^*_{max}$

where V^*_{max} is the actual model parameter for the particular extracellular enzyme (E_1) responsible for the rate limiting step (reaction 7.2).

The reaction rate then becomes

$$-r_s = \frac{V_{max} C E_T}{K_m + C} \tag{7.16}$$

A mass balance for 246-TCP over a CSTR at steady state with a residence time τ results:

$$C_{in} - C_{out} - r_S \tau = 0 \tag{7.17}$$

in which the rate of disappearance is given by a equation (7.16)

$$r_s = \frac{V_{max} C_{out}}{K_m + C_{out}} E_T \tag{7.18}$$

The resulting mass balance equation for a continuous flow reactor operating at steady state can be rearranged as

$$\frac{E_T \tau}{C_{in} - C_{out}} = \frac{K_m}{V_{max} C_{out}} + \frac{1}{V_{max}} \tag{7.19}$$

The kinetic parameters (V_{max} and K_m) can be obtained from these expressions by linear regression of the bench scale data. Equation (7.19) can be used to find the parameters K_m and V_{max} by plotting $1/C_{out}$ versus the group

$$\frac{E_T \tau}{C_{in} - C_{out}}.$$

Then by linear regression the inverse of the intercept would give V_{max} ,and K_m can be obtained from the slope of the straight line.

Similarly, the comparable equation for a batch reactor is:

$$K_m \ln\left(\frac{C_{in}}{C_{out}}\right)+(C_{in} - C_{out}) = V_{max} E_T t \qquad (7.20)$$

Once the parameters K_m and V_{max} are obtained, they can be used to predict the performance of other reactors.

CHAPTER 8

RESULTS FOR PACKED-BED CONTINUOUS REACTOR STUDIES

From the results of the batch experiments, a reaction scheme and kinetic model were formulated, as cited in Chapter 7. However, the model constants were found using a continuous packed-bed reactor, as described in this section.

8.1 Degradation of 246-TCP

After proving the degradative ability of the fungus against both 246-TCP and 245-TCPs, experiments were conducted in the packed-bed continuous reactor. The results of one typical run of such experiment are shown in Table 8.1 and Figure 8.1. This particular experiment was performed using reactor R1 and continued for 21 days. The retention time for this particular run was 19.6 hours. Steady state was reached after about 150 hours. It had been found that the time required to reach the steady state of the system is 7 - 9 times the residence time (R.T.) of the reactor. Initially, the biomass loss from the bed was slightly higher and slowly attained a steady state. The reason for this increased biomass loss was not understood. From Figure 8.1, one can observe that at steady state the 246-TCP concentration decreases from an inlet concentration of 46.5 ppm to 1.66 ppm at the outlet. This mineralization process was associated with chloride release. The chloride ion concentration increased from 6 ppm at the inlet to 28 ppm at the outlet. Thus 97% of stoichiometric amount of chloride is liberated in the process.

In general, the continuous packed-bed reactor can perform more effectively than a batch reactor since :

- Any inhibitor in the reactor can not buildup in a continuous system.

- The enzymes in the reactor remain active since the residence time in the reactor is less than the life time of enzymes which is around 30 to 45 hours, as seen in previous results in chapter 6.

8.2 Effect of Glucose Concentration on 246-TCP degradation

It was necessary to know the effect of glucose and nitrogen concentrations on the degradation of 246-TCP, and on the behavior of the packed-bed reactor. The steady state results for six run are given in Table 8.2 and Figure 8.2. Figure 8.2 shows a plot of the outlet glucose concentration vs. the outlet 246-TCP concentration. In these experiments the inlet 246-TCP concentration was maintained constant for all practical purposes. The figure shows for a glucose concentration within a range of 4 to 50 ppm, the degradation of 246-TCP remained constant. However, as the outlet glucose concentration reached concentrations above 60 ppm, the degradation rate of 246-TCP sharply decreased. No clear reason is known for this phenomenon. However, it is known that degradation of lignin requires starvation of nitrogen, carbohydrates and sulfur (39). It is most likely that above a certain range of glucose concentration, the metabolic characteristics of the microorganism change and this may change the type of enzyme liberated by the fungus.

Some other experiment (Table 8.3.1) indicated that a glucose concentration at the outlet as a low as 0.05 ppm is enough to maintain continued activity and viability of the microorganisms. The effect of glucose at higher concentration of glucose (above 109 ppm) was not studied.

Another very important feature of these experiments is the decrease in pH with increase in outlet glucose concentration, which is most likely associated with a change in metabolism. The same observation was made during the batch

culture, when the pH of the system dropped along with the consumption of glucose.

8.3 Effect of Nitrogen Concentration on 246-TCP Degradation

Experiments were also performed in which the nitrogen concentration was varied keeping all other parameters unchanged. Three sets of data are given in Table 8.3.1 to 8.3.3, and Figures 8.3.1 to 8.3.3. From Table 8.3.1 (Set 1) and Figure 8.3.1, one can observe that at an outlet concentration of 1.0 ppm of nitrogen, the system reached a steady state within 120 hours and degradation occurs as expected.

However in another experiment, in which the outlet concentration of nitrogen was maintained at 5 ppm (Set 2, Table 8.3.2), the system never reached steady state as seen in the Figure 8.3.2. In this Figure we see the pH slowly increased, with a loss of biomass from the reactor. In this case (set 2) the outlet biomass concentration was abnormally high in the range of 130 to 180 mg/l compared to 10.0 to 15 mg/l in normal conditions (Set 1). The process was monitored for 9 days, and did not attain steady state within this period. We visually observed a slow decay in biomass layer from the packed bed, and the white biofilm turned faint brownish color.

In this experiment (Set 3, Table 8.3.3), a much higher initial nitrogen concentration was used. Within 96 hours, complete washout of the system was observed and the pH reached 6.48. The outlet biomass concentration was as high as 486 mg/l, compared to 10 to 20 mg/l in normal operation.

8.4 Effect of Nitrogen Concentration at Constant pH

From the results of Section 8.3, we observed that at some high nitrogen concentration the packed-bed reactor did not attain steady state. We also

observed that during the transient phase, the biomass was depleted from the packed-bed with simultaneous increase in pH. It is important to know whether the biomass loss is due to increase in pH, to the high nitrogen concentration, or both. To answer this question, a single experiment was conducted at a controlled pH and the same inlet concentration as set #3. The results of this run are given in Table 8.4 and Figure 8.4. When the pH in the reactor was maintained at 5.6, the rate of biomass loss from the bed was smaller in comparison to the previous experiment (Table 8.3.3), and it took longer for complete washout; however washout still occurred.

Since It was not possible to achieve steady state at outlet nitrogen concentrations above 1 ppm, comparative data could not be obtained for the effect of nitrogen concentration on the degradation rate of 246-TCP.

8.5 Effect of Shear on 245-TCP Degradation

During the literature review (Chapter 2) it was mentioned that the fungus is very sensitive to shear. This is very important for operation of an industrial continuous reactor, and must be considered for bio-reactor design purposes. This experiment was conducted to find a critical volumetric flux (an indirect measurement of shear) beyond which shear has an impact on fungal growth and 245-TCP degradation. The results of this experiment are given in Table 8.5. Figure 8.5 is a plot of outlet 245-TCP concentration versus the volumetric flux. Below a volumetric flux of 1.2 ml/cm^2/min, the outlet concentration didn't change. However, at a volumetric flux of 1.9 ml/cm^2/min, the degradation was reduced drastically, and stopped completely at a volumetric flow rate of 2.8 ml/cm^2/min. In addition to reduced degradation, the glucose concentration at the reactor outlet also increased with increasing shear. This observation is in line with that observed by Toma et al. (67). These workers defined the shear-related reduced

metabolic activity as ***turbohypobiosis***. At high shear, a reduced DNA production was observed for various bacterial culture (67), and they concluded that above a certain shear, the catabolic and anabolic activity of most microorganism decreases. It was not possible to study the shear effect below a volumetric flux of 0.8, since below a recirculation rate of 6.0 ml/min, the reactor characteristics change and shift towards plug flow.

Thus it is concluded that for best degradation activity in packed-bed, a volumetric flux of close to 1.0 ml/min/cm^2 should be used. This critical volumetric flux (ml/min/cm^2) is an important criterion for designing a packed-bed immobilized type reactor.

8.6 Determination of Model Parameters

The packed-bed CSTR was used to determine the kinetic parameters V_{max} and K_m. According to batch studies, the enzymes are active for a period of 30 hours at pH 5.6 and 45 hours at of pH 4.6. Therefore to prevent enzyme deactivation, the residence time in the reactor was always maintained below 30 hours. The pH was varied and the kinetic parameters were found at different pH values. The method of finding the kinetic parameters was described in Chapter 7.

Experiments were conducted for both 246-TCP and 245-TCP at pH 3.6, 4.6 and 5.6. The results of the steady state experiments are tabulated in Table 8.6.1 through 8.6.6. The linearly regressed lines for each compound at three different pH values are shown in Figure 8.6.1 to Figure 8.6.6. For the case of 246-TCP at pH 5.6, 12 experimental runs were performed. Since each run takes almost a week, for other pH values, a fewer number (5 to 7) of experiments were conducted. The correlation coefficients varied from 0.977 in Figure 8.6.4, to 0.997 in Figure 8.6.3.

The V_{max} and K_m values at different pH are shown in Table 8.6.7 and 8.6.8 for 246-TCP and 245-TCP respectively. The V_{max} and the K_m values are strongly dependent on pH.

8.6.1 Effect of pH on V_{max}

The variation of V_{max} for both 245-TCP and 246-TCP is shown in Tables 8.6.7 and 8.6.8, and in Figure 8.6.7. For both 246-TCP and 245-TCP, V_{max} shows a similar trend. As the pH increases V_{max} also increases. However, at all pH values, V_{max} for 246-TCP is much higher than that of 245-TCP, and they differ by approximately one order of magnitude. As shown in Chapter 7, V_{max} is the rate constant for the rate limiting step, when the enzyme-substrate complex breaks down into its products according to reaction 7.2. According to the results obtained here, the rate constant for the rate determining step increases with increasing pH. These results imply that at higher pH the enzyme activity increases. This does not necessarily mean that the degradation rate would be higher at higher pH, since the overall rate of degradation depends on both V_{max} and K_m. The K_m also changes with pH as described in the next section.

8.6.2 Effect of pH on K_m

The K_m changed over the pH range 3.6 to 5.6. According to equation 7.5 and 7.14,

$$K_m = \frac{[E_1C]}{[E_1][C]}$$

If the complex E_1C has more than one oxidation state, (which is often the case), it will be a function of pH. This occurs via a zwiterion ion effect. Moreover, this type of phenomenon is commonly found for many enzymatic reactions (61).

The K_m values for both the TCP's are shown in Tables 8.6.7 and 8.6.8, and in Figure 8.6.8. Contrary to the trend of variation of V_{max} with pH, K_m varied with pH in a non-linear fashion. Furthermore, the pattern of changes in K_m with pH were different for 246-TCP and 245-TCP. For the case of 246-TCP, K_m decreased from 27.13 ppm to 16.74 ppm as the pH increased from 3.6 to 4.6. However for 245-TCP, the corresponding K_m value increased sharply from 2.20 ppm to 28.23 ppm as pH increased from 3.6 to 4.6. The K_m for 246-TCP increased from 16.74 ppm to 34.82 ppm as the pH increased from 4.6 to 5.6 whereas for of 245-TCP it decreases from 28.23 ppm to 9.63 ppm.

K_m is the inverse of the equilibrium constant for reaction 7.1. Thus, a low K_m, denoting a high equilibrium value for reaction 7.1, is desirable.

8.7 Verification of Model Parameters at Other pH Values

The model constants (V_{max} and K_m) were determined at three discrete pH values of 3.6, 4.6 and 5.6. At other pH values, V_{max} and K_m were determined by interpolation of the curves in Figures 8.6.7 and 8.6.8. Outlet reactor concentrations were then calculated and compared to experimental data. Results are given in Tables 8.7.1 and 8.7.2. The predictions matched reasonably well with the experimental values, with an average error of about 10%. Thus, the model can be used to predict the degradation of both model compounds within a range of pH from 3.6 to 5.6.

8.8 Degradation of Phenol

In this experiment, the degradative ability of the fungus against phenol was tested. Experiments were conducted at four pH values. The results are shown in Table 8.8 and Figure 8.8. Practically no degradation of phenol took place in any of the experiments. Degradation of phenol is also not reported in the

literature, in spite of the fact that lignin has a phenolic structure. One possible explanation is offered as follows:

According to Shoemaker et al. (64) the degradation process proceeds via a reduced oxygen species. Phenol is more electropositive compared to trichlorophenols, thus it may be possible that the electron transfer does not take place from the enzyme to the phenol. If this is true, then we should see higher degradation rates for more highly chlorinated phenols. So the next experiment was conducted to test the degradation of pentachlorophenol.

8.9 Degradation of Pentachlorophenol

The results of the pentachlorophenol degradation experiment is shown in Table 8.9. and Figure 8.9. Within 8.6 hours, the PCP concentration decreased from 23.4 ppm to 1.66 ppm. Compared to the results of Lin et al.(45), the rate of degradation obtained in the present study is more than 300 times greater. It is worth mentioning here that ligninase was not detected throughout this experiment.

CHAPTER 9

VALIDATION OF THE MODEL IN BATCH SYSTEM AND OPTIMIZATION OF PH

In this chapter, the model was used to predict the rate of degradation of 246-TCP in batch systems. These values were compared with the experimental data. In addition, a sensitivity analysis was performed to optimize the operating pH.

9.1 Comparison of the model with Batch experimental data

As described in the previous chapters, the kinetic parameters were found from continuous reactor experiments. However, to check the validity of the model, it was tested against batch experimental results. To have a batch system with fresh enzymes, these experiments were performed by separated biomass as described in Chapter 5. The results for these experiments with 246-TCP are shown in Figures 9.1.1 to 9.1.3. The predicted concentrations of 246-TCP were calculated using equation 7.20 as shown in the same Figures.

In Figure 9.1.1 (pH 5.6), we observe that the predicted result agrees with the experimental data for about 30 hours. A similar observation was also made with separated biomass experiments. As a possible explanation we suggest that the enzyme gets deactivated after a certain period. The time for which the enzymes remain active (life time of enzymes) depends on the pH of the system. The approximate life time for these enzymes are 32 hours at pH 5.6, and 45 hours at a pH 3.6 and 4.6. Considering the error involved in the measurements of TCP concentration and protein concentration, the experimental results were in good argument with the predicted results. These results not only validate the

model, but also prove that the enzyme has an definite life time, and the rate of deactivation depends on the pH of system.

9.2 Optimization of pH

Many contradictory reports have been published regarding the optimum pH for degradation by *Phanerochaete chrysosporium*. The range of pH varied from 3.0 to 6.0. For example, according to Kersten and Kirk (36), the optimum pH for this degradation process is around 6.0. On the contrary, Aitken and his co-workers reported the optimum pH to be around 3.0 for o-cresol. Harvey et al. (27), reported the optimum pH to be around 4.6. Here we suggest that most likely all of them are right, and the optimum pH depends on the compound and its concentration.

According to the proposed model, the rate of degradation depends on V_{max} and K_m. The kinetic constants V_{max} and K_m are functions of pH, and the life time of the enzymes also depends on pH. To optimize pH for any particular degradation process, results can be obtained by simulation, using the previously obtained kinetic parameters. In these simulations, a constant concentration of protein of 10 ppm was used in all cases. The time for which the enzyme remains active is 32 hours for pH 5.6, and 45 hours for pH 3.6 and 4.6. The initial concentrations of either 246-TCP and 245-TCP were 2.0, 10.0, 25.0 and 50.0 ppm. Three different pH values (3.6, 4.6 and 5.6) were studied. The results are shown in Figure 9.2.1 to 9.2.8.

In Figure 9.2.1, for the case of 246-TCP (having a initial concentration of 2.0 ppm) we observe, that for both pH 4.6 and 5.6, the degradation rate is practically same and for pH 3.6 the degradation is lower. Thus, a suitable pH would be either 4.6 or 5.6. So one can choose between either pH 4.6 or pH 5.6 for the degradation of 246-TCP with an initial concentration of 2.0 ppm.

However, as has been shown previously, the enzyme can retain its effectivness for about 45 hours at a pH of 4.6 compared to only 32 hours at pH 5.6. Therefore, pH 4.6 would be the right choice in this case.

In the case of 245-TCP in Figure 9.2.2 for the same initial concentration, the choice is between pH 3.6 or 5.6. Due to longer activity of the enzyme at pH 3.6, this would be a better choice. So the suitability of pH varies from case to case, depending upon the initial concentration of the toxic compound.

However, in case of both 246-TCP and 245-TCP, as the initial concentration goes up, higher pH (5.6) becomes the obvious choice as seen in Figures 9.2.3 to 9.2.8.

CHAPTER 10

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

10.1 Conclusions

- It was experimentally verified that *Phanerochaete chrysosporium* can degrade both 246-trichlorophenol and 245-trichlorophenol, and pentachlorophenol, but not phenol.
- Degradation of tri-chlorophenols and pentachlorophenol can occur in a ligninase-depleted system.
- The fungus can not retain its degradative ability for more than 5-7 days under nutrient-depleted conditions. As the nutrients are depleted, the pH of the system rapidly increases.
- The optimum ratio of sodium nitrate to glucose in both growth medium and induction medium is about 1:170 by weight.
- For maximum degradation, the optimum concentration of glucose should be below 50 ppm, that of nitrogen should be below 1.0 ppm and the volumetric flux below 1.0 ml/cm^2/min.
- Neither biomass nor the extracellular enzyme alone can degrade 246-TCP or 245-TCP. Both biomass and extracellular enzyme are necessary to mineralize these compounds.

- The concentration of the extracellular enzyme secreted by the biomass is rate limiting in this process.
- An enzymatic model has been developed, and the model parameters determined from continuous reactor experiments.
- The model parameters developed in the continuous reactor were able to predict the batch performance fairly well.
- A packed-bed on which the fungus was immobilized can be operated continually for 8 to 10 months.
- The values of the model parameters V_{max} and K_m strongly depends on pH. The optimum pH depends on the concentration of the compound to be treated.

10.2 Recommendations for Future Work

- This study indicates that higher nitrogen concentration has a deleterious effect on both the microorganism and degradation the rate. However, the use of slow releasing nitrogenous material may be helpful in eliminating the problem.
- Studies must be done on fundamental understanding of enzyme deactivation.
- Studies should be performed with mixed substrates.
- Pilot plant studies, using industrial effluents, should be conducted

APPENDIX I

TABLES

Table 6.1

Effect of Air Stripping on Nitrogen Source in Shaker Flask

pH = 5.0

Time (Hrs)	NH_4Cl (ppm)	$NaNO_3$ (ppm)
0	31	34.6
1	30	34.6
2	28.7	34.6
3	27	34.6
4	26.1	34.5
15	24	34.3
25	18	34.2
43	12	34.2
79	7	34.1

Table 6.2

Parameter Changes During Growth of *Phanerochaete chrysosporium*

Time (hrs)	pH	N as $NaNO_3$ (ppm)	N as NH_4Cl (ppm)	Glu (ppm)	Prot. (ppm)	BM (ppm)
0	4.55	29.0	0.4	6916	1.0	0
1.5	4.54	25	0.8	6889		0
5.25	4.51	16.0	1.0		33	10
25.0	3.39	4.5	2.0	5575	300	17
49.0	3.30	1.3	1.2	5100	758	30
75.0	3.20	0.7	1.0		947	
80.0	3.12	0.3	1.3	4416	823.3	190
104.0	2.94	0.2	1.4	3369	870	
128.0	2.91	0	0.9	2834	1106	310
152.0	2.88	0	1.3	2090	1292	
178.0	2.85	0	2.1	2000	1341	330
204.0	2.82	0	0.2	1990	-	
228.0	2.81	0	1.0	1307	2011	300
252.0	2.80	0	1.4	492	2083	
276.0	2.79	0	2.0	316	2024	280
300	2.81	0	3.7	0	-	
324.0	2.82	0	9.0	0	1911	260
328.0	2.83	0	11.0	0	-	
376.0	2.86	0	9.2	0	1851	
424.0	3.11	0	6.2	0	1830	239

Table 6.2.4

Relationship Between pH and Biomass Concentration

Time (hrs)	pH	N (ppm)	Glu (ppm)	Prot. (ppm)	BM (ppm)
0	4.42	31	5990	470	3000
2.0	4.2	9	5950	700	
5.0	3.7	3	4740	1000	3050
10.0	3.2	1	4000	3670	
24.0	2.9	0.8	1300	4200	4800
30.0	2.7	0.4	400	4700	
36.0	2.63	0	0	-	5300
40	2.82	0	0	-	
42	3.4	0	0	4610	-
53.0	5.8	0	0		5130
72.0	6.4	0	0	-	
75.0	6.5	0	0	3850	
80.0	6.58	0	0		4990
96.0	6.62	0	0		
108.0	6.66	0	0		
132.0	6.68	0	0	3650	4740

Table 6.3.1

Degradation of 246-TCP by Whole Slurry in Shaker Flask

Time (Hrs)	246- (ppm)	Prot (ppm)	pH	Glu (ppm)	N (ppm)	B.M (ppm)	Cl (ppm)	Lig (AU)	Cont (ppm)
0	22.40	326	3.73	37	1.5	147	17	0	26.43
12	22.07	327	3.70	26	1.2		17.1	0	
24	21.15	329	3.63	17	0.2	149	17.3	0	
36	20.88	330	3.61	7	0		17.6	0	
48	20.08	334	3.61	0	0	149	17.8	0	
72	19.54	341	3.60	0	0		18.6	0	
96	18.74	349	3.60	0	0		18.9	0	26.33
120	17.97	359	3.61	0	0		-	0	
143	17.13	367	3.62	0	0	138	19.3	0	
168	16.84	379	3.64	0	0		19.8	0	
192	16.53	386	3.67	0	0		20.1	0	
216	16.11	404	3.71	0	0		-	0	25.81
240	15.98	390	3.76	0	0	133	20.2	0	
265	15.79	392	3.82	0	0		20.2	0	
288	15.59	394	3.88	0	0		20.3	0	25.70
311	15.44	387	3.94	0	0	120	20.4	0	
336	15.38	380	4.10	0	0		20.4	0	
384	15.40	360	4.11	0	0	119	20.45	0	25.44

Table 6.3.1b

Degradation of 246-TCP by Whole Slurry with Adjusted pH

pH adjusted to 5.6 by KOH solution,
Initial Biomass Concentration=270 ppm

Time (hrs)	2,4,6 (ppm)	Protein (ppm)	pH	Glu (ppm)	N (ppm)	Lig (AU/5 min)	Cl (mg/l)	Cl Rec (%)
0	44.89	217	5.6	60	1	0.011	8	-
6	44.16	219	5.56	26	0.4	0.009	N.R.	N.R.
10	43.86	223	5.54	11	0.2	0.007	8.3	54.02
22	43.06	229	5.52	6	0.1	0.002	8.8	81.08
31	42.26	231	5.47	3	0	0	9.2	84.62
48	41.72	247	5.39	1	0	0	9.6	93.61
72	41.07	261	5.28	0	0	0	9.8	87.39
96	40.83	287	5.27	0	0	0	9.9	86.79
108	40.43	298	5.36	0	0	0	10	83.17
133	40	304	5.47	0	0	0	10.5	94.82
157	39.7	289	5.49	0	0	0	10.7	96.48
205	39.2	267	5.5	0	0	0	10.9	94.52
220	38.97	258	5.5	0	0	0	11	93.98
256	38.57	250	5.53	0	0	0	11.2	93.90

Table 6.3.4

Degradation of 246-TCP by a Whole Slurry in a Substrate-Depleted Medium

Initial Biomass Concentration=2.42 gm/l Final Biomass Concentration 2.04 gm/l

Time (Hrs)	246 (ppm)	Prot (ppm)	pH	Glu. (ppm)	N (ppm)	Cl (ppm)	Lig. (AU)	BM (ppm)	Cont. (ppm)
0	49.6	2473	4.31	0	0	51.0	0.009	2420	51.4
24	47.8	2474	4.09	0	0	52.0	0.009	-	51.4
36	45.71	2489	4.56	0	0	53.0	0.008	-	
72	43.42	2546	4.98	0	0		0.003	2400	
96	41.16	2600	5.6	0	0	54.0	0	-	51.1
108	40.0	2689	5.69	0	0		0	-	
120	39.21	2711	5.70	0	0	55.7	0	-	
144	38.60	2800	5.72	0	0	56.4	0	-	50.66
168	37.08	3158	5.72	0	0	57.0	0	-	
192	36.84	3087	5.75	0	0	57.2	0	-	50.08
216	36.60	3000	5.80	0	0	57.2	0	-	
240	36.48	2922	5.82	0	0	57.3	0	2040	49.88
242	36.49	2940	5.80	4000	8	57.3	0	-	-
250	35.84	3000	5.41	3830	2	-	0	2134	-
266	35.00	3300	4.62	2160	1	58.6	0	-	-
290	34.49	3500	4.30	700	0.3	-	0	2300	49.72
310	34.00	3797	4.20	0	0	59.1	0	2200	-
316	33.78	3544	5.74	0	0	59.1	0	-	-
340	33.60	3382	6.30	0	0	59.2	0	2100	49.37

Table 6.3.5

Degradation of 245-TCP by Whole Slurry in Shaker Flask

pH adjusted to 5.6 by 0.1 N KOH solution

Time (Hrs)	246 (ppm)	Prot (ppm)	pH	Glu. (ppm)	N (ppm)	Cl (ppm)	Lig. (AU)	BM (ppm)	Cont. (ppm)
0	33.5	200.1	5.60	92	1.8	0.4	0.009	240	35.0
6	33.16	201	5.57	74	1.3	0.4	0.009	-	
10	32.00	203	5.56	33	0.3	0.7.	0.008	-	
22	31.51	209	5.54	11	0.1	1.2	0.003	-	
31	31.13	211	5.49	4	0	1.3	0	-	
48	30.72	217	5.43	0	0	1.9	0	267-	35.0
72	28.59	228	5.2	0	0	2.2	0	-	
96	27.17	240	5.18	0	0	2.9	0	-	
108	26.88	238	5.24	0	0	3.0	0	232	
132	26.50	229	5.43	0	0		0	-	
156	26.00	223	5.54	0	0	3.3	0	-	
180	25.89	210	5.60	0	0	3.4	0	-	34.89
248	25.75	205	5.62	0	0	3.4	0	-	
256	25.68	205	5.64	0	0	-	0		
280	25.49	189	5.68	0	0	3.5		225	34.60

Table 6.4.1

Degradation of 246-TCP by Supernatant at pH 5.6

Time (Hrs)	246 (ppm)	Prot (ppm)	Cl (ppm)	pH	Cont (ppm)
0	45.06	210	5	5.6	44.85
2	44.86			5.6	
5	44.65	210	5.0		
6	44.51				
8	44.33	211		5.6	
16	43.29	209	5.0		44.7
24	44.77			5.6	
32	44.0	212	5.0		
40	43.67			5.6	
52	43.39	211	5.05		43.98
70	43.28	208	5.1	5.64	43.60

Table 6.4.1b

Degradation of 246-TCP by Supernatant at pH 4.6

Time (Hrs)	246 (ppm)	Prot (ppm)	Cl (ppm)	pH	Control. (ppm)
0	32.33	177	5.6	4.6	34.54
1	32.50				
2	32.32				
3	32.35	176			
4	32.32			4.6	
6	32.27	177	5.6		
8	32.36				
10	32.22		5.6	4.60	34.58
11	32.18				
22	31.68	178			34.40
26	31.40				
36	31.1				
48	30.94	175	5.7	4.60	34.28
53	30.78				
61	30.58				
70	30.55	177			
80	30.59	176	5.73	4.60	34.03

Table 6.4.2

Degradation of 246-TCP by Separated Biomass at pH 5.6

Biomass Concentration=44 ppm

Time (hrs)	246-TCP (ppm)	Protein (ppm)	pH	Cl (ppm)	Cum. Cl Recovery (%)
0	45.13	0	5.6	4	0
1	45	0	5.6	4	0
2	44.89	0	5.6	4	0
3	44.93	0	5.6	4	0
4	44.97	0	5.6	4	0
5	44.88	0.7	5.6	4	0
6	44.73	1.9	5.6	4	0
8	44.78	2.2	5.6	4.15	79.48
9	44.03	2.2	5.6	4.25	42.15
10	43.99	2.27	5.6	4.3	48.81
12	43.1	2.33	5.61	4.35	31.98
14	42.8	N.R.	5.61	N.R.	N.R.
16	42	2.4	5.61	5.17	69.33
18	41.39	N.R.	5.62	N.R.	N.R.
27	40.71	2.48	5.62	5.6	67.13
32	40	2.52	5.62	5.75	63.27
36	39.88	2.58	5.63	5.95	68.89
40	39.54	2.63	5.63	6.1	69.67
54	39.4	2.78	5.64	6.1	67.97
70	39.42	2.8	5.65	6.2	71.46

Table 6.4.2b

Degradation of 246-TCP by Separated Biomass at pH 4.6

Biomass Concentration=69.0 ppm

Time (Hrs)	246-TCP (ppm)	Protein (ppm)	Cl (ppm)	pH	246-TCP Control. (ppm)
0	31.63	0	1.0	4.6	34.54
1	31.60	0	1.0	4.6	
2	31.62	0	1.0	-	
3	31.31	0	1.0	-	
4	31.62	0.19	1.0	4.6	
6	31.57	3.7	1.0	-	
8	31.56	4.96	1.1	-	
10	31.22	5.0	1.3	4.6	34.58
11	31.0	5.07	-		
22	30.0	5.11	2.6	4.62	34.40
26	29.0	5.18	-		
36	28.0	5.18	2.6	4.62	
48	26.41	5.13	-		34.28
53	26.18	5.29	3.8	4.63	
61	26.03	5.40	-		
70	25.89	5.37			
80	25.92	5.63	4.0	4.63	34.03

Table 6.4.3

Table 6.4.3

Degradation of 246-TCP with Additional Biomass at pH 5.6

With Addition					Without Addition				
Time (Hrs)	246 (ppm)	Prot (ppm)	BM	Cl (ppm)	246 (ppm)	Prot (ppm)	BM	Cl (ppm)	Cont. (ppm)
0	54.6	0	24	0	54.63	0	24	0	53.1
2	54.7	0	24	0	54.6	0	24		
3	54.68	0	24	0	54.7	0	24	0	53.3
4	54.70	0	24	-	54.6	0	24		
5	54.72	0	24	-	54.6	0	24	0	
6	54.30	0.3	24	-	54.36	0.3	24		53.1
8	54.1	1.7	60	0.2	54.07	1.75	24	0.2	
10	53.51	1.9	60		53.36	2.0	24		
12	53.21	2.0		0.6	53.14	2.0	24	0.4	
14	52.74	2.0	60		52.83	2.0			52.90
16	52.45	3.7		1.1	52.68	2.0			
18	52.30	4.1	60		52.58	2.1	24		
32	48.1	4.2			51.86	2.11		1.0	
48	45.0	4.4	60	5.8	51.74	2.2	24	1.1	52.80

Table 6.4.3b

Degradation of 246-TCP with Additional Biomass at pH 4.6

With Addition					Without Addition				
Time (Hrs)	246 (ppm)	Prot (ppm)	BM (ppm)	Cl (ppm)	246 (ppm)	Prot (ppm)	BM (ppm)	Cl (ppm)	Cont (ppm)
0	21.9	0	89	0	21.9	0	89	0	21.9
1	22.0	0		0	-	0		0	
2	21.87	0		0	21.9	0		0	
3	21.9	0		0	-	0		0	
4	22.07	0.5		0	21.0	0.9		0	
6	22.0	-		0	22.0	4.5		0	
8	21.7	8.6	154	0	21.80	9.1	-	0	21.9
10	21.4	9.2		0.2	21.50	9.20		0.2	
12	20.8	9.4		0.5	21.0	9.17		0.4	
14	20.4	13.1		0.4	20.81	9.40	-	0.4	
16	19.2	17.0		0.7	20.30	9.20		0.7	
18	18.4	18.7		2.0	19.52	9.30		0.9	
20	17.7	19.0	154	2.4	19.5	9.60	89	1.2	21.8

Table 6.4.4

Degradation of 246-TCP with Additional Supernatant at pH 5.6

Initial Biomass Concentration= 22 mg/l
10 ml Supernatant Added

Time	With Additional				Without Addition				
(Hrs)	246 (ppm)	Prot. (ppm)	Cl (ppm)	pH	246 (ppm)	Prot (ppm)	Cl (ppm)	pH	246 (ppm)
0	49.6	0	0	5.6	49.64	0	0	5.6	44.85
1	49.6	0	0		49.58	0	0	5.6	
2	49.6	0	0	5.6	49.67	0	0	-	
3	49.7	0	0		49.63	0	0	-	
4	49.67	0	0	5.6	49.67	0	0	-	
5	49.62	0.1	0		49.62	0	0	-	44.91
6	49.60	0.9	0	5.6	49.60	0.9	0	5.6	
7	49.52	1.17	0.1	5.60	49.60	1.9	0.2	5.6	
8	44.20	3.32	0.2	5.56	49.30	2.10	0.4	-	
9	43.49	3.40	0.3	5.56	48.90	2.12	0.7	-	
10	42.97	3.38	0.4	5.56	48.54	2.13	1.0	5.6	44.71
12	41.58	3.35	0.71	5.56	47.8	2.14	1.2	5.6	44.82
14	40.32	3.33	1.37	5.56	47.10	2.14	1.5	5.6	
16	39.08	-	2.0	5.56	46.10	2.14	1.9	-	
18	38.54	3.29	2.37	5.57	45.30	2.16	3.4	-	44.83
20	37.41	3.31	2.60	5.57	42.00	2.18	3.5	5.60	
30	35.16	3.37	3.0	5.57	41.3	2.18	3.6.	-	
32	34.62	3.36	4.0	5.57	40.40	2.18	-	5.60	
34	32.87		5.0	5.57	39.90	2.18	5.0	5.60	44.70
36	32.22		5.1	5.57	38.7	2.19	5.0		
40	31.85	3.39	5.50	-	36.90	2.20	5.1	5.61	
54	30.9	3.39	5.80	5.57	36.73	2.20	-		
56	30.5	3.41	5.90	-	36.40	2.22	5.1	5.61	
60	29.63	3.43	-	5.58	36.20	2.22	-	-	44.47
68	29.11	3.47	6.0		36.00	2.22	5.2	5.62	
70	28.89	3.41	6.1	5.58	36.00	2.24			
72	28.80	3.44	6.3		35.89	2.27	5.3	5.62	
80	28.88	3.60	6.3	5.60	35.83				44.52
108	28.63	3.89	6.4	5.62	35.73	2.30	5.4	5.63	

Table 6.4.4b

Degradation of 246-TCP by Additional Supernatant at pH 4.6

Biomass Concentration=89 mg/l
5 ml of supernatant added

	With Additional Supernatant				Without Additional Supernatant				Cont.
	246 (ppm)	Prot (ppm)	Cl (ppm)	pH	246 (ppm)	Prot (ppm)	Cl (ppm)	pH	246 (ppm)
0	22.0	0	0	4.6	21.9	0	0	4.6	21.9
1	-	0	0		-	0	0		
2	22.0	0	0		21.9	0	0		
3		0	0	4.6	-	0	0		
4	21.9	0.5	0		21.0	0.9	0		
6	21.94	5.0-	0		22.0	4.5	0		
8	21.75	17.4	0	4.6	21.80	9.1	0		21.9
10	21.21	17.6	0.1		21.50	9.20	0.2	4.6	
12	20.50	17.7		4.6	21.0	9.17	0.4		
14	19.72	17.8	1.4		20.81	9.40	0.4		
16	19.0	17.8		4.6	20.30	9.20	0.7	4.6	
18	18.0	18.0	2.1		19.52	9.30	0.9		
20	17.5	18.1	2.4	4.6	19.5	9.60	1.2	4.6	21.8

Table 6.5

Oxygen Requirement During Degradation of 246-TCP by Whole Slurry

Biomass concentration=137 mg/l; pH adjusted to 5.6

In Presence of Air in Shaker Flask					In absence of Air in air tight bottle				
Time (Hrs)	246 (ppm)	Prot (ppm)	DO (ppm)	Cl (ppm)	246 (ppm)	Prot (ppm)	DO (ppm)	Cl (ppm)	Cont (ppm)
0	29.6	185	7.0	2.0	30.7	186	0	2.0	30.3
2	29.6	187	6.7	2.0	30.4	186	-	2.0	30.3
6	29.41	190	6.5	2.0			-		
17	28.61	190	6.5	2.4	30.30	187	-	2.0	
20	28.2	190	6.4	2.5			-		
36	27.4	198	6.4	3.1			-		
48	26.7	211	6.4	3.2	30.3	185	-	2.0	30.22
54	26.11	221	6.4	-			-		
60	25.40	227	6.4	4.0	30.28	183	-		30.34
70	24.0	230	6.3	-	30.32	189	-		
81	23.73	235	6.4	4.3			-		
96	23.00	239	6.4	4.4	30.3	188	-		
108	22.30	229	6.4	4.5			-		
140	22.51	226	6.4	4.6	30.25	185	-	2.0	30.30

Table 8.1

Degradation of 246-TCP in Packed-bed Continuous Reactor

Measured concentrations at the outlet of the packed bed reactor during a particular run in which the residence time was 19.6 hours, at a flow rate of 1 ml/min,inlet 2,4,6-TCP concentration was 46.5 mg/l, Chloride concentration 6 ppm at a steady state outlet pH of 5.6 *

Time (hrs)	246 (ppm)	Glu (ppm)	N (ppm)	Lig	Prot (ppm)	BM (ppm)	Cl (ppm)	D.O (ppm)
0	0	170	4	N.D	56	6	6	6.7
96	1.2	14	1	Det	144	11	14	6.6
146	1.5	13	0.7	N.D	174	16	26	6.7
192	1.6	11	0.6	Det	180	13	28	6.4
242	1.5	10	0.6	N.D	181	13	27	6.5
290	1.55	10	0.7	DET	179	12	28	6.4
338	1.50	11	0.7	N.D	183	12	28	6.6
390	1.46	10	0.7	Det	179	13	28	6.6

Table 8.2

Effect of Glucose Concentration on Degradation of 246-TCP in Continuous Reactor

Void volume 700 ml; Flow rate=1.698 ml/min;

Retention time=6.87 hours: Recirc.rate = 7.0 ml/min

All steady state values at the outlet

Inlet					Outlet					
Glu.	**N.**	**Cl**	**pH**	**246**	**Glu.**	**N.**	**Cl**	**pH**	**246**	**Prot.**
460	3	1.0	3.97	38.9	4	0.	14.4	4.67	6.33	89
400	3	1.2	4.57	41.0	17	0.1	15.8	4.62	7.11	98
500	3	1.2	4.60	40.7	50	0.1	16.3	4.38	7.0	101
600	3	1.2	4.58	39.4	45	0.03	17.5	3.84	6.93	93
1000	3	1.3	4.62	42.0	70	0	16.8	2.86	9.0	120
1500	3	1.3	4.60	40.6	109	0.0	12.8	2.73	14.6	135

Table 8.3.1

Effect of Nitrogen Concentration on Degradation of 246-TCP

SET # 1

Retention Time =16.08 hours; Inlet Flow rate=0.7719 ml/min.
Internal recirculation rate=7.0 ml/min;
Inlet Gcose=800 ppm, N=5 ppm, 246=10.6 ppm ; Cl=1.0 ppm; pH=4.10;

Time (Hrs)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)	246 (ppm)	pH	BM (ppm)
0	180	1.0	3.0	28	0	4.2	31
24	54	1.1	2.0	39	0	4.4	38
48	11	1.3	2.8	63	0.5	4.59	69
72	0.10	1.8	3.0	76	1.4	4.63	37
96	0.12	1.6	4.3	80	1.7	4.61	21
120	0.09	1.7	4.9	86	2.0	4.62	16
144	0.05	1.7	5.3	83	2.2	4.62	16
168	0.14	1.7	5.3	85	2.0	4.63	11
190	0.11	1.68	5.4	83	1.8	4.62	12

Table 8.3.2

Effect of Nitrogen Concentration on Degradation of 246-TCP

SET # 2

Inlet Glucose concentration = 800 ppm; Nitrogen=10 ppm; pH=4.10;
Chloride=1.0 ppm; 246-TCP=11.4 ppm, Retention Time=16.08 hours.

Time (Hrs)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)	246 (ppm)	pH	BM (ppm)
0	70	0.2	3.0	97	0	4.4	17
24	7.0	0.7	3.1	126	0	4.5	98
48	0	2.0	3.5	80	0.4	4.5	54
96	0	2.4	3.6	56.43	2.8	4.6	101
120	0	7	4.0	48.0	2.4	4.8	130
144	0	2.6	3.5	85	2.9	5.0	129
168	0	3.0	3.0	43	2.5	5.0	177
196	0	7	2.8	56	2.2	5.2	144
220	0	11	3.0	35	2.7	5.3	119

Table 8.3.3

Effect of Nitrogen Concentration on Degradation of 246-TCP

SET # 3

Inlet Glu=800 ppm; Nitrogen=30 ppm; chloride=1.0 ppm; pH 4.7 ; 246-TCP=10.5 ppm;
Retention time= 16.08 hours

Time (Hrs)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)	246 (ppm)	pH	BM (ppm)
0	67	1.0	3.1	78	0	4.4	12
24	8.0	2.9	2.1	198	2.4	4.9	40
48	0.01	21	1.6	220	3.6	5.3	120
72	0.0	23	1.8	251	3.8	5.7	320
96	0.0	20	2.0	270	3.0	6.48	486

Table 8.4

Effect of Nitrogen Concentration on Degradation of 246-TCP at Constant pH

Time (Hrs)	246 (ppm)	BM (ppm)	N (ppm)	pH
0	0	17	0.0	5.5
24	0.7	67	1.4	5.61
48	2.0	53	3.0	5.62
72	3.7	178	18	5.58
96	5.0	260	23.7	5.60
120	7.0	300	27.0	5.63
150	7.4	239	26.1	5.59

Table 8.5

Effect of Shear on Degradation of 245-TCP in Continuous Reactor

All steady state values at different recirculation rate

Void volume=770 ml; I.D=4.9 cm; Total Reactor volume=1430 ml.

Length of reactor= 76.2 c.m. Retention time= 6.337 hours

Inlet					Outlet			
pH	245 (ppm)	N (ppm)	Glu (ppm)	RR	pH (ppm)	245 (ppm)	N (ppm)	Glu. (ppm)
4.16	10.6	4	600	7	4.6	1.7	0.2	20
4.16.	10.6	4	600	10	4.61	1.6	0.1	17
4.15	10.0	4	600	19	4.37	5.7	2.0	239
4.15	10.0	4	600	30	4.23	10.0	3.7	569

Steady-State Experimental Results for Degradation of 246-TCP at pH 3.6

Inlet						Outlet					
pH	246 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	RT (hrs)	pH	246 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)
2.8	60.5	800	4	1.0	6.37	3.62	18.0	14	0	20.4	120
2.30	60.5	900	4	1.0	12.34	3.63	15.60	7	0.04	23.0	77
2.40	41.5	860	4	2.0	12.34	3.59	10.33	10	0	15.3	77
2.8	60.5	800	4	1.0	16.08	3.60	10.0	12	0.1	27.0	99.5
3.4	29.0	400	3	1.8	6.37	3.65	6.60	0	0	11.0	141.5
3.3	33.7	1000	6	1.0	16.08	3.58	5.68	0	0.08	12.8	80

Table 8.6.2

Steady-State Experimental Results for Degradation of 246-TCP at pH 4.6

Inlet						Outlet					
pH	246 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	RT (hrs)	pH	246 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)
4.3	42.6	880	4.5	2.0	7.34	4.63	15.67	35	0.4	16.0	50.5
4.2	27.5	816	4.0	1.0	8.337	4.62	10.0	27	0	15.6	44
4.3	27.5	816	4.0	1.0	8.337	4.60	9.0	29	0.2	16.2	51
4.2	13.17	800	5.0	1.6	13.39	4.56	3.0	0	0.8	5.2	31
4.3	27.5	816	4.0	0.31	9.90	4.59	2.70	30	0.3	12.9	138
4.27	15.9	800	4.0	3.0	13.70	4.66	2.3	0	0	9.1	57.7

Table 8.6.3

Steady-State Experimental Results for Degradation of 246-TCP at pH 5.6

Inlet						Outlet					
pH	246 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	RT (hrs)	pH	246 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)
5.3	58.11	500	3.0	2.5	5.87	5.6	26.60	0	0	17.0	38.0
3.9	85.0	750	4.0	1.0	14.28	5.61	22.80	41	0.3	33.0	44.25
3.9	85.0	750	4.0	1.0	16.74	5.63	15.0	0	0	40.0	48
4.7	85.0	750	4.0	1.0	9.72	5.60	14.83	66	0.2	36	87.5
4.7	84	750	4.0	1.0	9.72	5.60	14.0	47	0.1	36.0	81.0
4.2	57	700	4.0	1.0	12.34	5.60	10.0	20	0.3	26.2	55.0
5.4	10.58	800	6.0	2.8	6.86	5.64	3.80	0	1.3	4.3	35.0
5.1	14.0	800	4.0	12.0	9.27	5.60	3.30	0	0	17.0	44
5.0	13.17	800	4.0	18.0	13.5	5.59	2.95	5	0.2	23.0	33.0
3.9	46.5	780	3.0	6.0	19.6	5.60	1.66	0	0	29.7	181

Table 8.6.4

Steady-State Experimental Results for Degradation of 245-TCP at pH 3.6

Inlet						Outlet					
pH	245 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	RT (hrs)	pH	245 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)
3.4	49.22	800	4	1.0	7.64	3.58	21.0	0	0	16.0	200
3.4	45.5	656	3	1.0	7.40	3.57	17.07	0	0	15.3	253
3.47	44.72	900	4	1.0	10.0	3.60	12.11	0	0	15.9	203.5
3.4	32.02	750	3	1.0	10.0	3.62	9.0	16	0.6	12.0	148.3
3.4	25.50	720	4	1.0	7.64	3.63	5.0	0	0	10.3	193.7
3.0	15.3	600	3.5	1.0	12.50	3.60	3.70	0	0.2	6.1	79

Table 8.6.5

Steady-State Experimental Results for Degradation of 245-TCP at pH 4.6

Inlet						Outlet					
pH	245 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	RT (hrs)	pH	245 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)
4.1	36.65	800	4	1.0	8.448	4.65	18.2	12	0.52	8.3	85.37
3.95	49.22	790	3	0.4	9.2	4.59	17.26	43	0.2	17.4	153.3
3.80	26.75	656	3	1.4	10.0	4.63	13.6	13	0	11.0	94.3
3.3	34.79	700	4	0.5	16.8	4.61	10.0	0	0.7	12.7	109.5
3.4	13.5	700	3	0.01	10.05	4.59	6.7	30	0	3.1	71.8
4.3	13.50	1000	6	6.0	9.143	4.58	4.72	20	0	10.0	125.0
3.6	16.02	1000	7	1.0	11.6	4.59	4.0	0	0	8.5	162

Table 8.6.6

Steady-State Experimental Results for Degradation of 245-TCP at pH 5.6

Inlet						Outlet					
pH	245 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	RT (hrs)	pH	245 (ppm)	Glu (ppm)	N (ppm)	Cl (ppm)	Prot (ppm)
3.9	38.9	772	5	0.5	6.4	5.56	15.51	30	1.0	12.2	64.5
3.9	35.0	780	4	2.5	13.6	5.63	12.2	25	0.1	14.2	35.12
4.9	22.5	800	3.8	2.5	6.4	5.6	10.0	0	0	8.0	48.5
2.6	73.0	700	4	2.7	16.8	5.57	7.304	0	0	37.1	120.4
3.0	29.32	750	4	14	12.82	5.61	5.0	0	0.3	28.0	70.0
5.3	12.45	900	7	2	6.337	5.65	3.0	0	1.1	10.0	75.0

Table 8.6.7

Model Parameters For 246-Trichlorophenol at Different pH

pH	V_{max} (1/hr)	K_m (ppm)
3.6	0.1223	27.13
4.6	0.1408	16.74
5.6	0.286	34.82

Table 8.6.8

Model Parameters For 245-Trichlorophenol at Different pH

pH	V_{max} (1/hr)	K_m (ppm)
3.6	0.0189	2.20
4.6	0.0516	28.23
5.6	0.08176	9.65

Table 8.7.1

Predicted Vs Experimental Results of 246-TCP at Different pH

pH	246 at inlet (ppm)	246 at outlet (ppm)	Prot (ppm)	R.T (hrs)	V_{max} (1/hr)	K_m (ppm)	Pred. 246 outlet (ppm)	Δ (Pred-Exp) (ppm)
3.80	23.5	3.0	142.08	12.337	0.12	25.4	2.7	+0.3
4.0	27.50	3.2	143.0	9.97	0.125	23.0	3.6	-0.4
4.2	27.20	2.8	146.0	9.97	0.13	21.0	3.1	+0.3
5.4	35.0	13.0	38.0	10.0	0.24	27.5	10.25	2.75

Table 8.7.2

Predicted Vs Experimental Results of 245-TCP at Different pH

pH	245 at Inlet (ppm)	245 at outlet (ppm)	Prot (ppm)	R.T (hrs)	V_{max} (1/hr)	K_m (ppm)	Pred. 245 outlet (ppm)	Δ (Pred-Exp) (ppm)
3.7	44.78	12.75	184.0	10.0	0.023	5.0	12.9	+0.15
3.8	29.22	15.0	137.7	7.927	0.025	7.0	13.2	+1.8
4.2	26.7	9.0	150.0	9.97	0.038	18.0	9.0	0
5.4	54.0	8.88	166.0	10.0	0.075	15.8	8.90	+0.02

Table 8.8

Degradation of Phenol in Packed-Bed Reactor

Inlet					Outlet				
pH	Phe (ppm)	Glu (ppm)	N (ppm)	RT (hrs)	pH	Phe. (ppm)	Glu (ppm)	N (ppm)	Prot (ppm)
3.0	86.0	600	4	6.4	4.7	85.8	0	0.2	78
4.1	86.0	600	4	10.34	5.58	86.2	0	0	120
5.0	25.0	800	4.0	16.00	6.0	24.97	0	0	167
4.5	25.0	800	4.0	3.37	4.6	23.7	39	0.16	42

Table 8.9

Degradation of Pentachlorophenol in Packed-Bed Reactor

Initial PCP conc.=23.4 ppm, Glucose=800 ppm, Nitrogen= 4.2 ppm, Chloride=1.0 ppm, pH=4.9

Void Volume=718 ml, Flow Rate= 1.139 ml/min. Recirculation Rate=7.5 ml/min.

Time (hrs)	PCP (mg/l)	Glu (mg/l)	Nitrogen. (mg/l)	Lig (AU)	Prot (mg/l)	Cl (mg/l)	BM (mg/l)
0	0	170	4	N.D	56	1.8	6
24	0.2	14	1	ND	90	2.7	
48	1.0	13	0.7	N.D	134	4.9	16
72	1.6	11	0.6	ND	144	11.7	
96	1.4	10	0.6	N.D	142	14.04	13
120	1.55	10	0.7	ND	143	14.6	
144	1.70	9	0.4	N.D	146.2	14.5	
168	1.63	8	0.3	ND	142.7	14.6	
197	1.60	11	0.2	N.D	143.0	14.3	13
220	1.458	12	0.2	ND	143.5	14.6	12

APPENDIX II

FIGURES

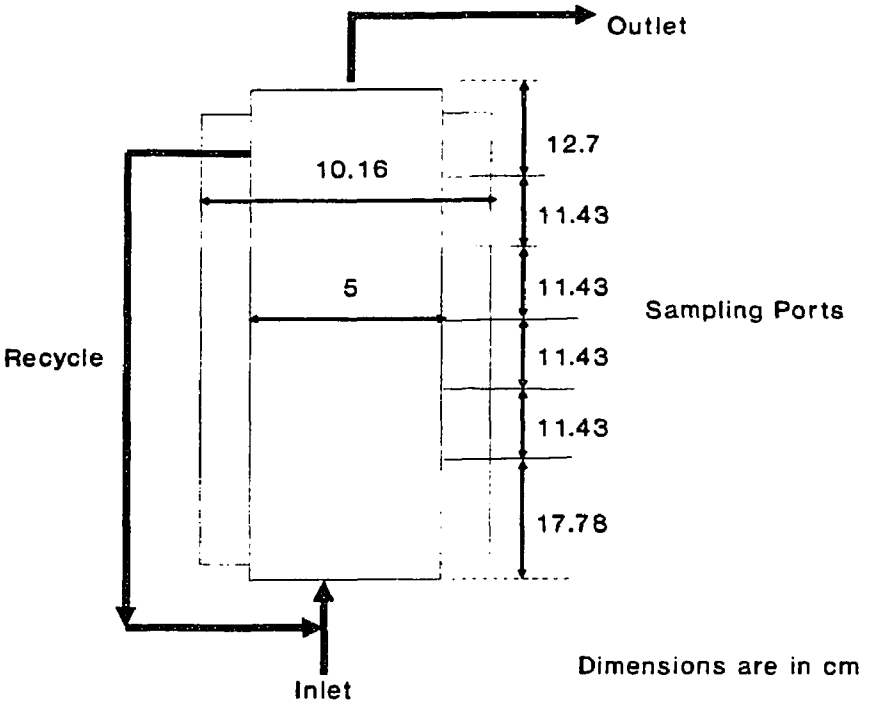


Figure 5.5.5 Dimensions of Reactor R 1

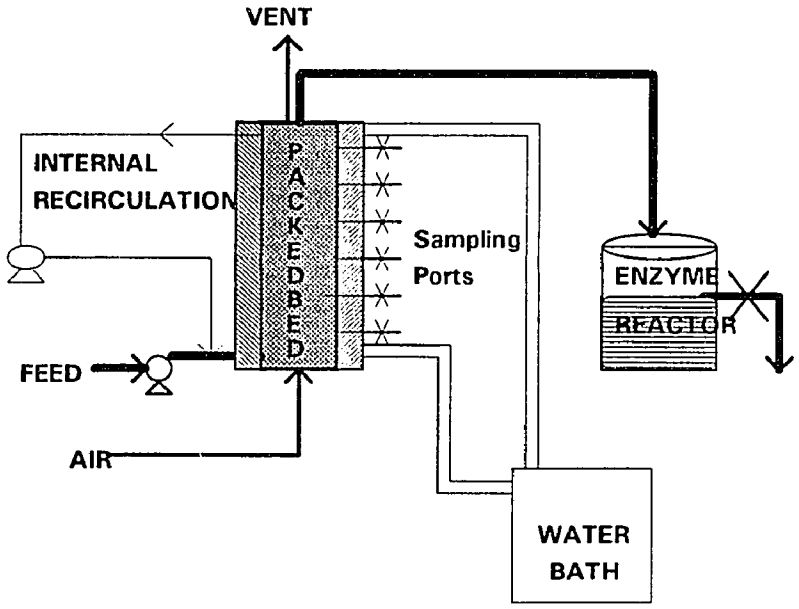


Figure 5.5.6 Schematic of combined packed-bed / enzyme reactor system

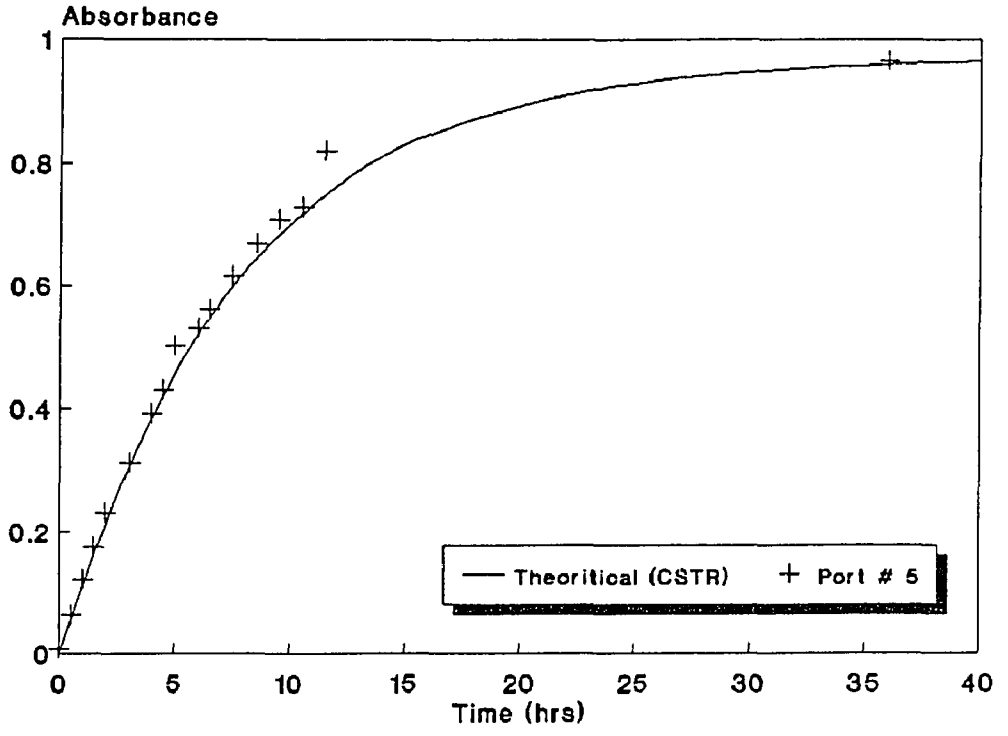


Figure 5.8.1 A typical Residence Time Distribution (RTD) curve for a packed-bed reactor.

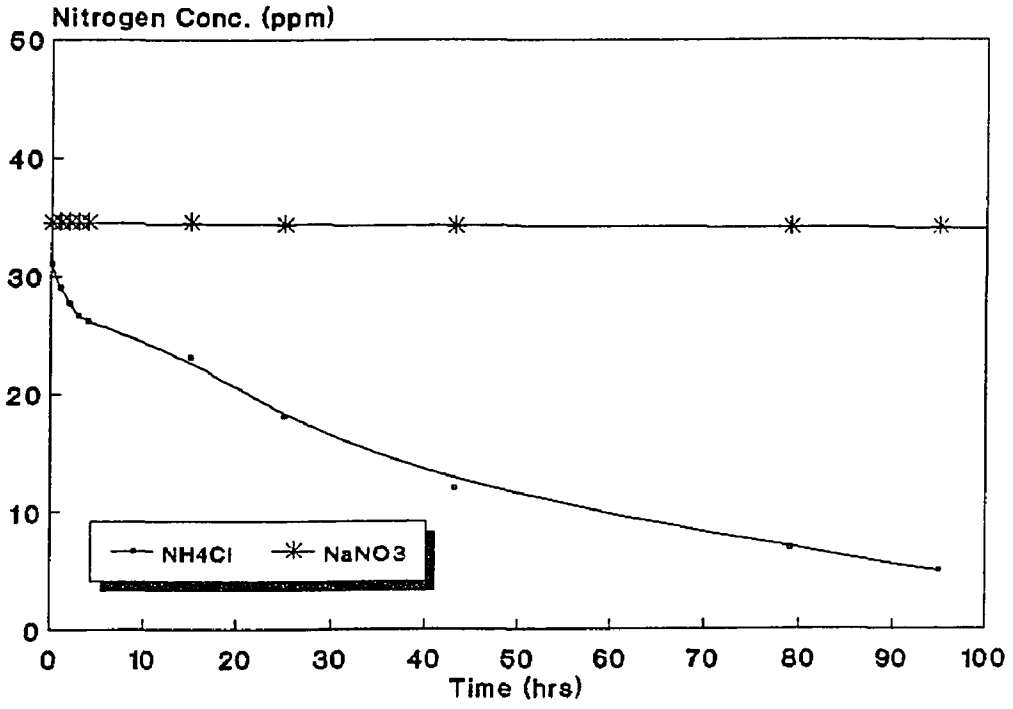


Figure 6.1 Effect of air stripping on nitrogen source (shaker flask experiment at pH 5.0)

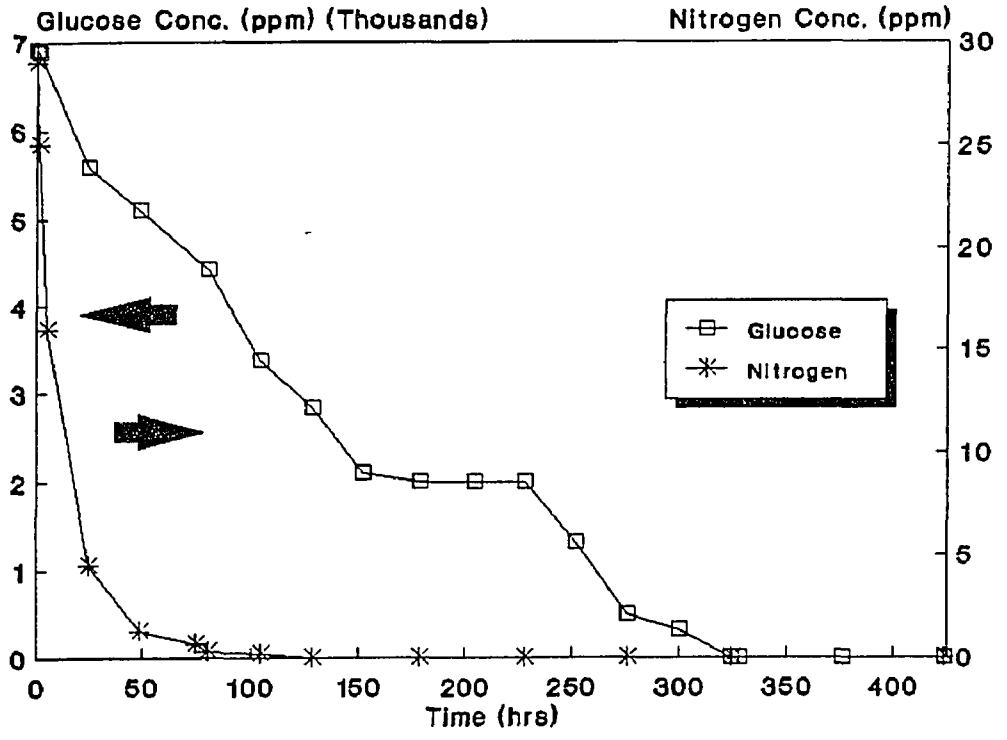


Figure 6.2.1 Glucose and nitrogen depletion during growth

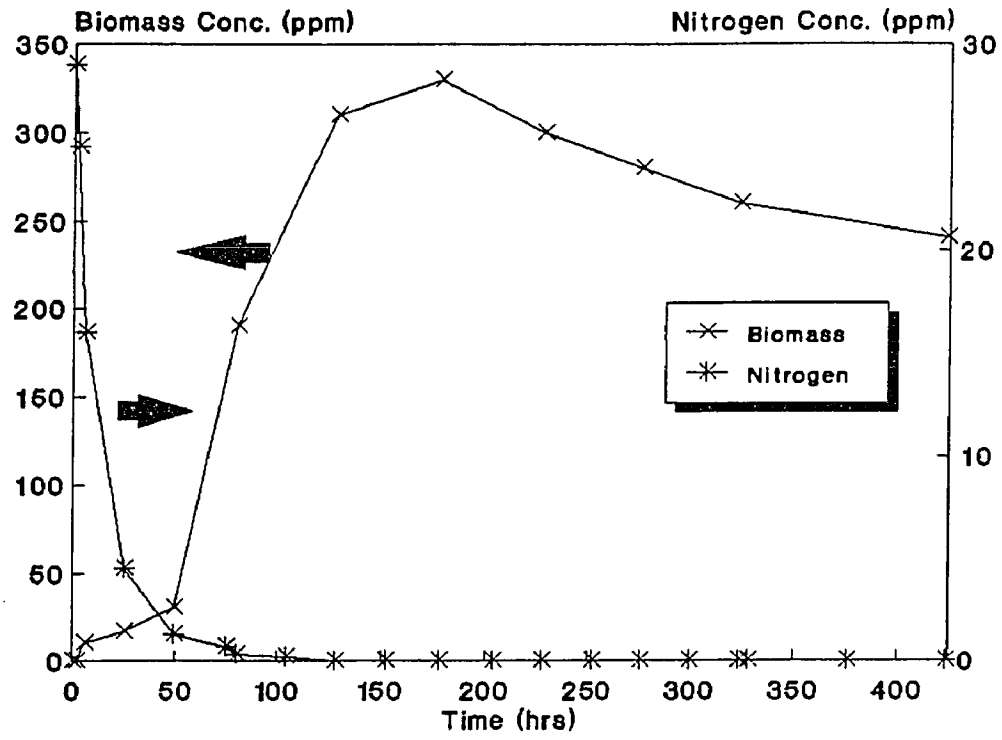


Figure 6.2.3 Change in biomass and nitrogen concentration during growth

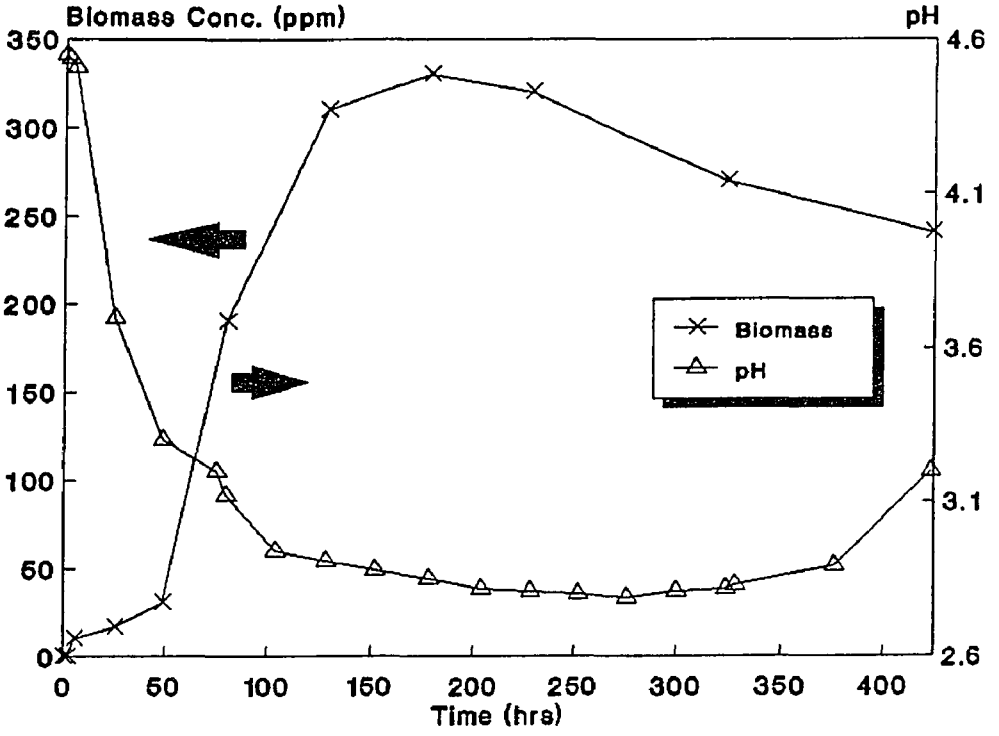


Figure 6.2.4a Change in biomass concentration and pH during growth

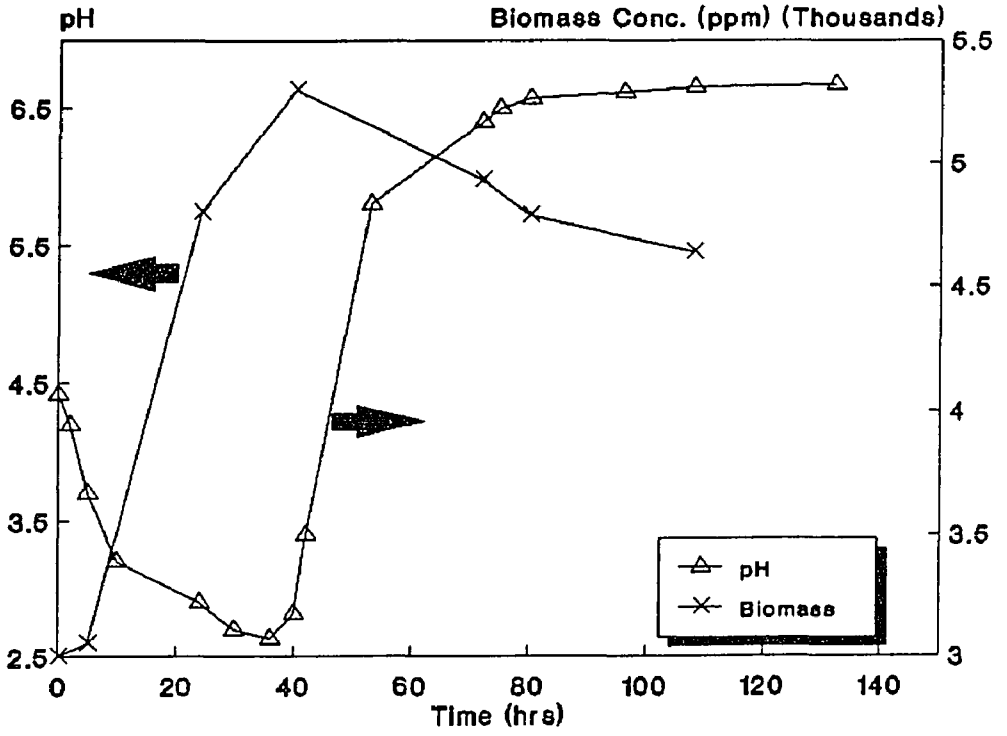


Figure 6.2.5 Change of biomass and protein concentration during growth

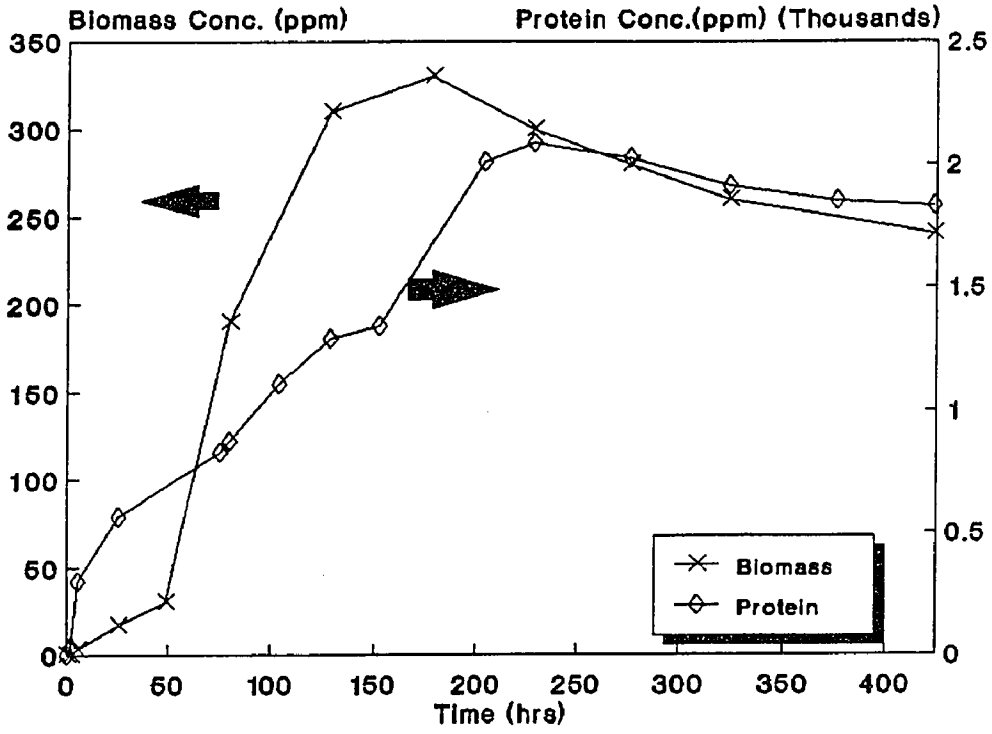


Figure 6.2.4b The inverse relationship between pH and biomass concentration

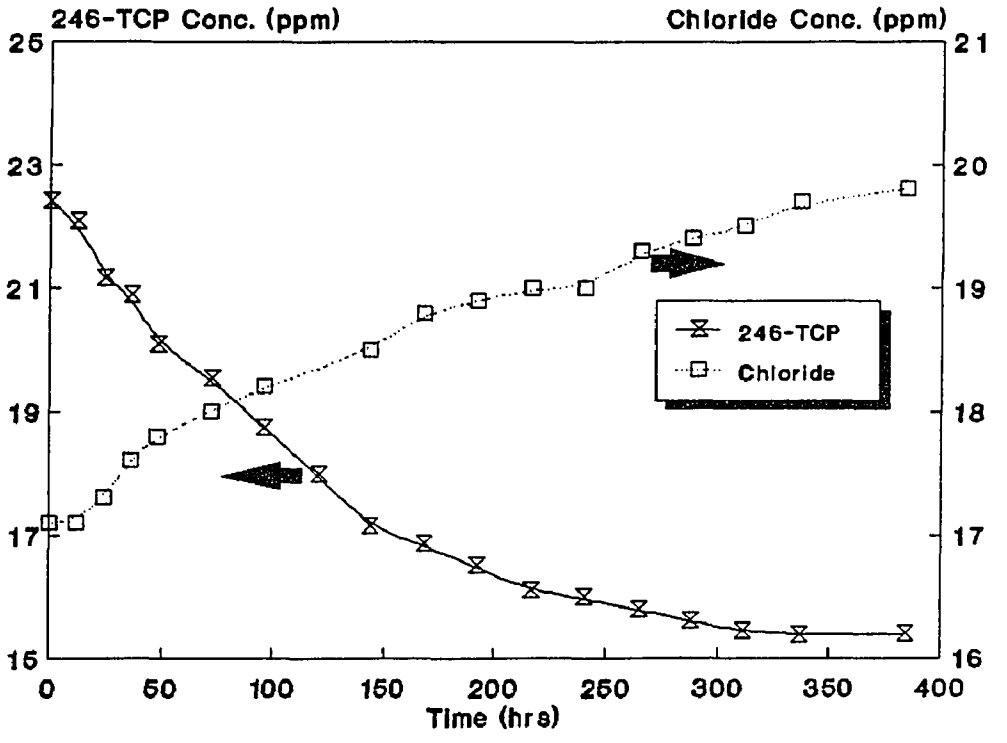


Figure 6.3.1 Degradation of 246-TCP by whole slurry in shaker flask experiments without pH adjustment

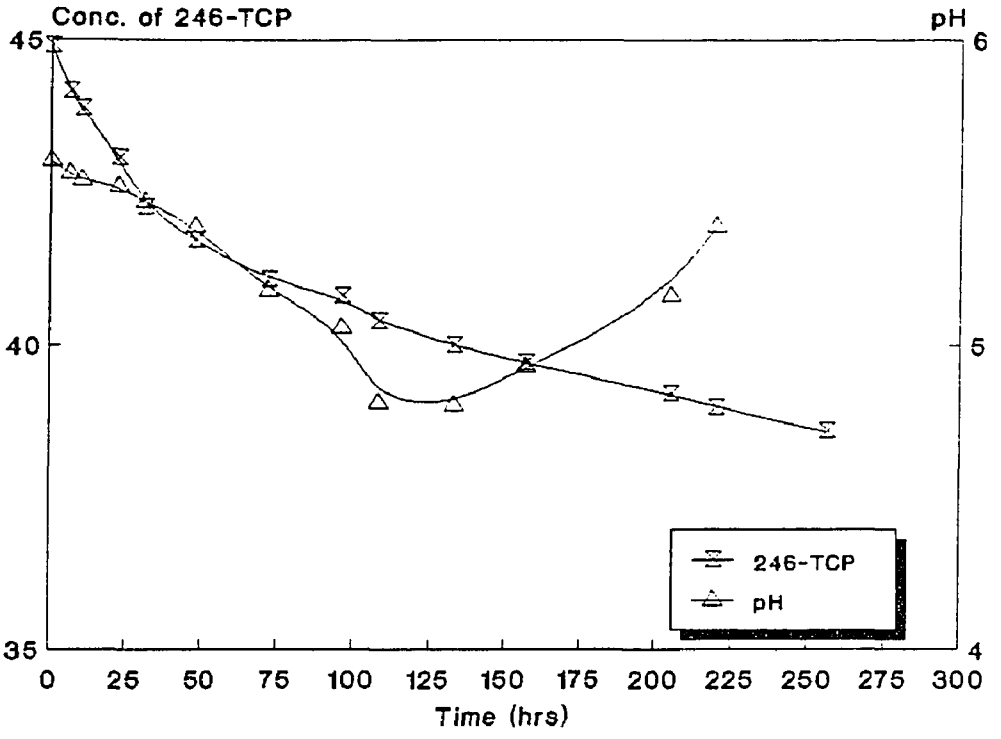


Figure 6.3.1b Degradation of 246-TCP by whole slurry in shaker flask experiments with pH adjustment

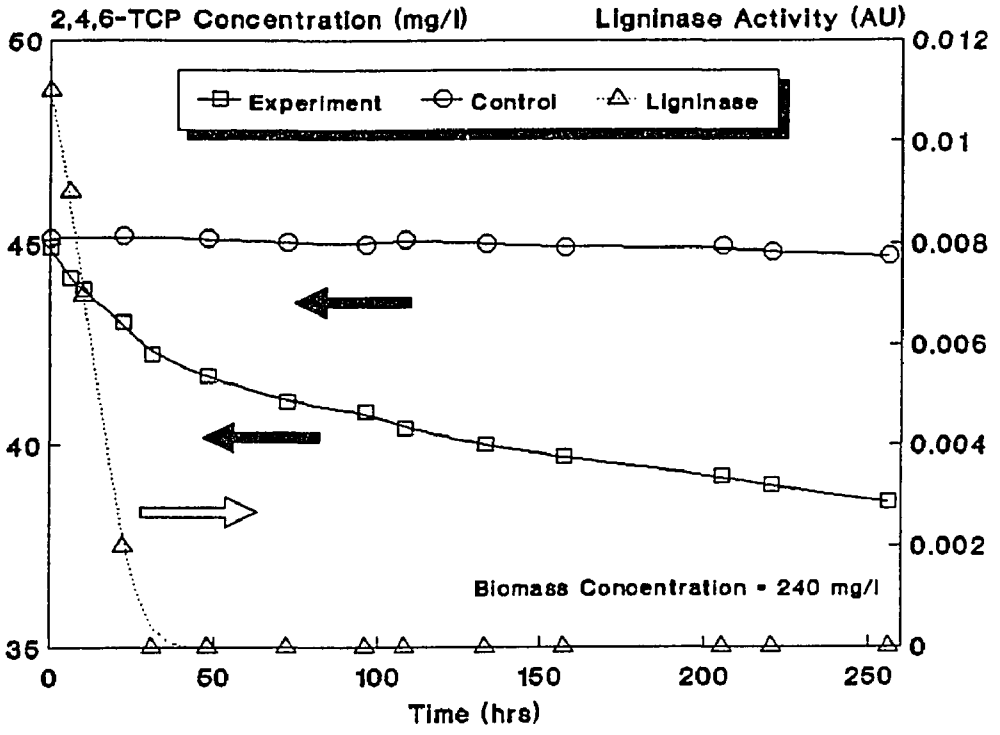


Figure 6.3.2 Degradation of 246-TCP in ligninase-depleted system

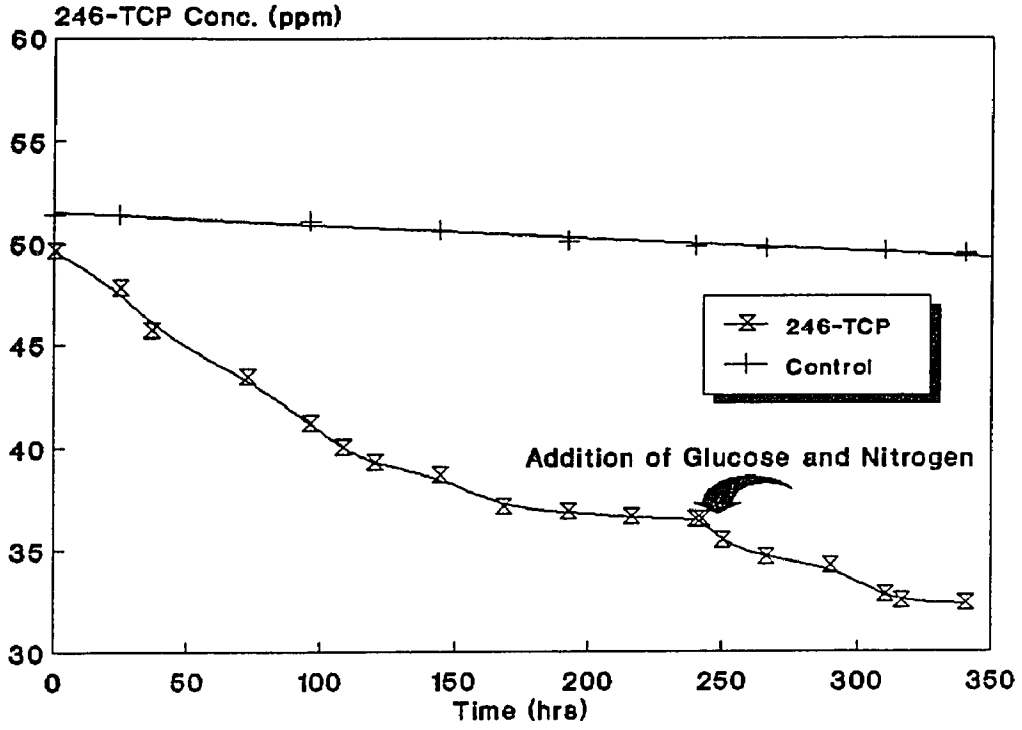


Figure 6.3.4 Restart of degradation of 246-TCP after addition of substrates in a depleted culture

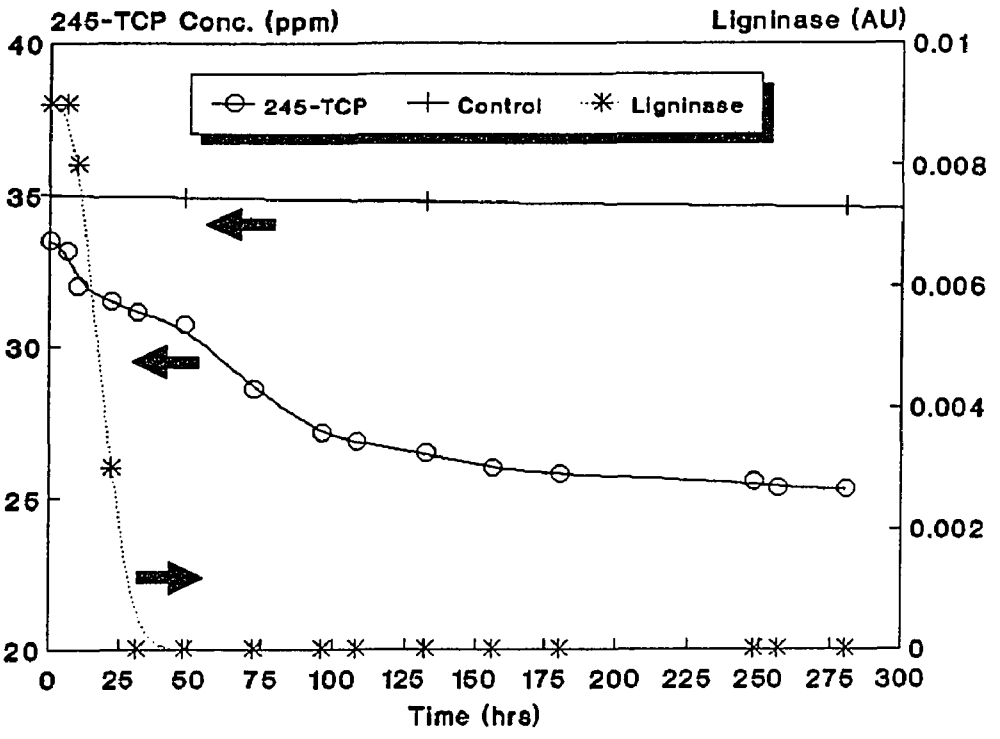


Figure 6.3.5 Degradation of 245-TCP by whole slurry in shaker flask

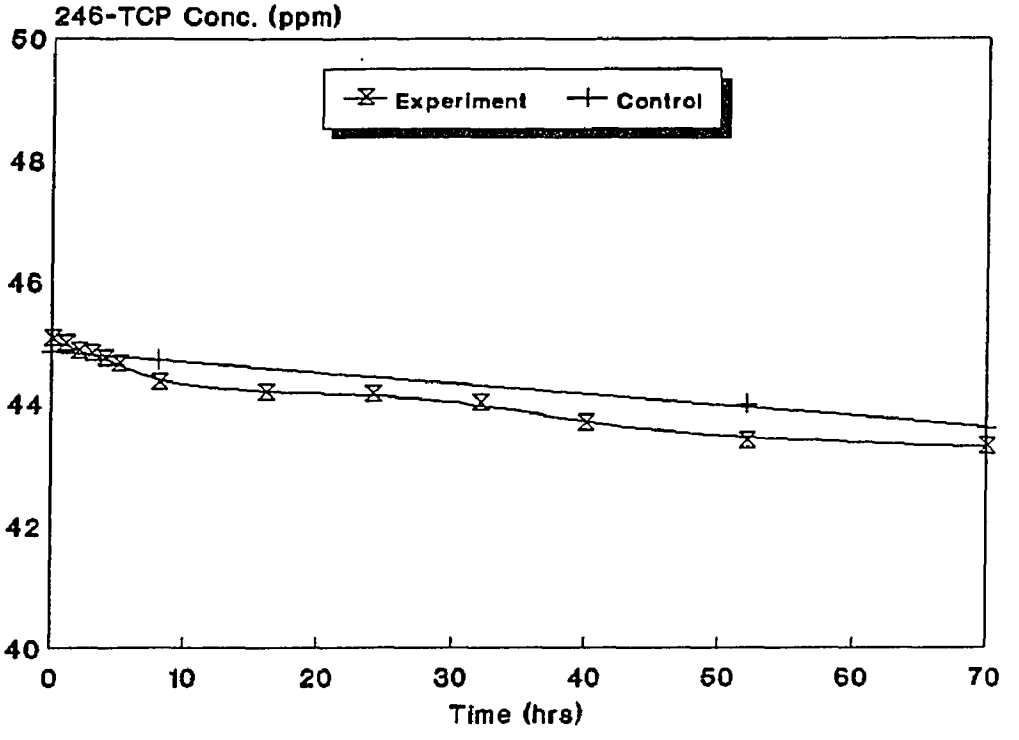


Figure 6.4.1 Degradation of 246-TCP by separated supernatant in shaker flask at pH 5.6

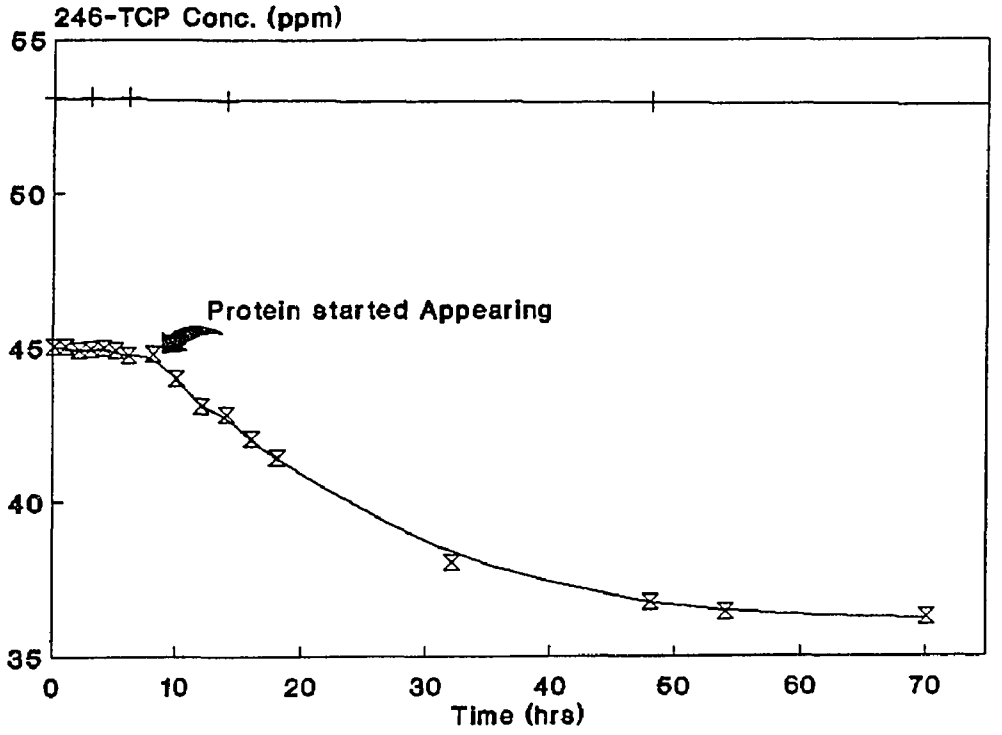
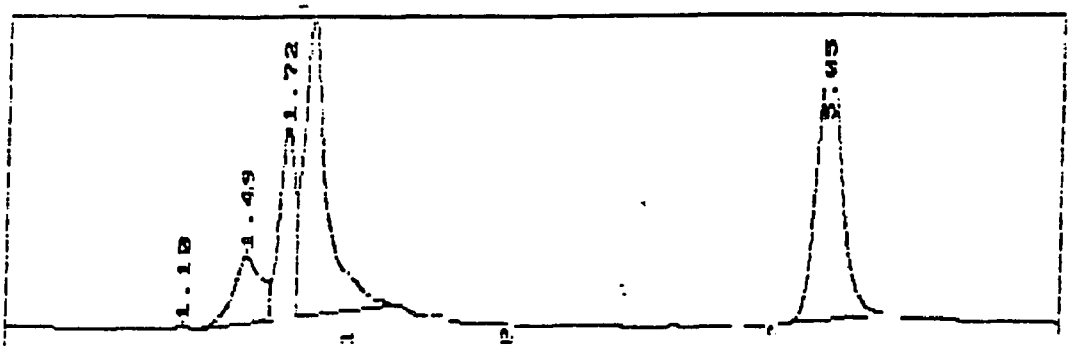
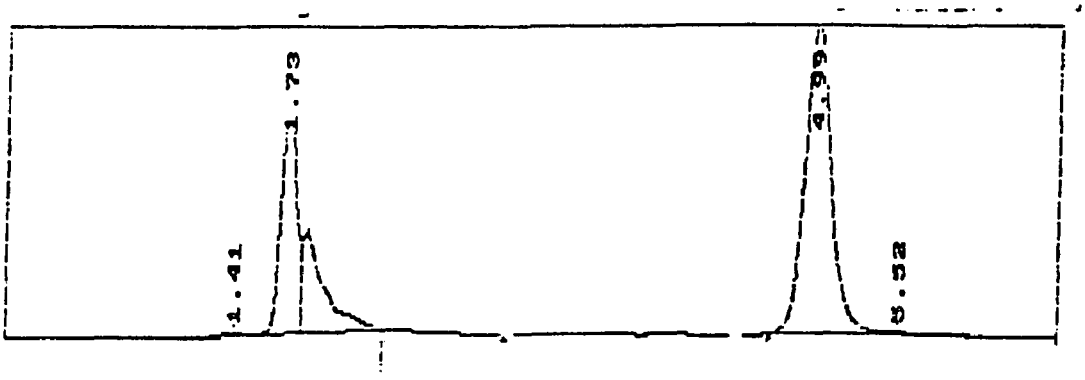


Figure 6.4.2 Degradation of 246-TCP by separated biomass in shaker flask at pH 5.6

Chromatograms for the Experiments with Separated Biomass



Chromatogram at 0 hour



Chromatogram at 12th hour

Chromatogram at 40 th hour

Figure 6.4.2b Chromatogram showing stable intermediates from separated biomass biomass, possibly due to inactivation of enzymes

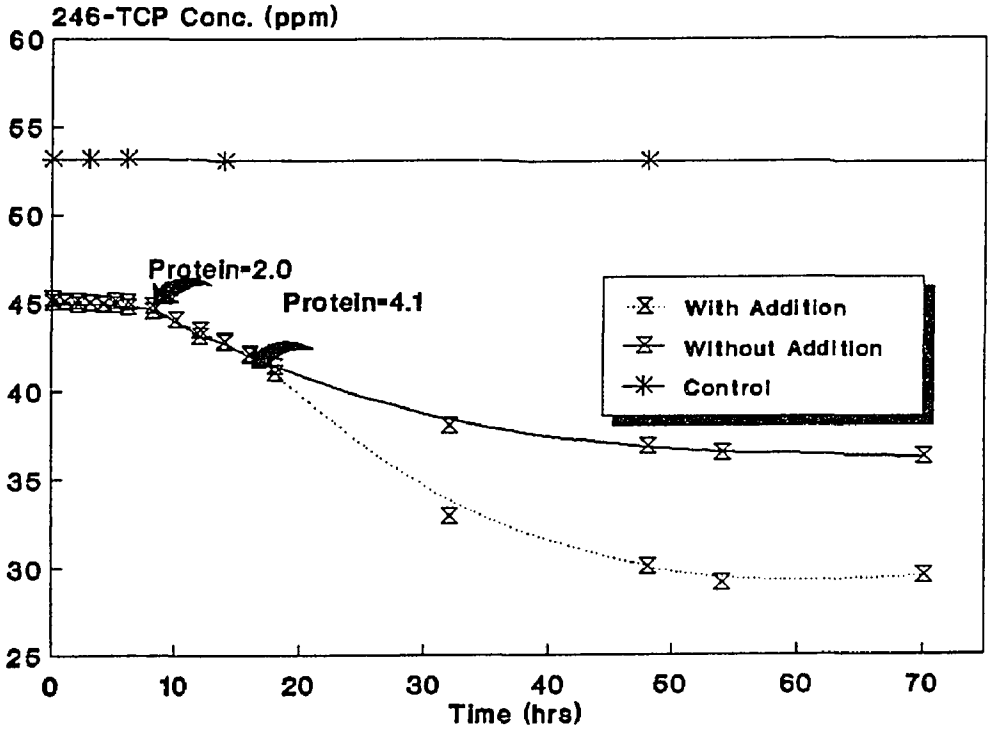


Figure 6.4.3 Degradation of 246-TCP by additional biomass in shaker flask at pH 5.6

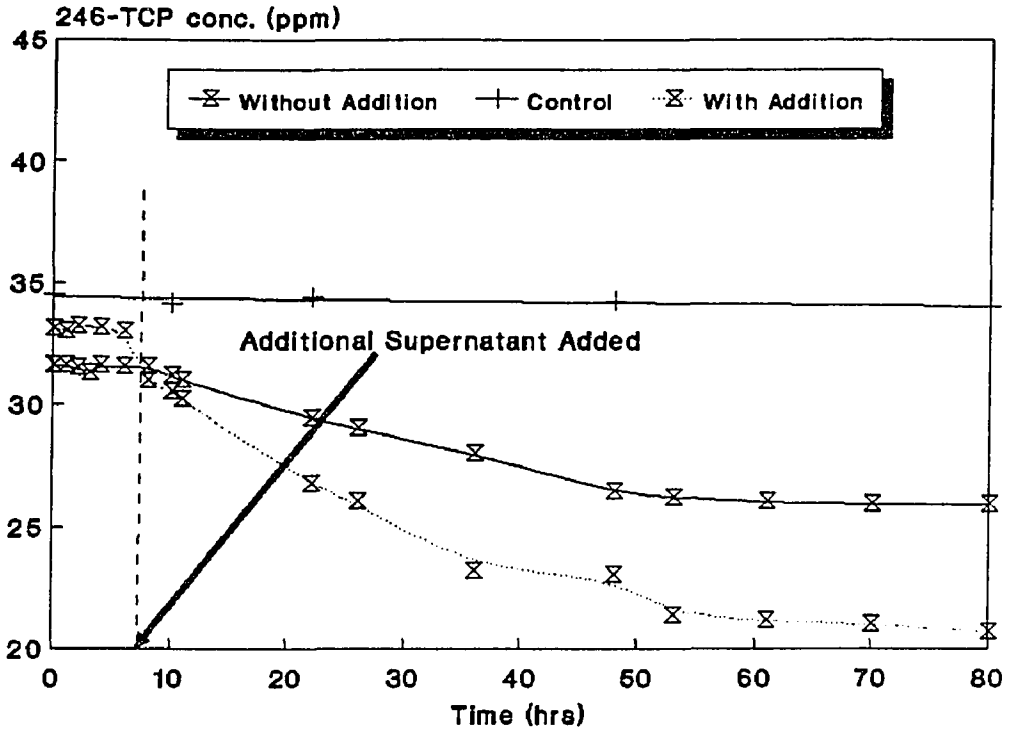


Figure 6.4.4 Degradation of 246-TCP by additional supernatant in shaker flask at pH 5.6

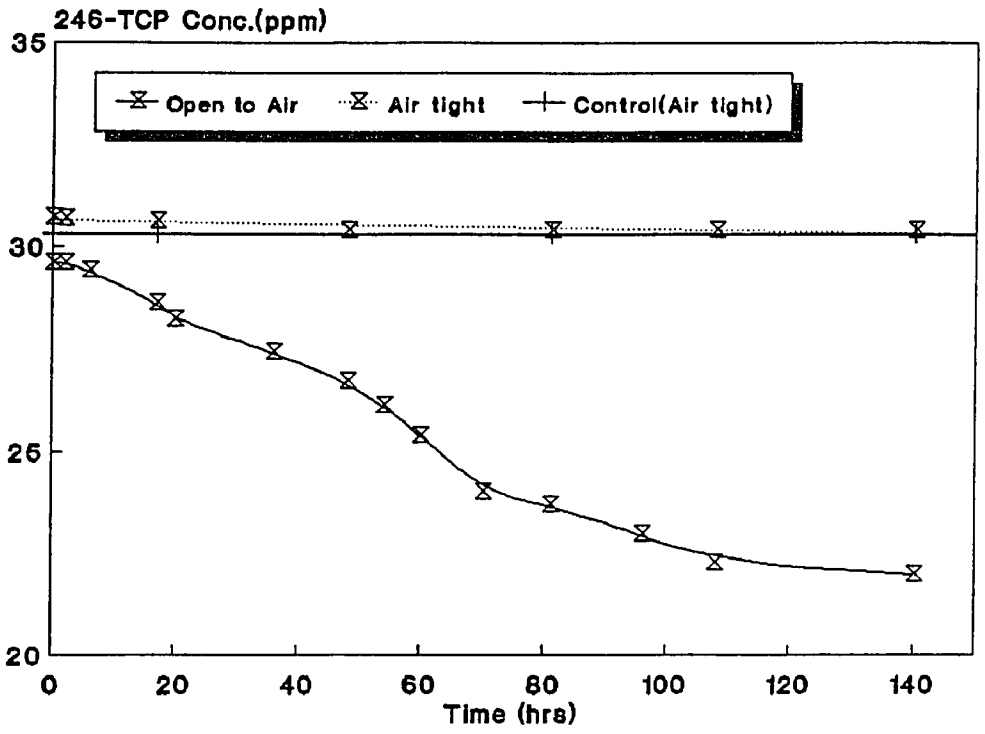
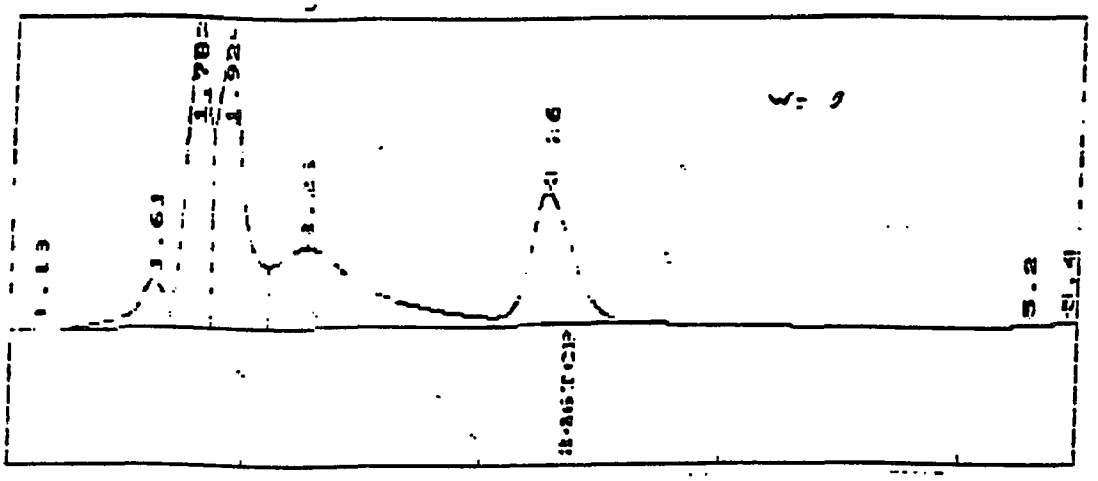
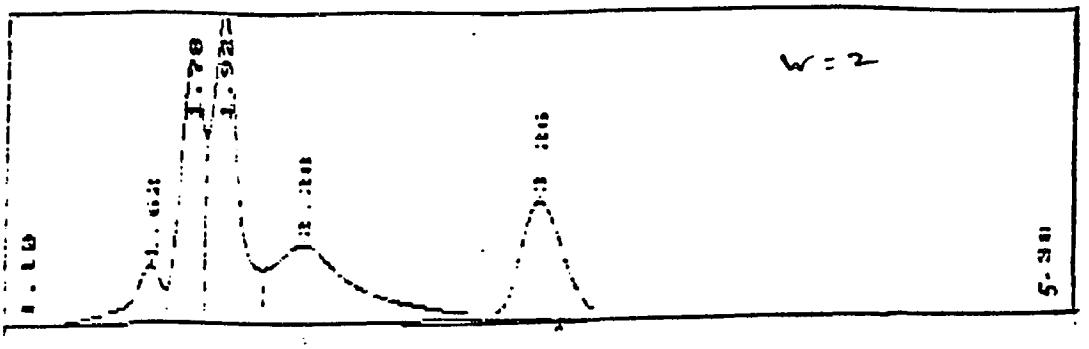


Figure 6.5 Oxygen requirement during the degradation of 246-TCP by whole slurry

Chromatograms For Experiments With Whole Slurry
Retention Time of 246-TCP= 3.26 minutes



Chromatogram at 15 minutes
Intermediate detected at 5.2 min, Area= 2279.8
Intermediate detected at 5.4 min, Area= 2900.25

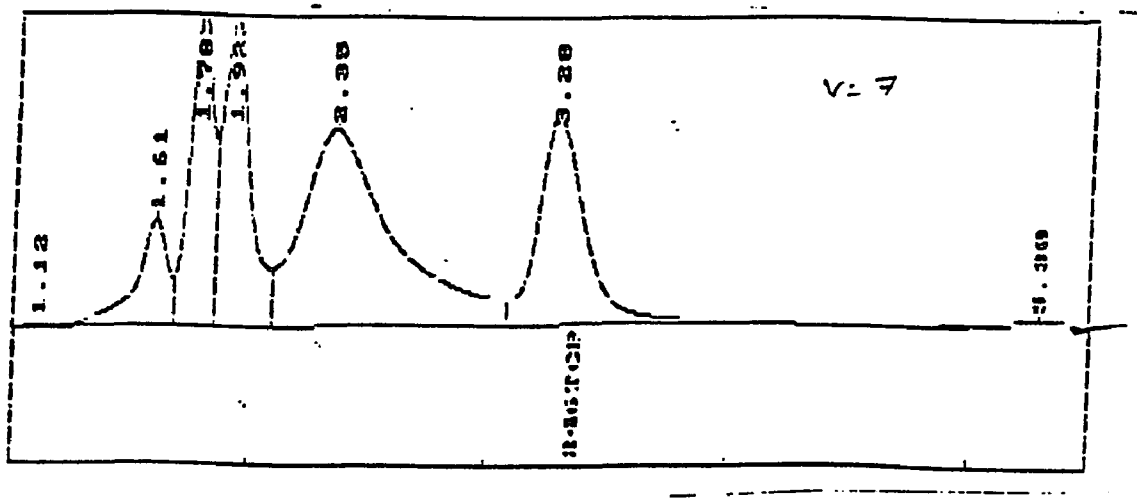


Chromatogram at 2.0 hr
Intermediate detected at 5.38 hours, Area=15353.10

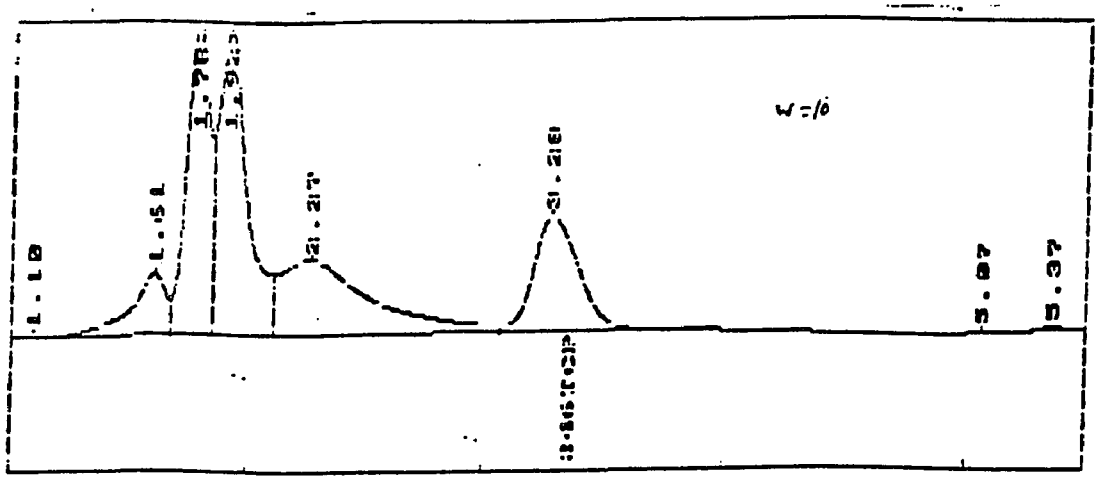
Figure 7.1

Chromatograms For Experiments With Whole Slurry

Retention Time of 246-TCP= 3.26 minutes



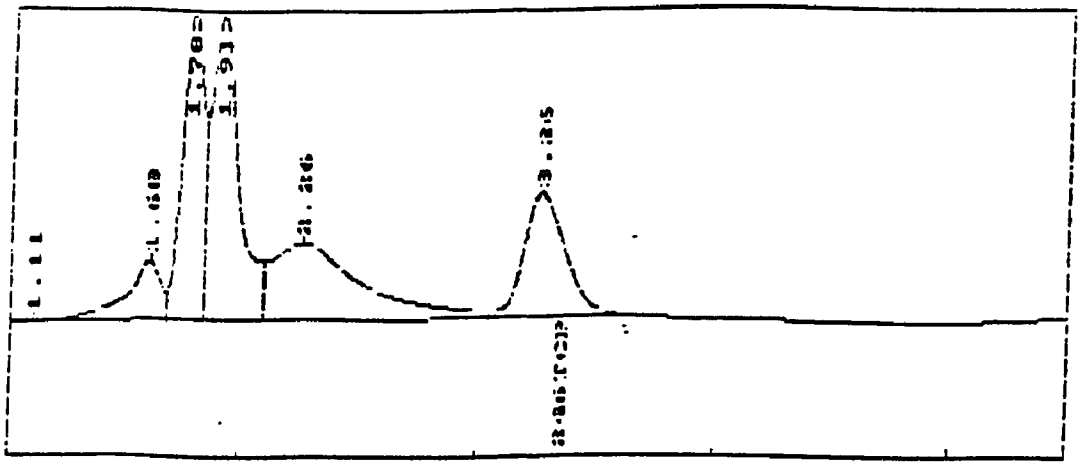
Chromatogram at 7.0 hours
Intermediate detected at 5.30 min, Area= 3475.90



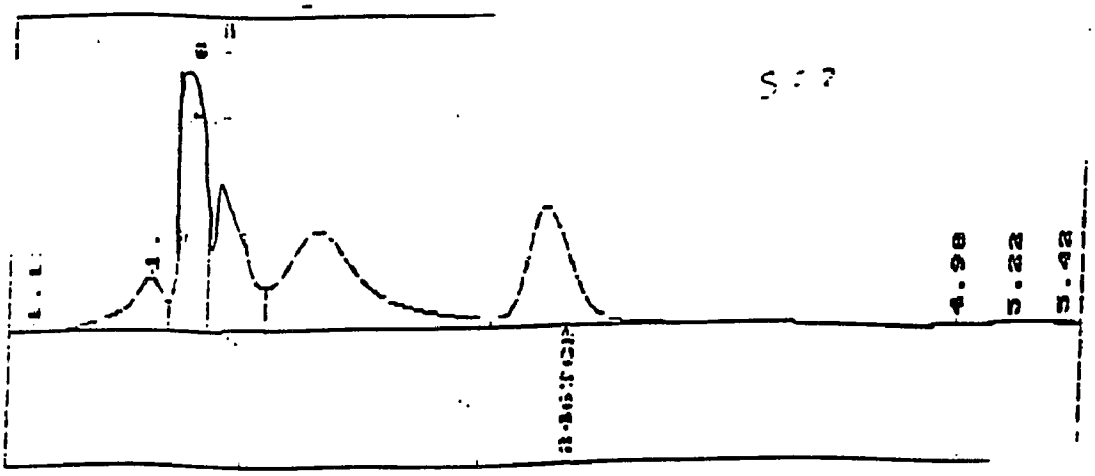
Chromatogram at 10.0 hr
Intermediate detected at 5.07 minutes, Area= 1435.3
Intermediate detected at 5.38 minutes, Area= 7745.6

Figure 7.2

Chromatograms of Separated Supernatant



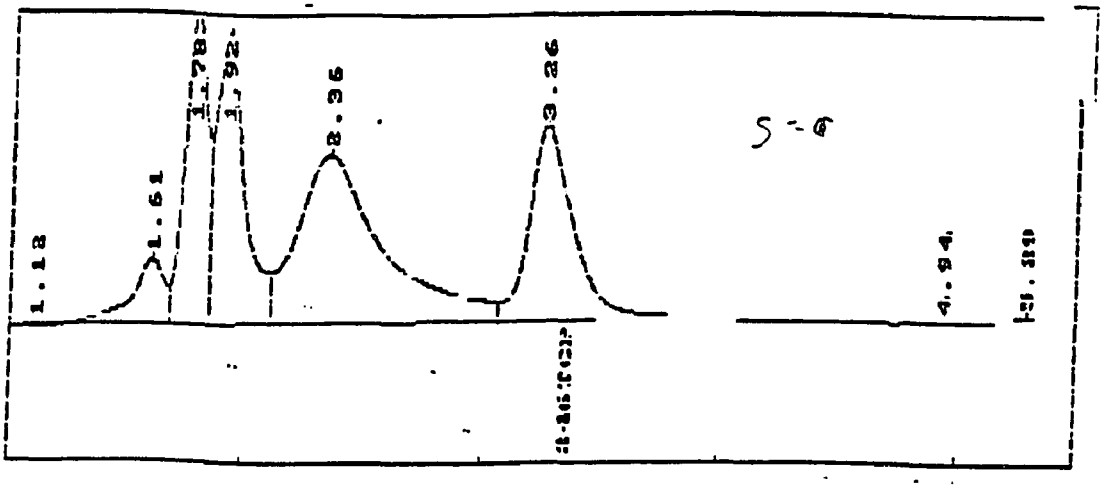
At 15 minutes



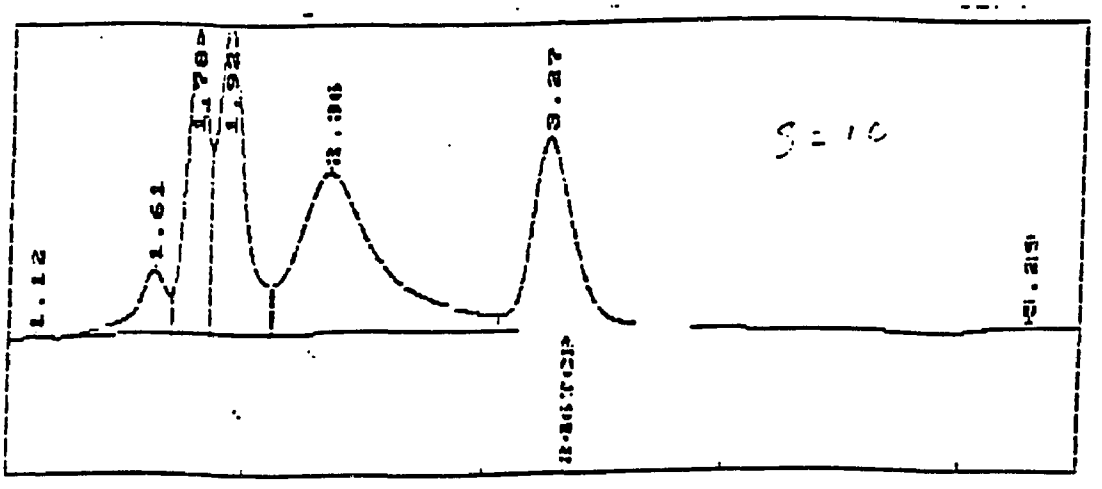
At 2.0 hours

Figure 7.3

Chromatograms of Separated Supernatant



At 7.0 hours



At 10.0 hours

Figure 7.4

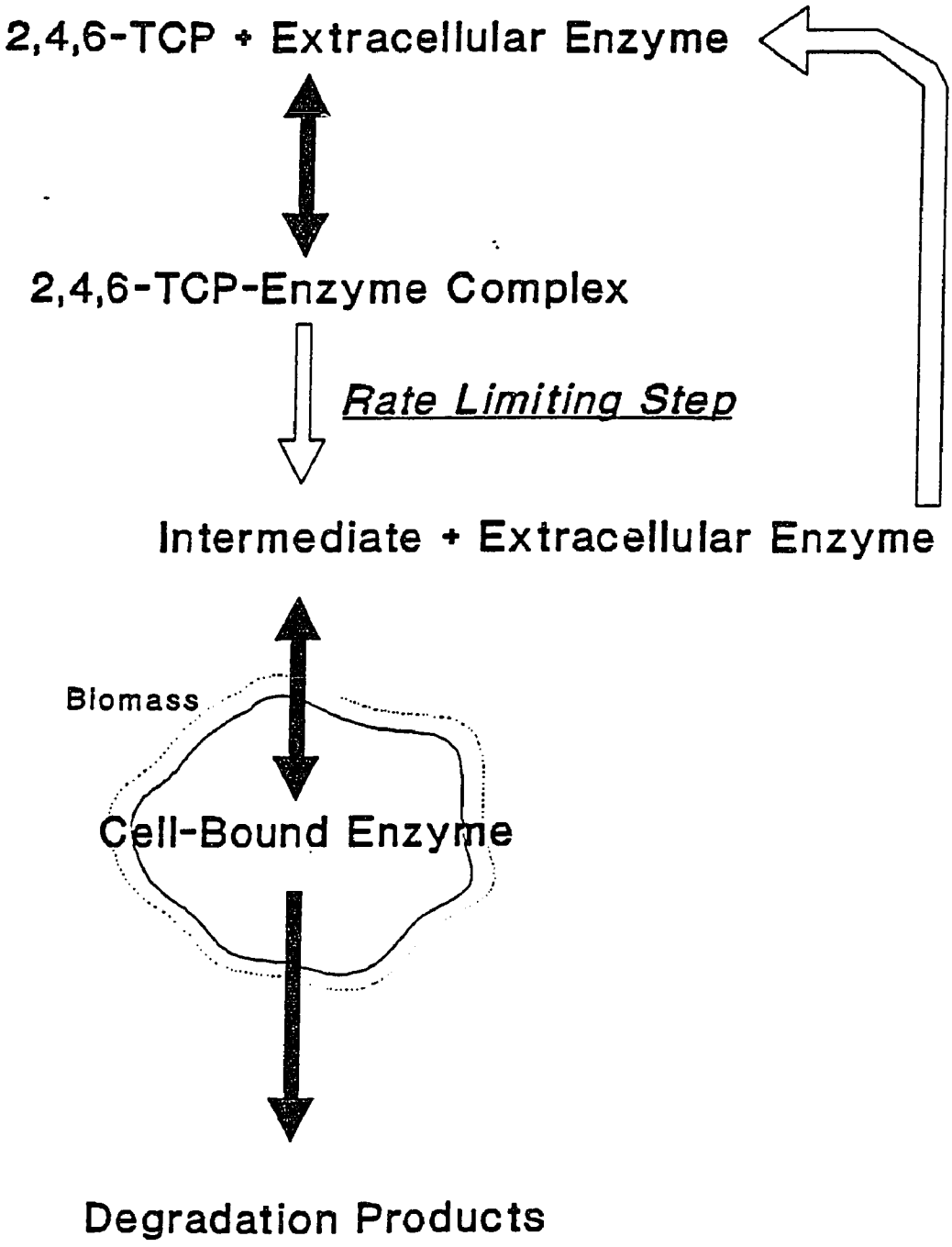


Figure 7.5 Proposed Reaction Scheme for 246-TCP Degradation

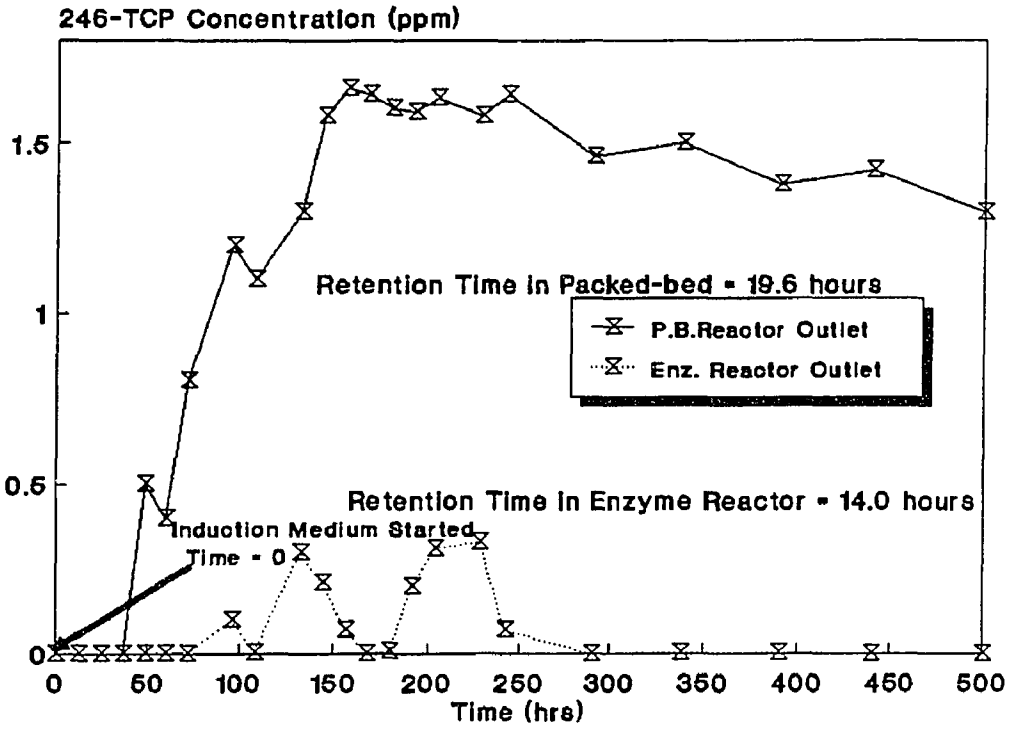


Figure 8.1 Degradation of 246-TCP in packed-bed continuous reactor with a retention time of 19.6 hours

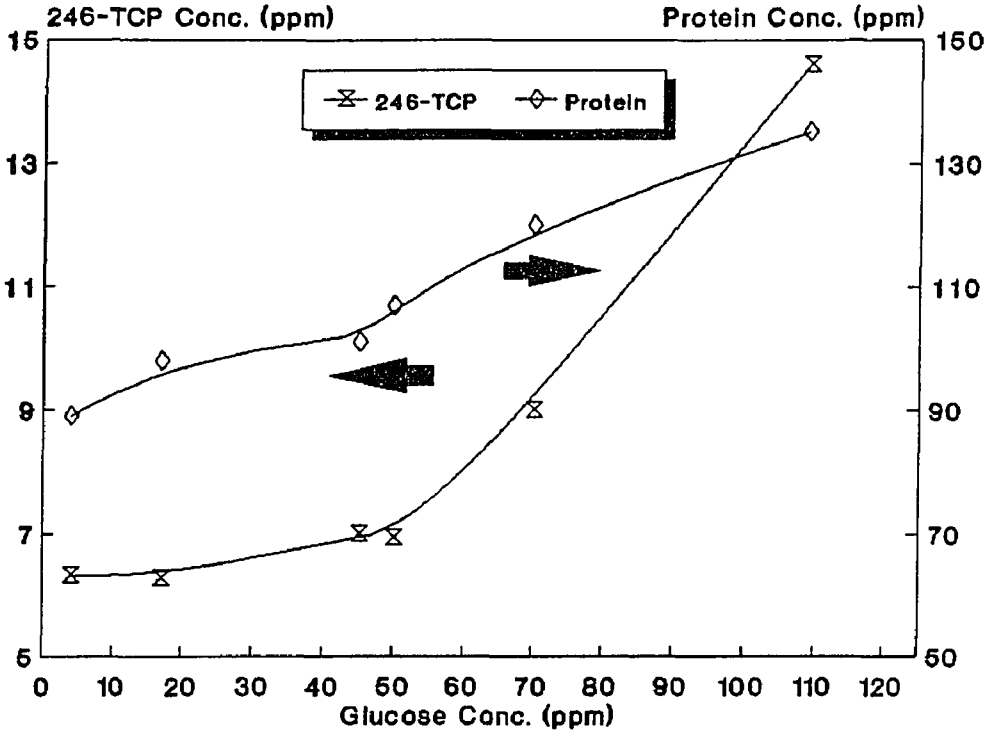


Figure 8.2 Effect of glucose concentration on degradation of 246-TCP in packed-bed continuous reactor system

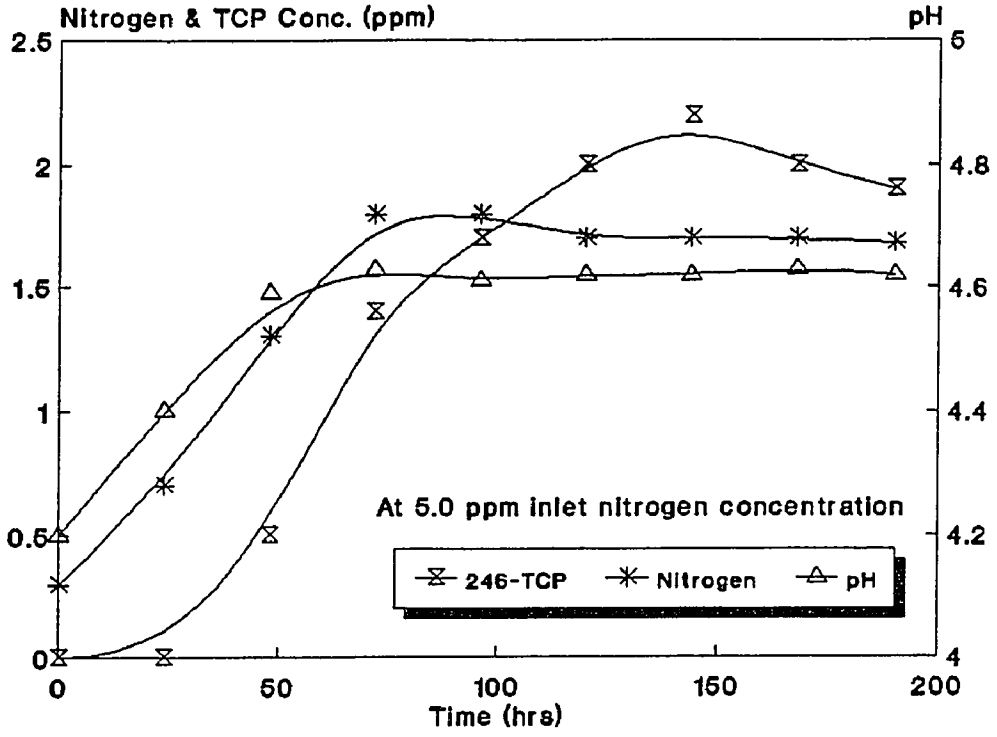


Figure 8.3.1 Effect of nitrogen concentration on degradation of 246-TCP in packed-bed continuous reactor system (SET # 1)

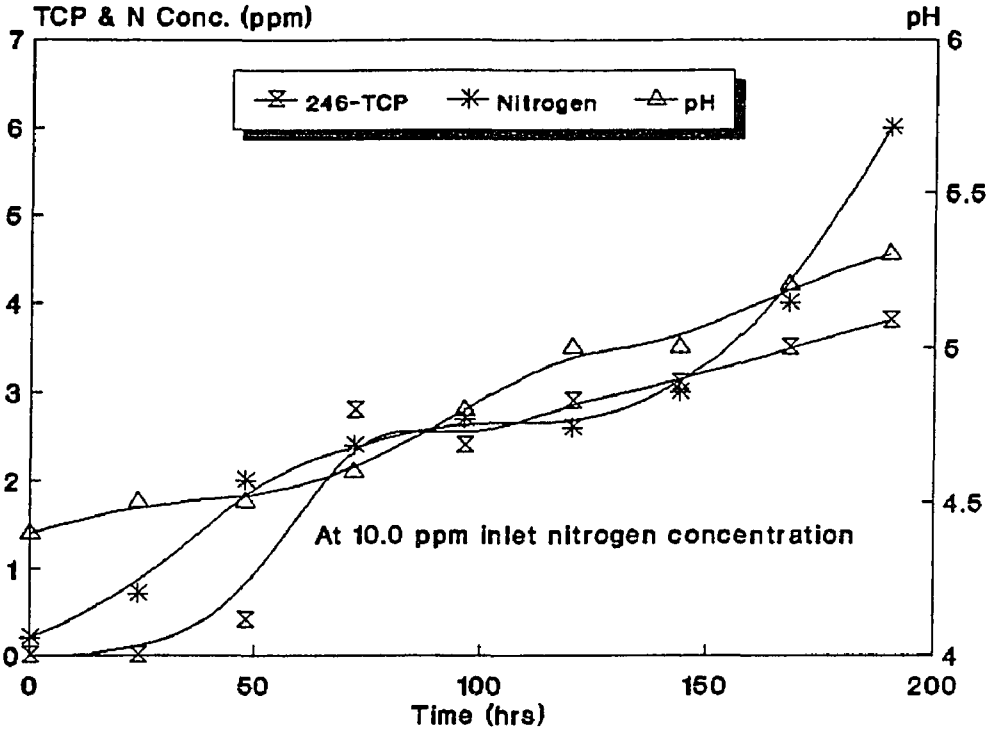


Figure 8.3.2 Effect of nitrogen concentration on degradation of 246-TCP in packed-bed continuous reactor system (SET # 2)

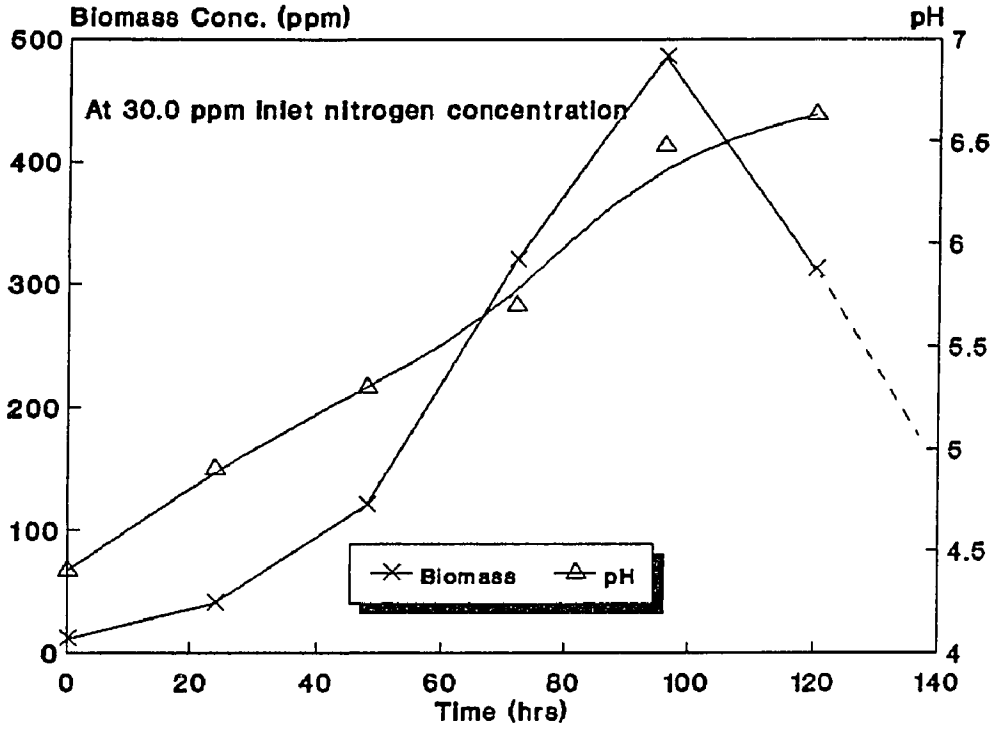


Figure 8.3.3 Effect of nitrogen concentration on degradation of 246-TCP in packed-bed continuous reactor system (SET # 3)

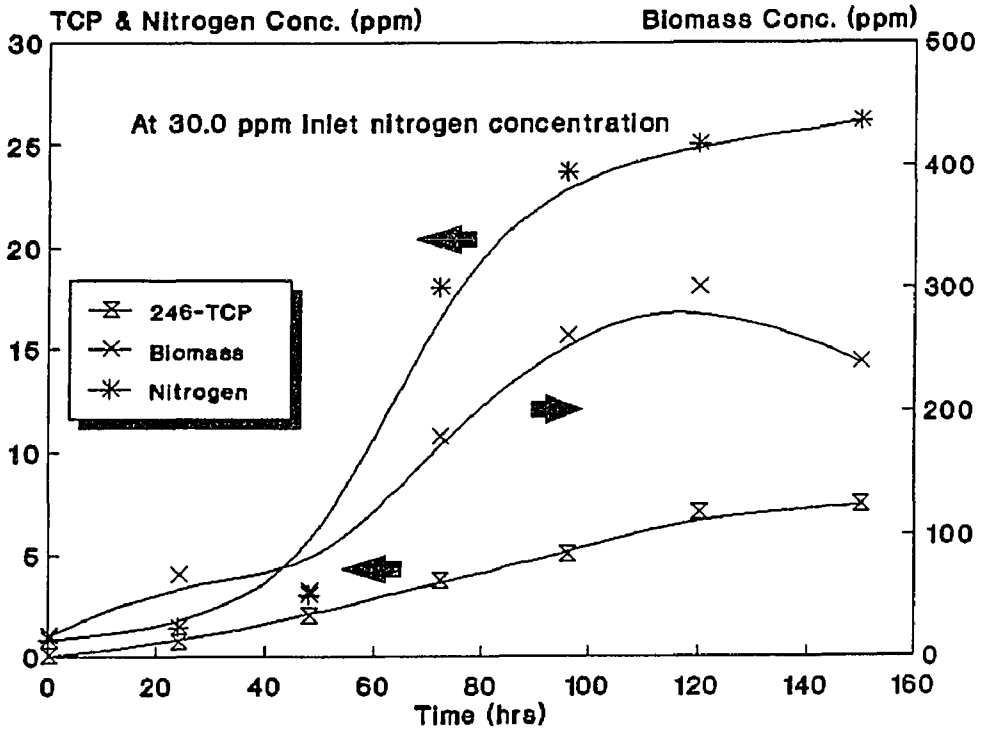


Figure 8.4 Effect of nitrogen concentration on degradation of 246-TCP in packed-bed continuous reactor system at constant pH of 5.6

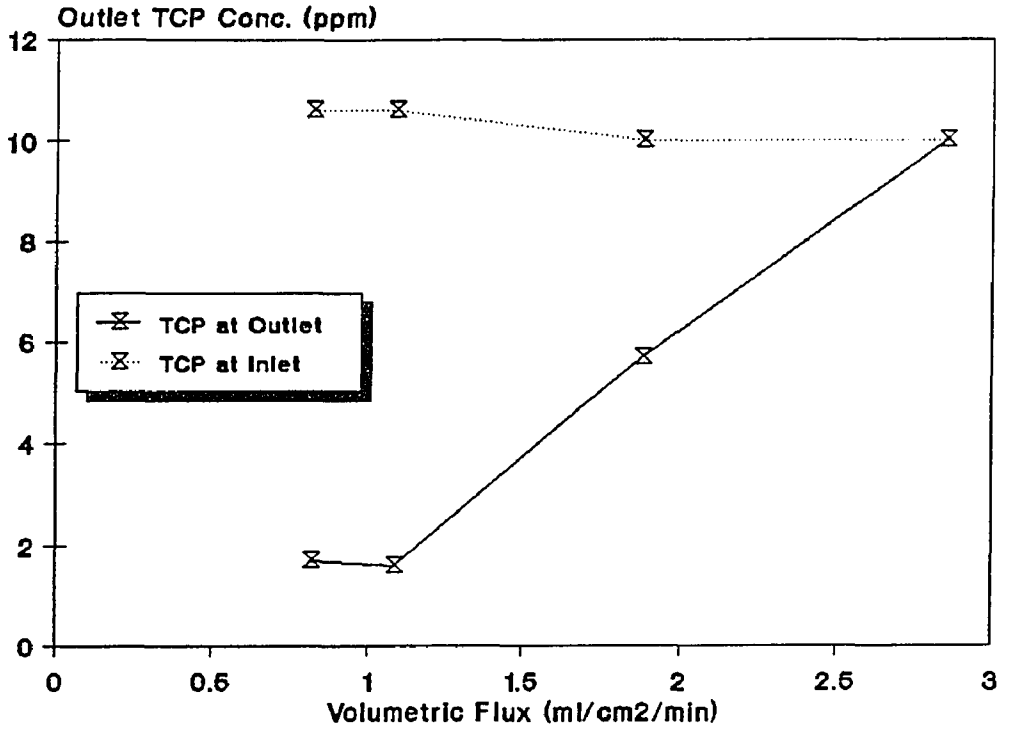


Figure 8.5 Effect of shear on degradation of 246-TCP in packed-bed continuous reactor

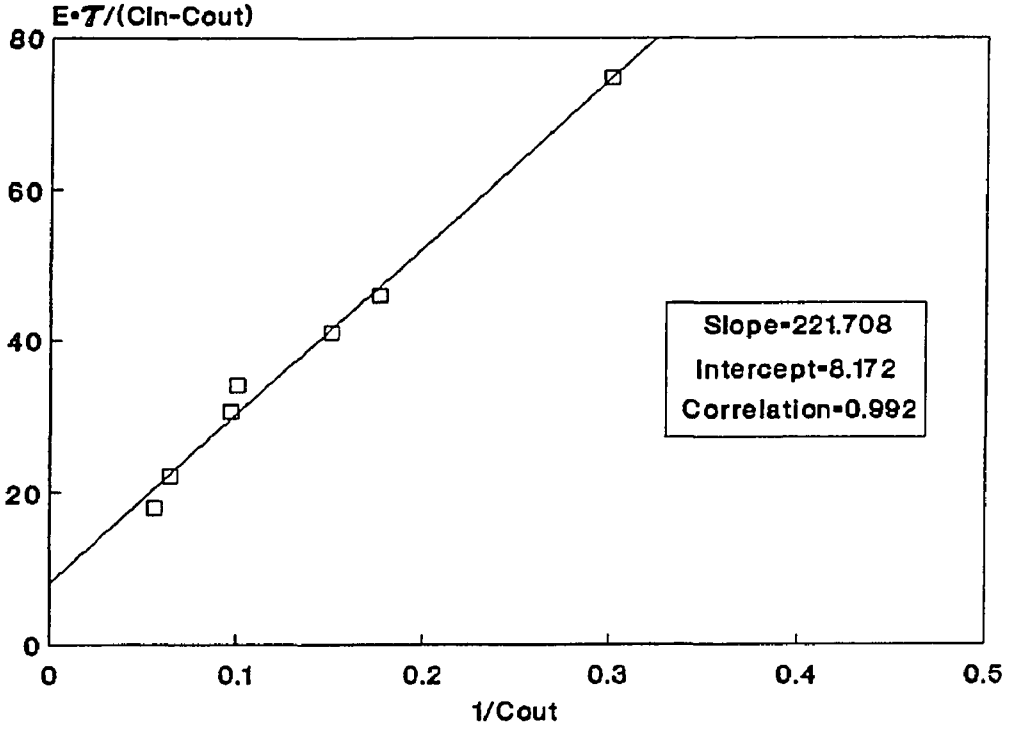


Figure 8.6.1 Determination of model parameters for 246-TCP at pH 3.6

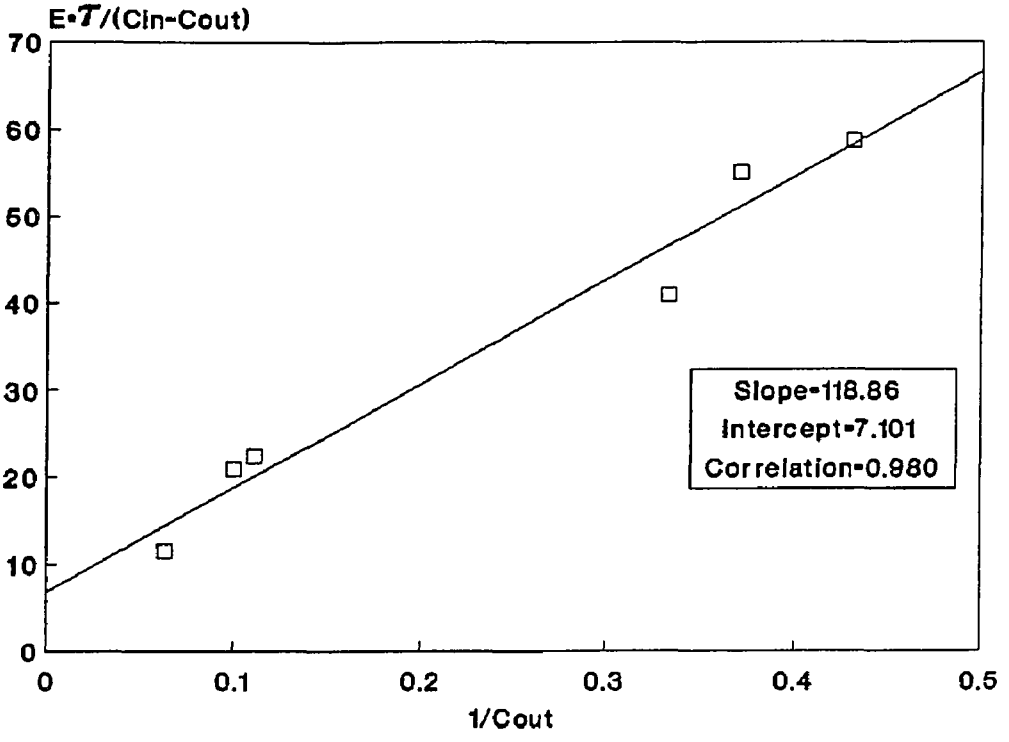


Figure 8.6.2 Determination of model parameters for 246-TCP at pH 4.6

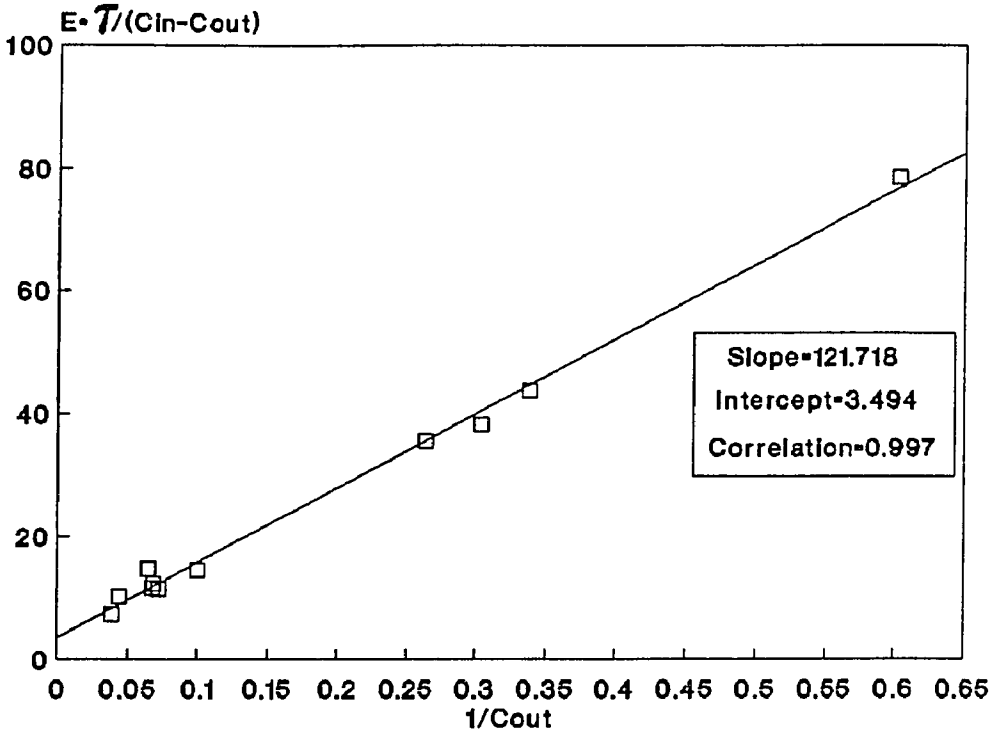


Figure 8.6.3 Determination of model parameters for 246-TCP at pH 5.6

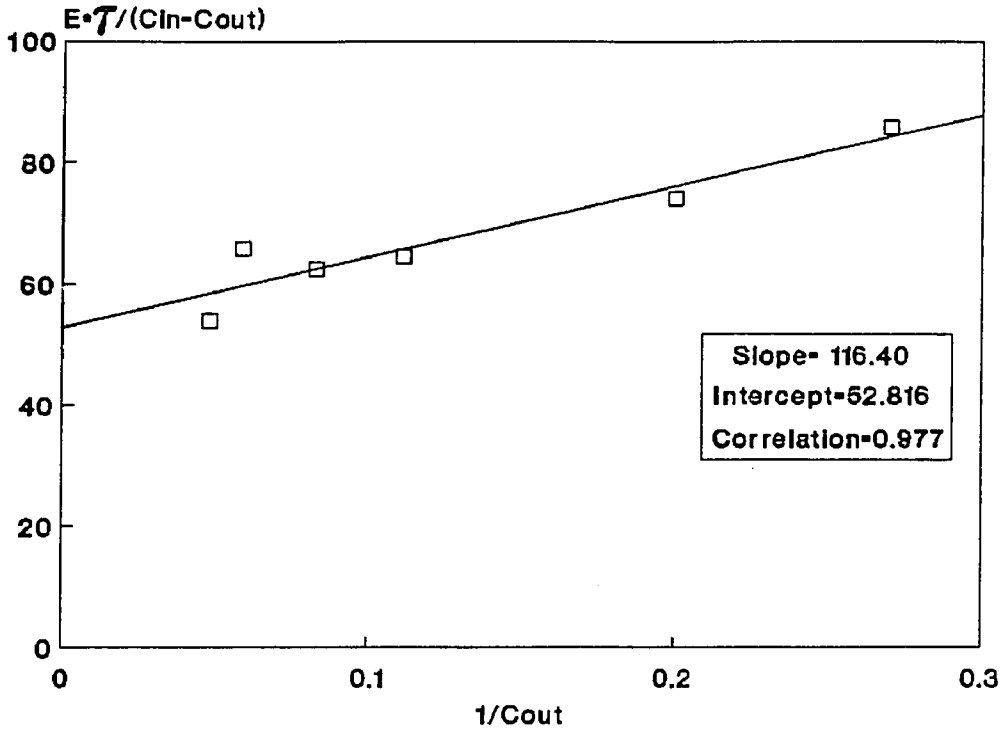


Figure 8.6.4 Determination of model parameters for 245-TCP at pH 3.6

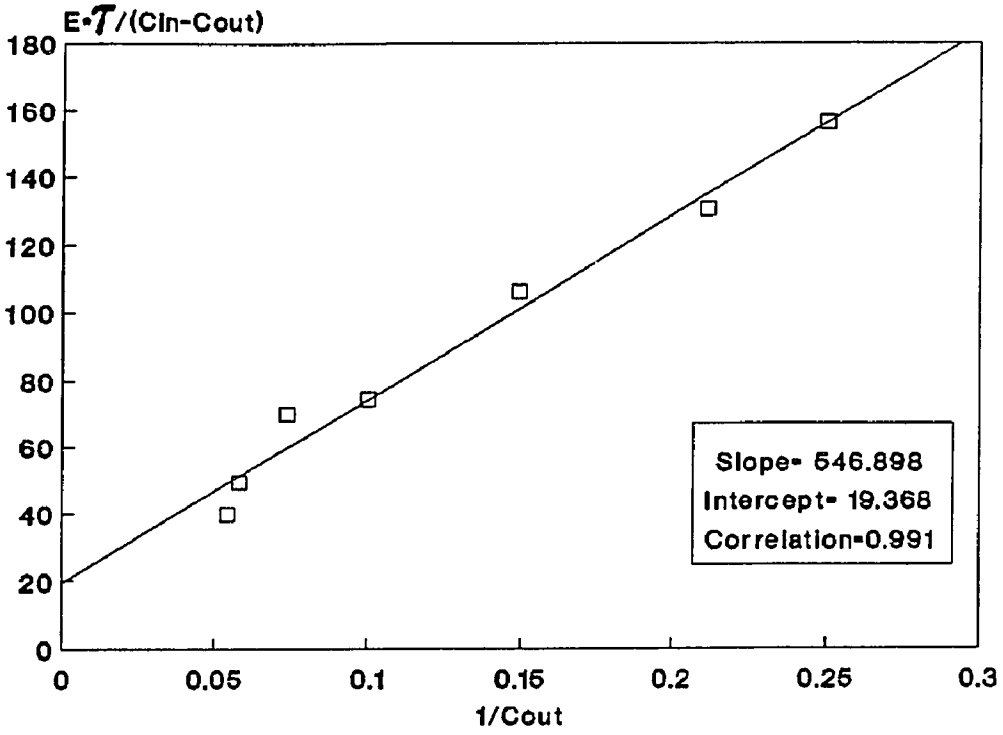


Figure 8.6.5 Determination of model parameters for 245-TCP at pH 4.6

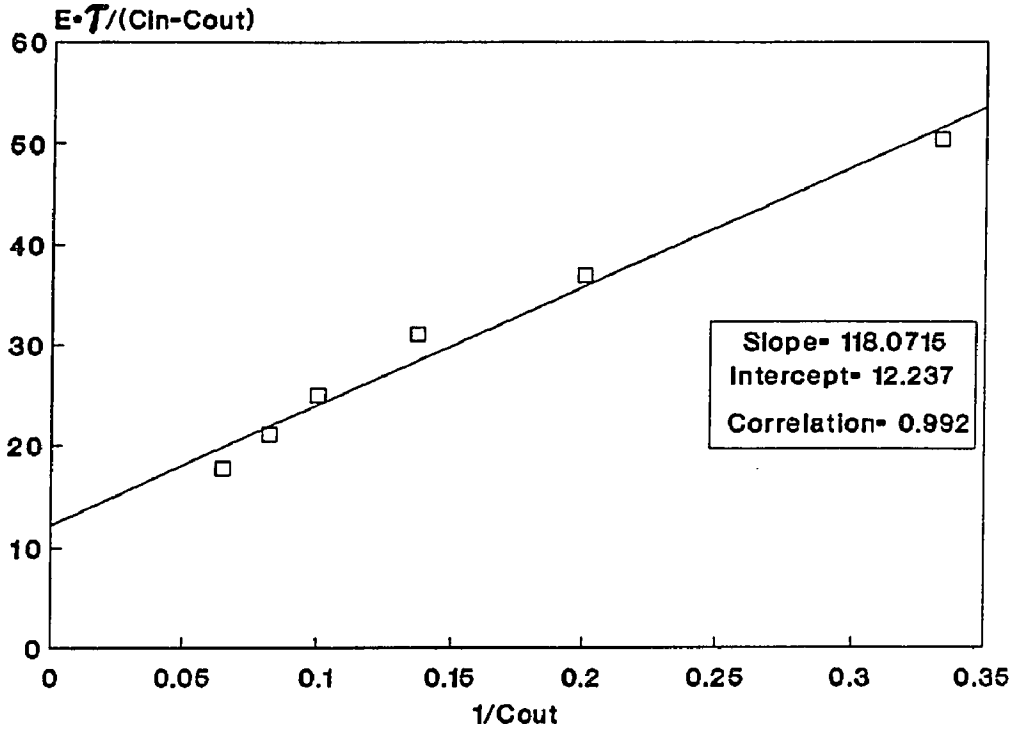


Figure 8.6.6 Determination of model parameters for 245-TCP at pH 5.6

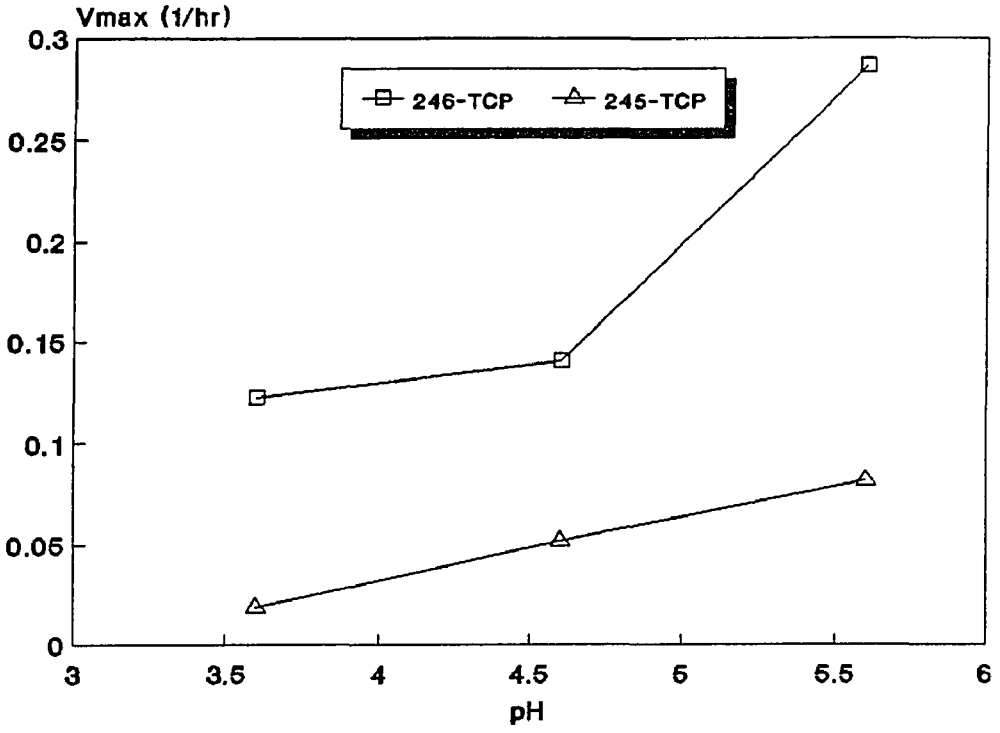


Figure 8.6.7 Variation of V_{max} with pH for 246-TCP and 245-TCP

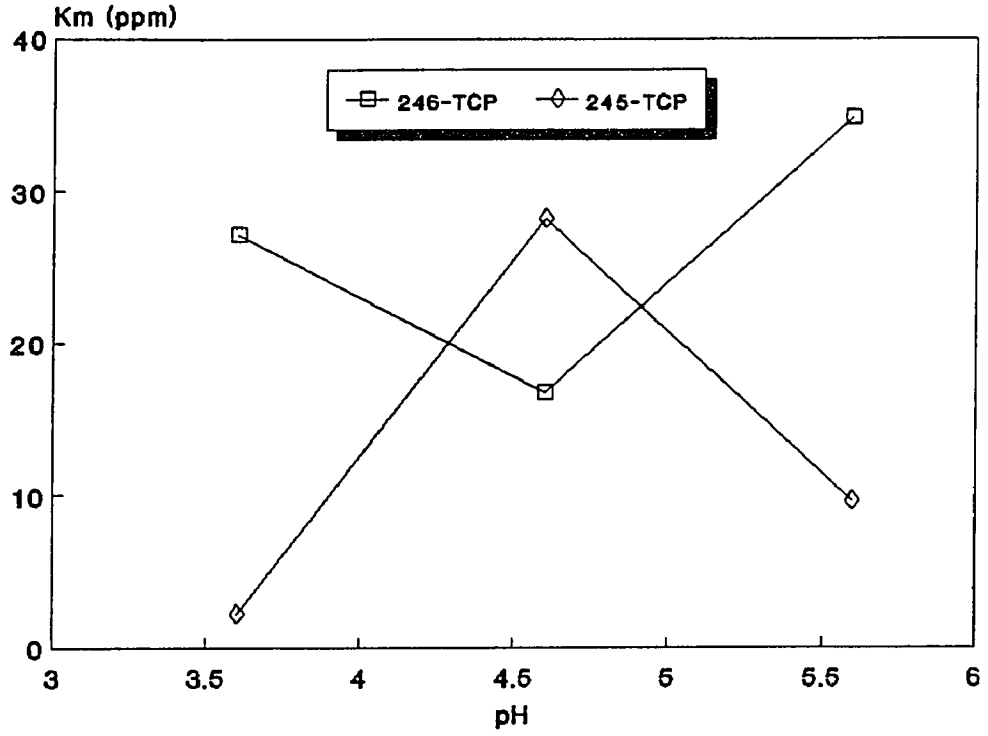


Figure 8.6.8 Variation of K_m with pH for 246-TCP and 245-TCP

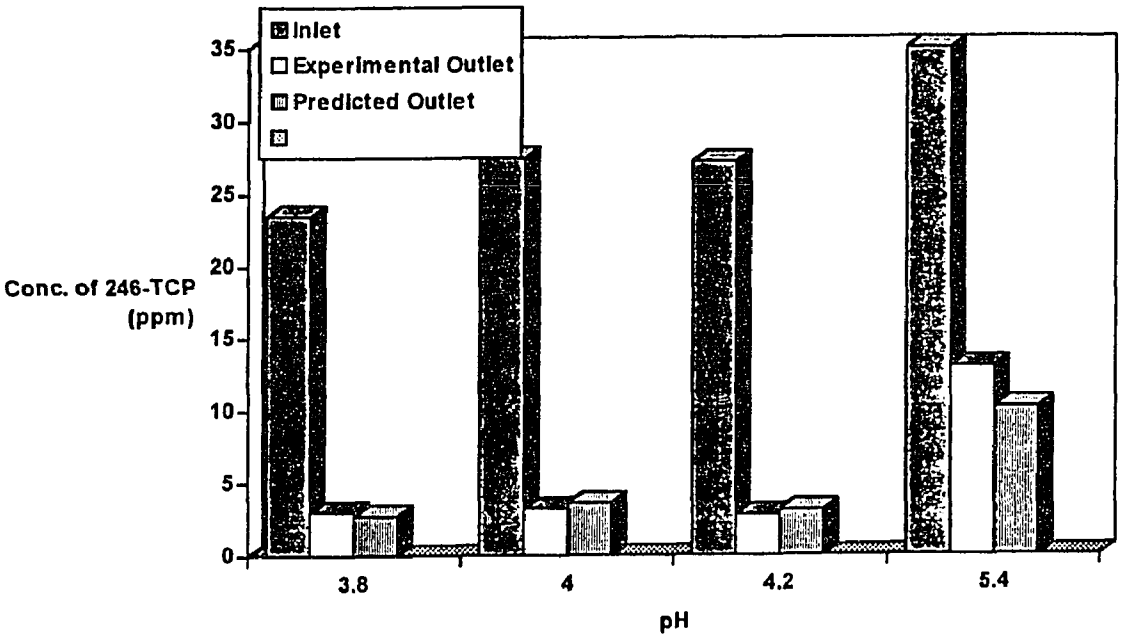


Figure 8.7.1 Prediction of experimental results for 246-TCP at various pH values by interpolation of kinetic data

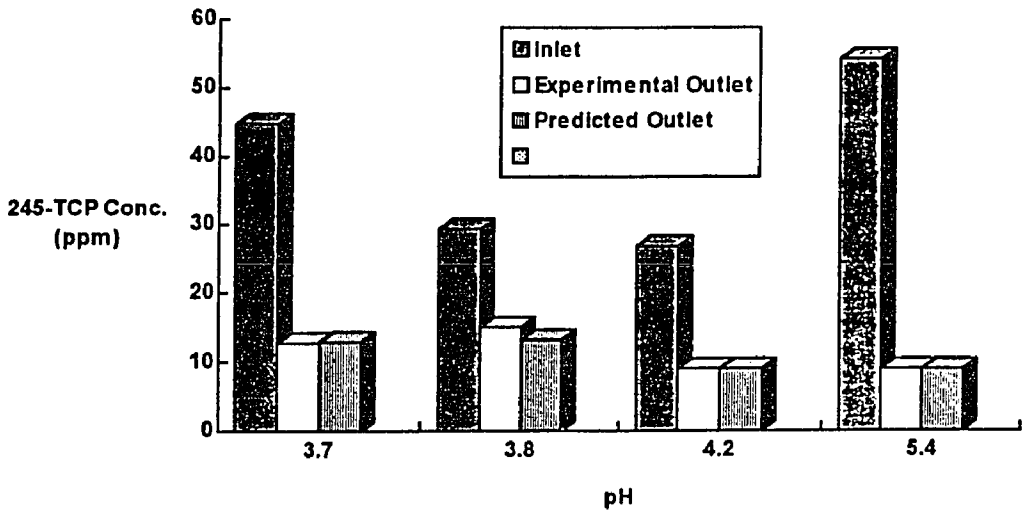


Figure 8.7.2 Prediction of experimental results for 245-TCP at various pH values by interpolation of kinetic data

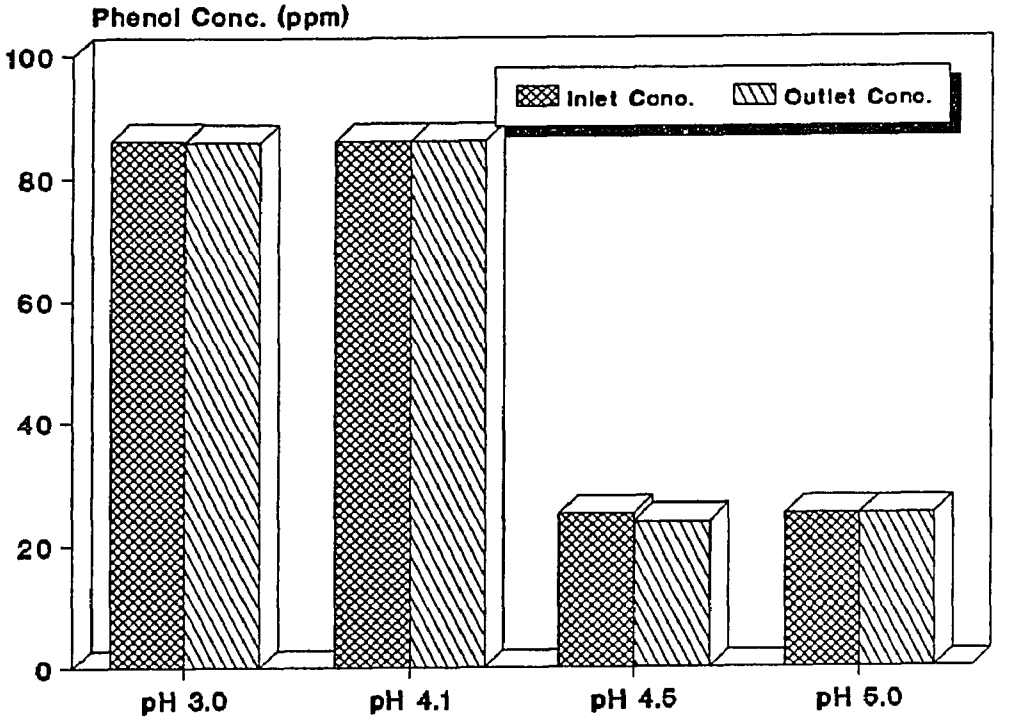


Figure 8.8 Degradation of phenol in packed-bed continuous reactor at different pH values

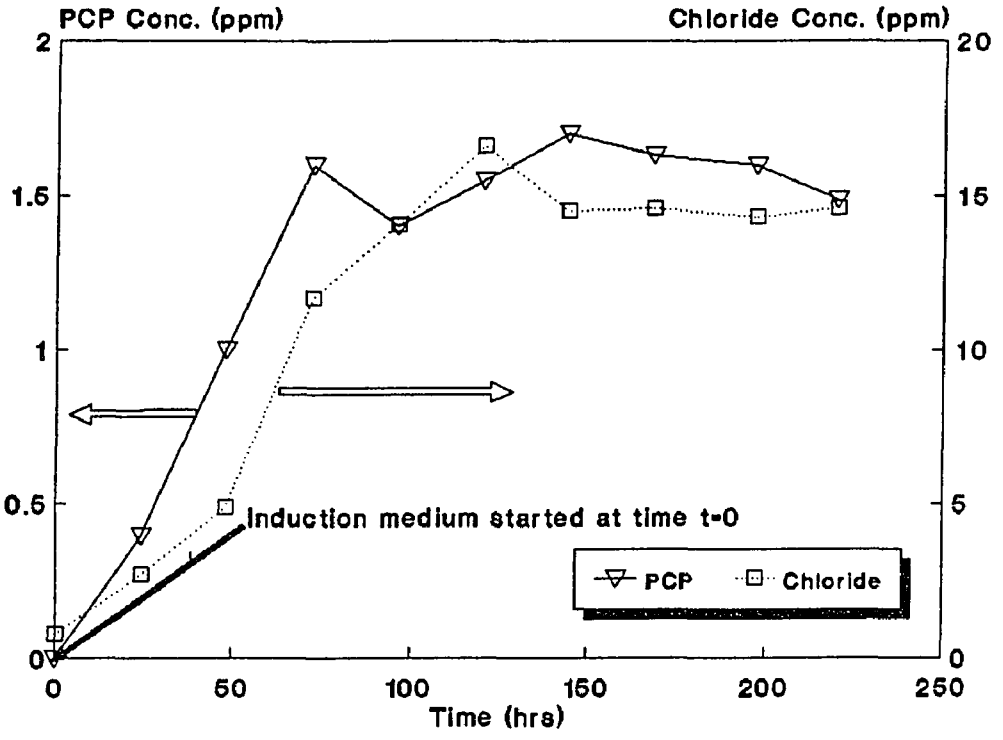


Figure 8.9 Degradation of pentachlorophenol (PCP) in packed-bed continuous reactor

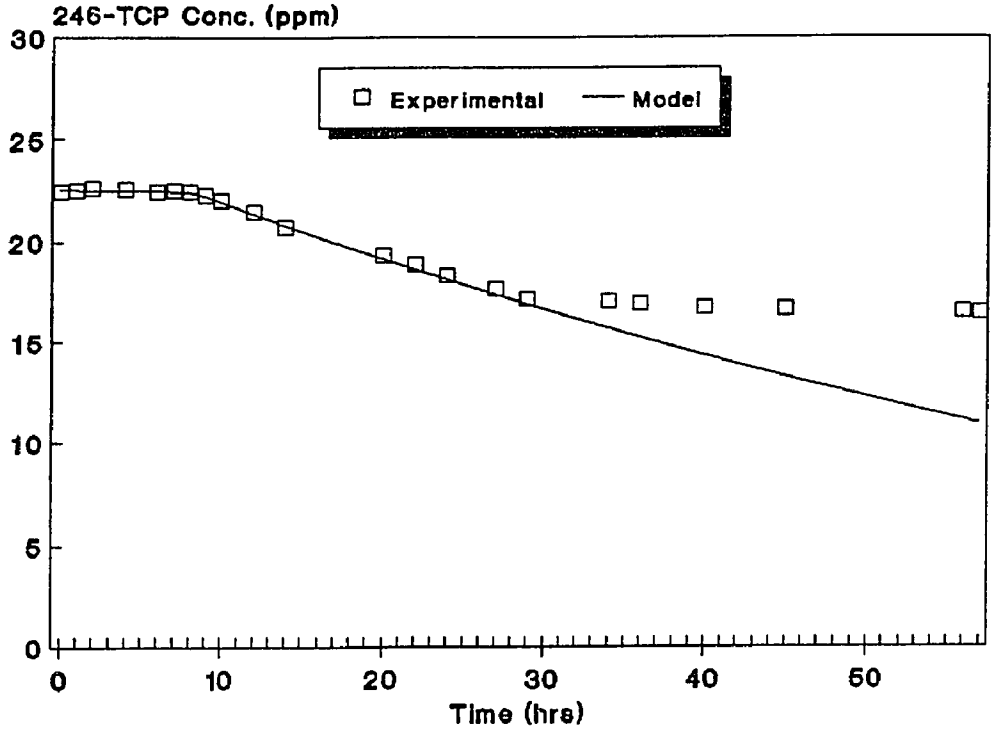


Figure 9.1.1 Comparison of predicted vs. experimental results in batch reactor with separated biomass at pH 5.6

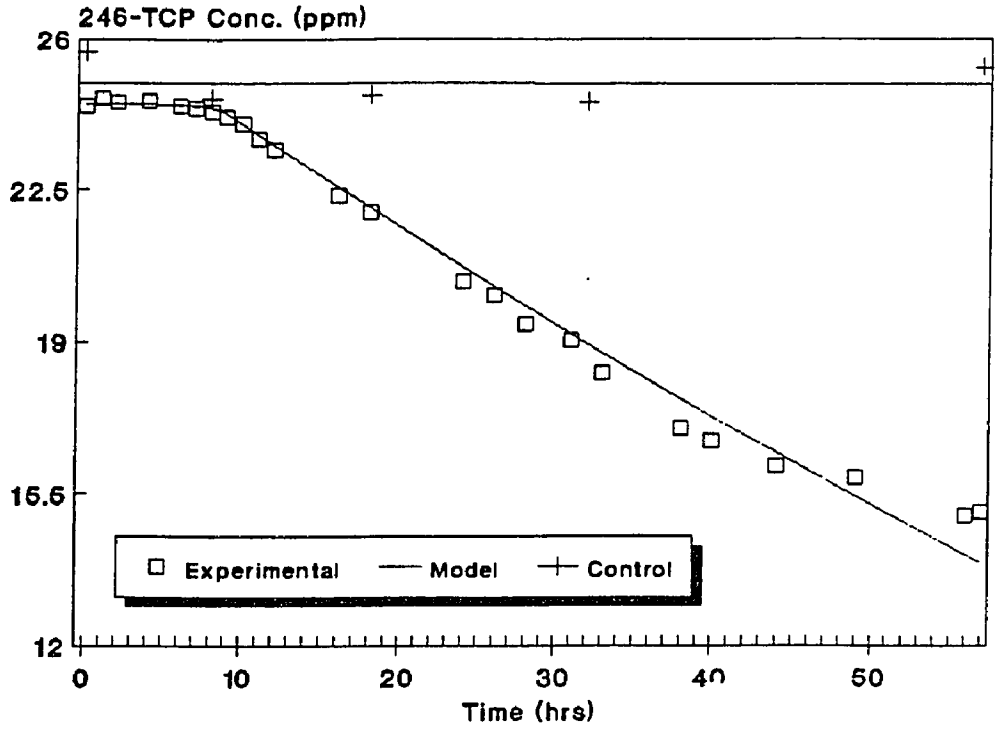


Figure 9.1.2 Comparison of predicted vs. experimental results in batch reactor with separated biomass at pH 4.6

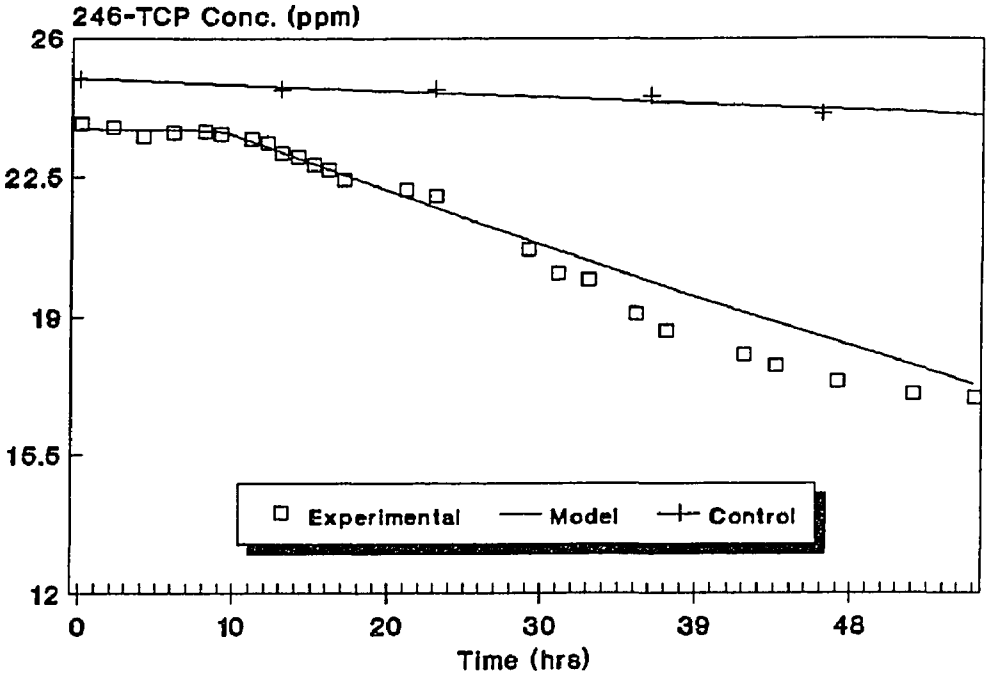


Figure 9.1.3 Comparison of predicted vs. experimental results in batch reactor with separated biomass at pH 3.6

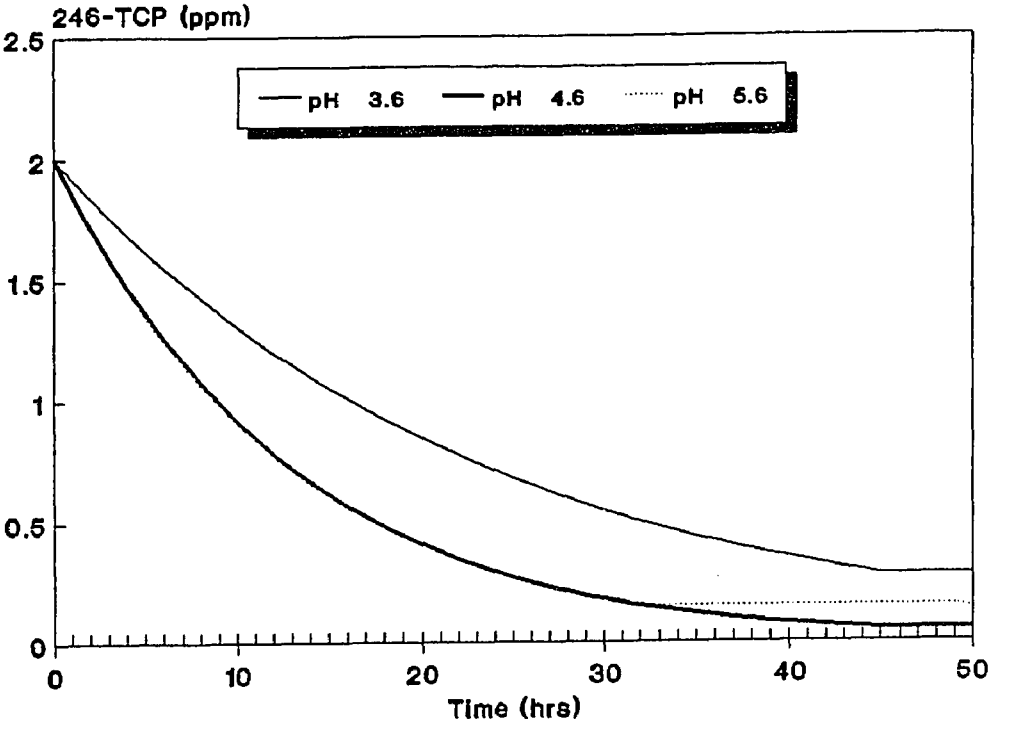


Figure 9.2.1 Optimization of pH for degradation of 246-TCP at an initial concentration of 2.0 ppm

The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

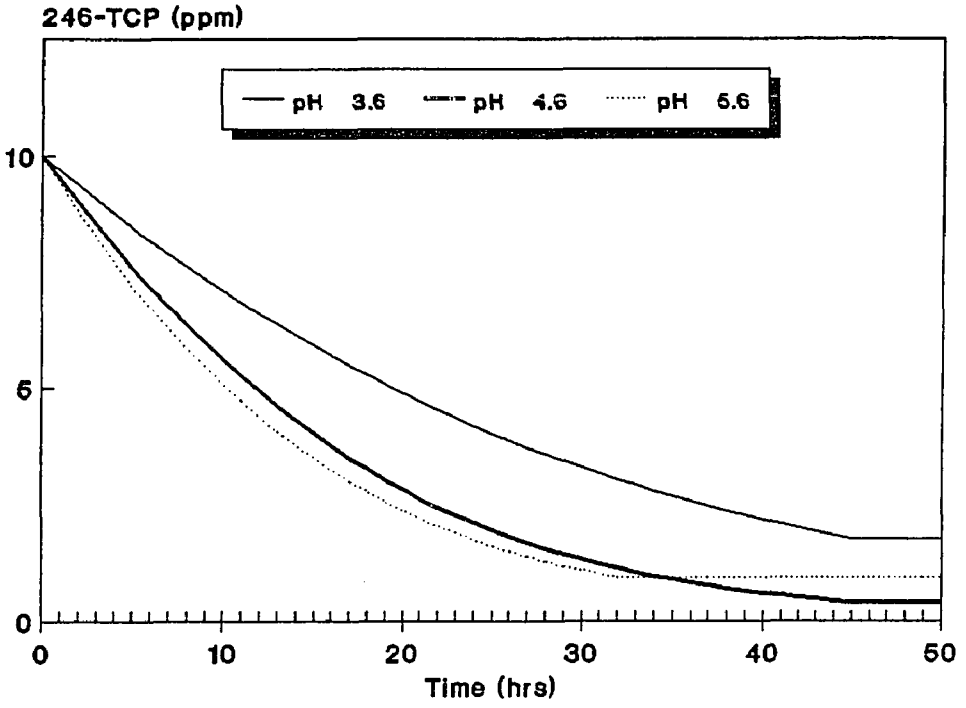


Figure 9.2.2 Optimization of pH for degradation of 246-TCP at an initial concentration of 10.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

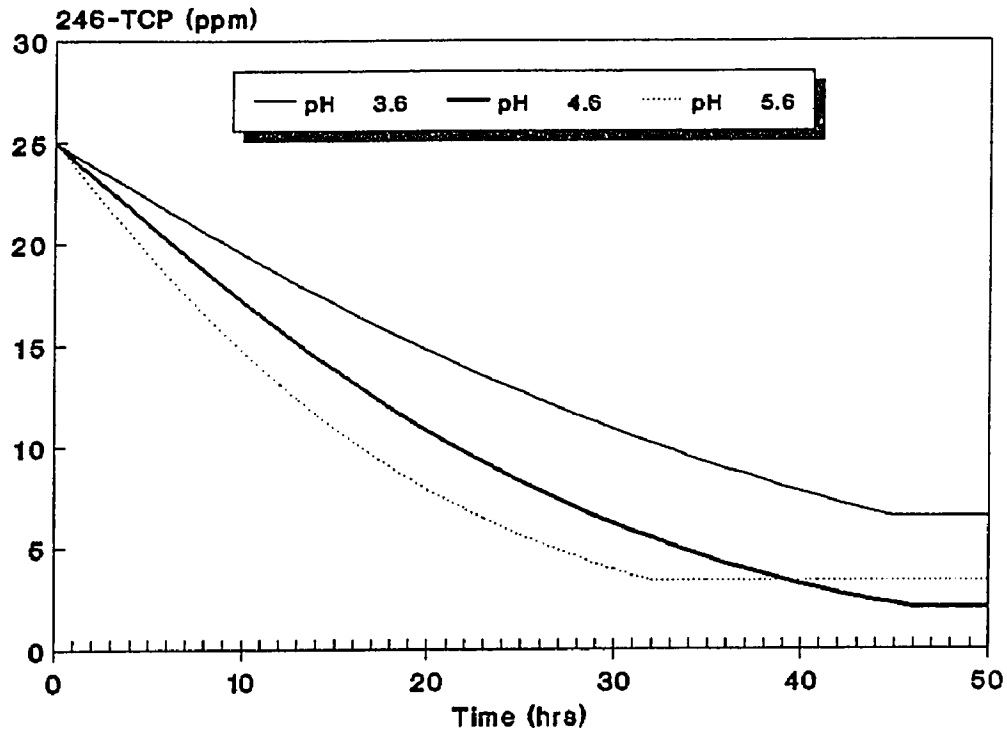


Figure 9.2.3 Optimization of pH for degradation of 246-TCP at an initial concentration of 25.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

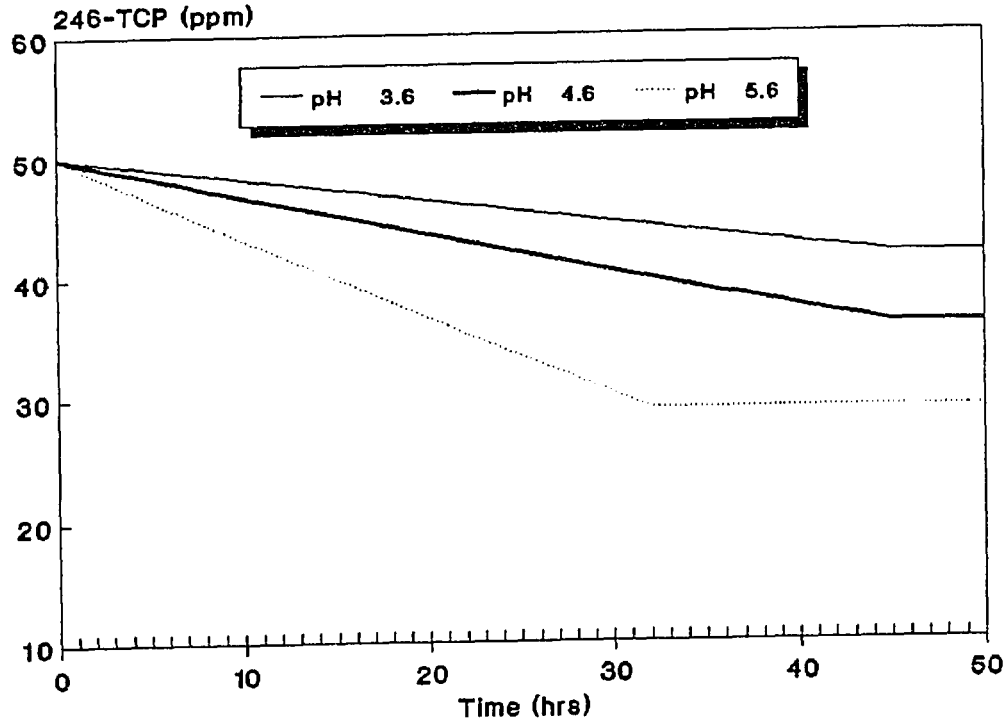


Figure 9.2.4 Optimization of pH for degradation of 246-TCP at an initial concentration of 50.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

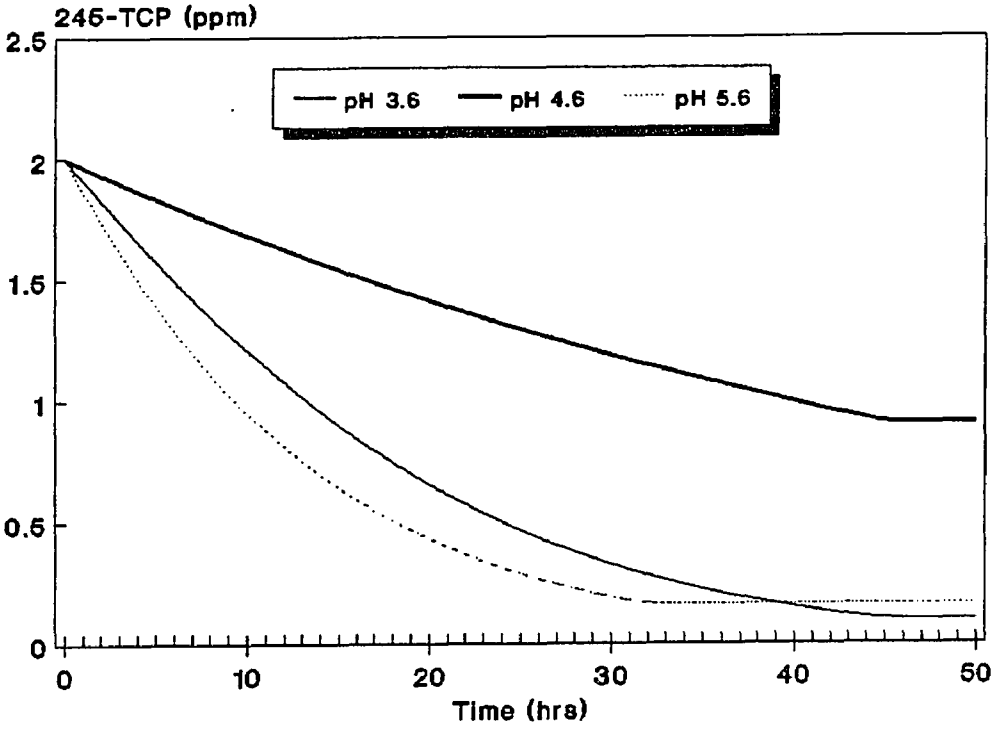


Figure 9.2.5 Optimization of pH for degradation of 245-TCP at an initial concentration of 2.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

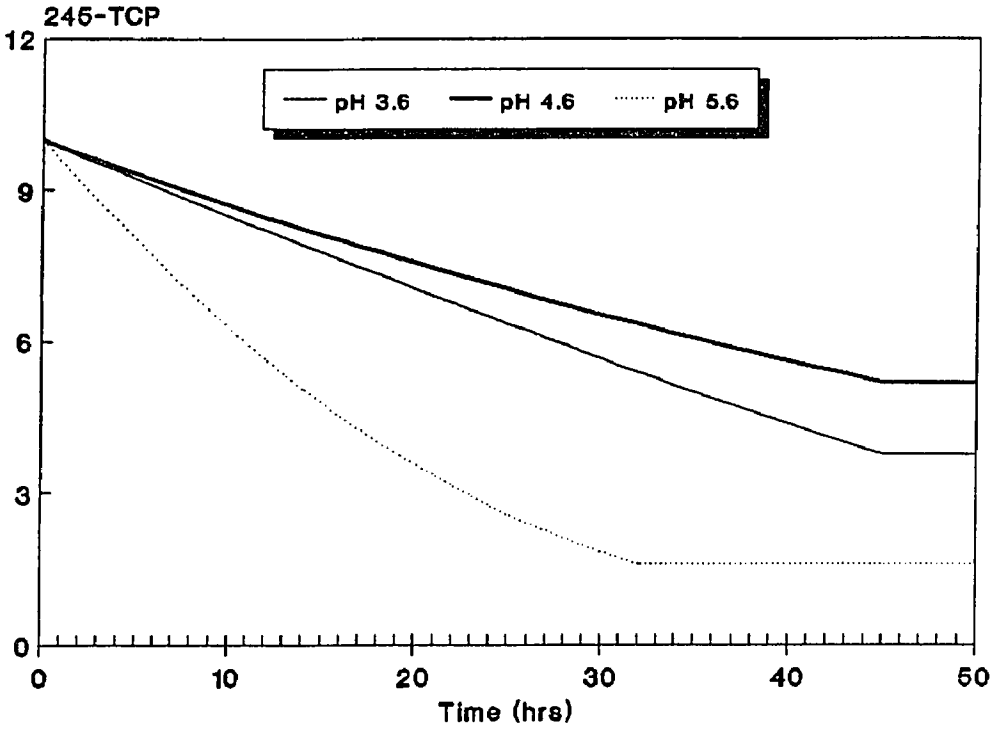


Figure 9.2.6 Optimization of pH for degradation of 245-TCP at an initial concentration of 10.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

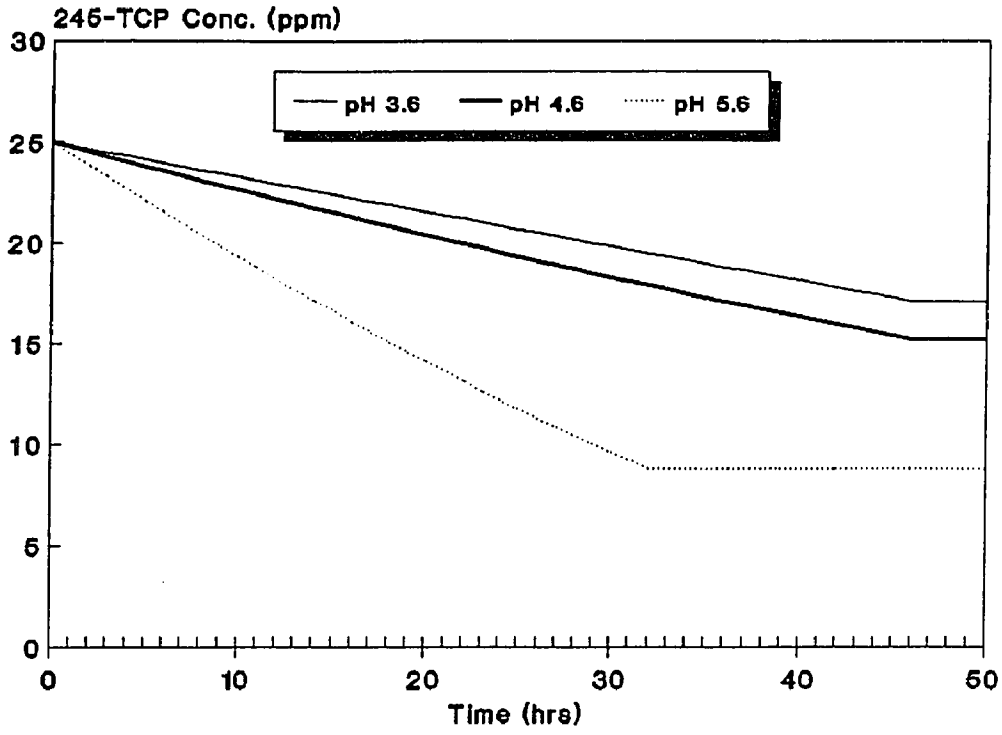


Figure 9.2.7 Optimization of pH for degradation of 245-TCP at an initial concentration of 25.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

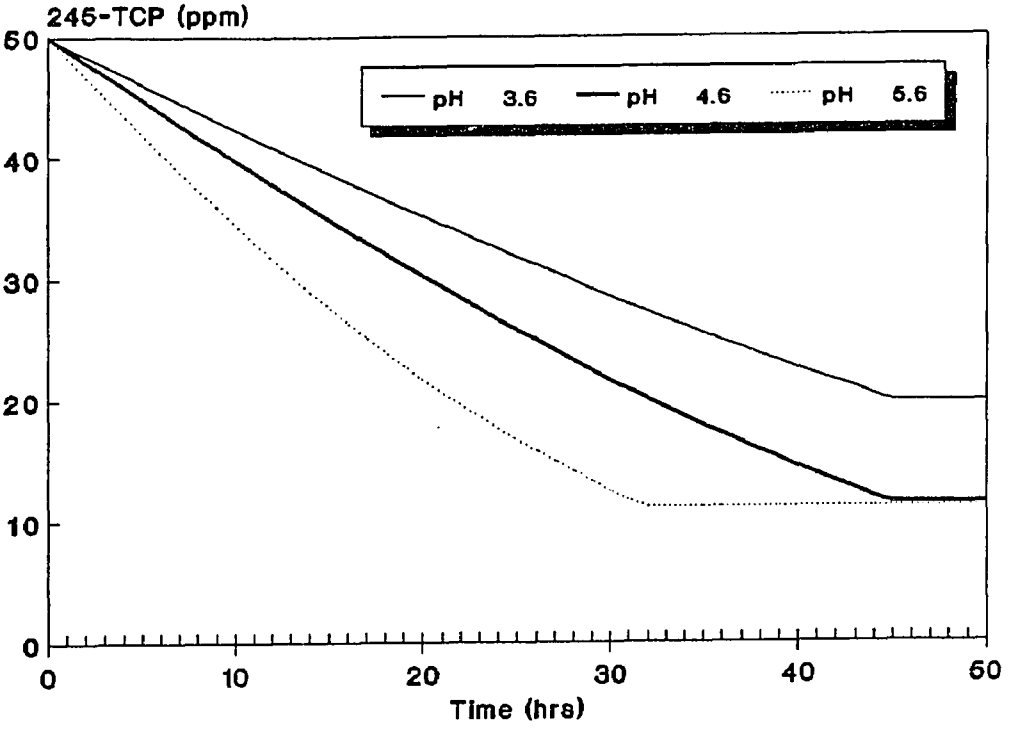
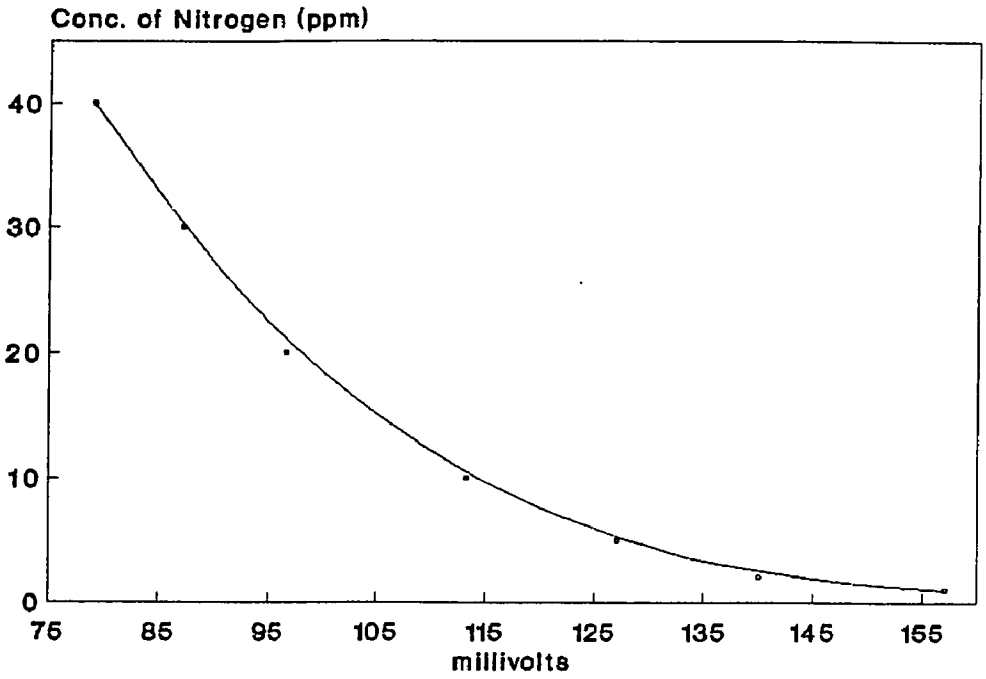


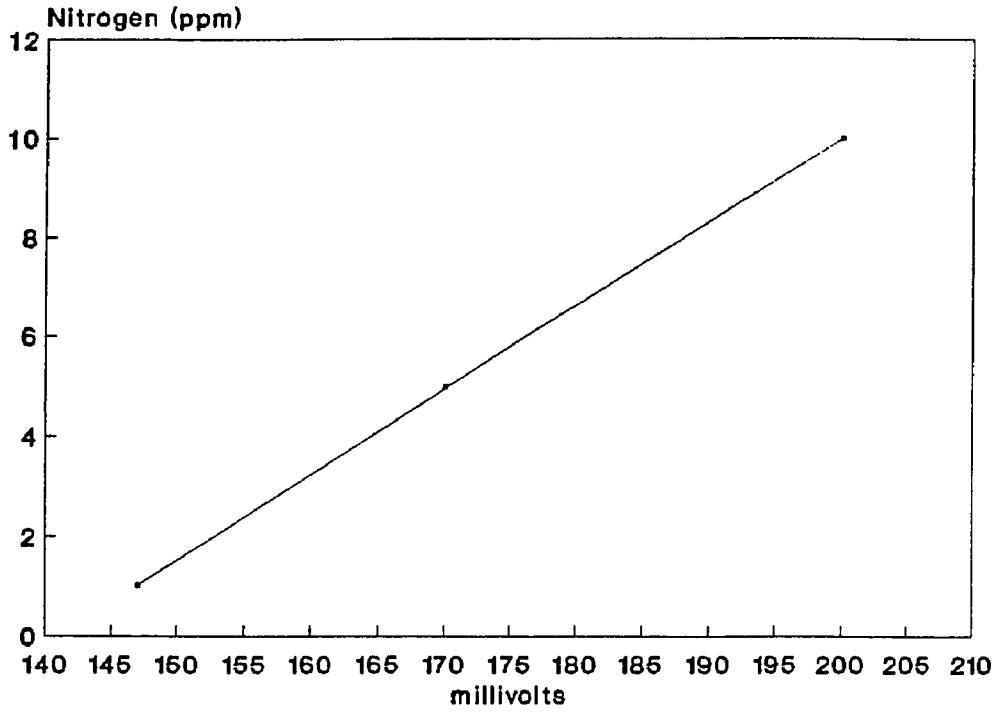
Figure 9.2.8 Optimization of pH for degradation of 245-TCP at an initial concentration of 50.0 ppm
The discontinuity in the curve is due to the assumption that the enzyme activity is a step function. According to this assumption, enzyme(s) remains fully active for 32 hours at pH 5.6 and 45 hours at pH 3.6 and 4.6; and then looses its activity instantaneously.

APPENDIX III

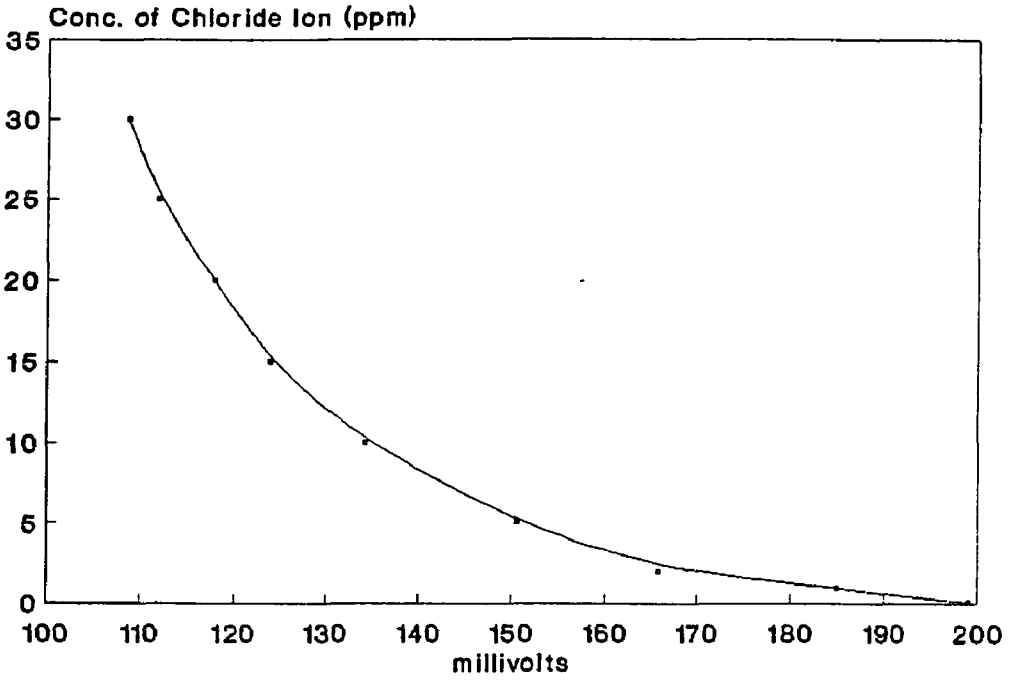
CALIBRATION CURVES



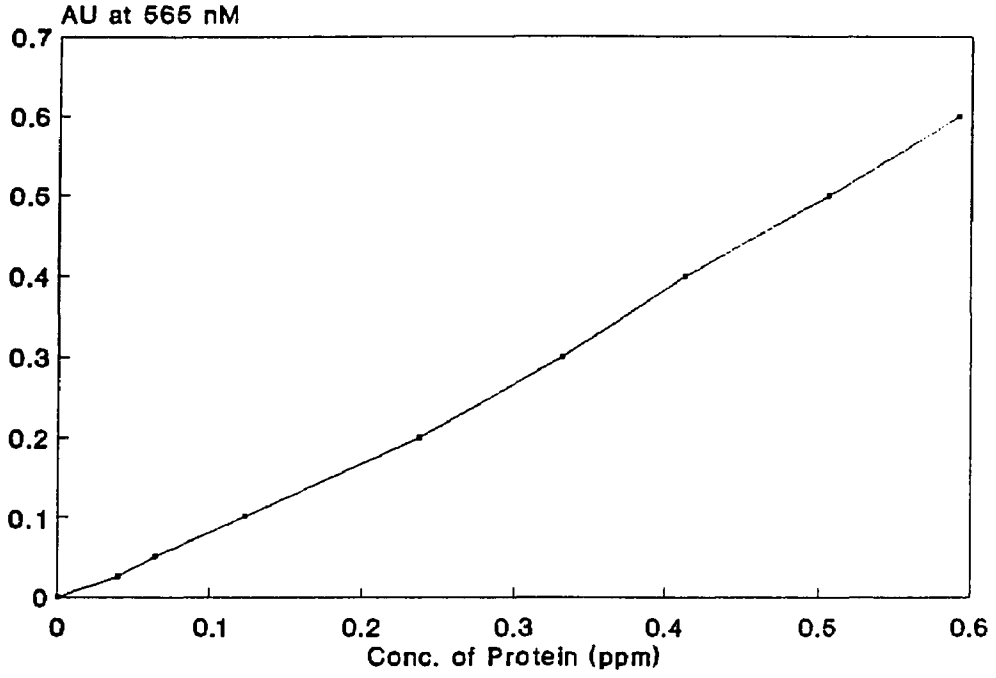
Calibration Curve for Nitrogen as Nitrate



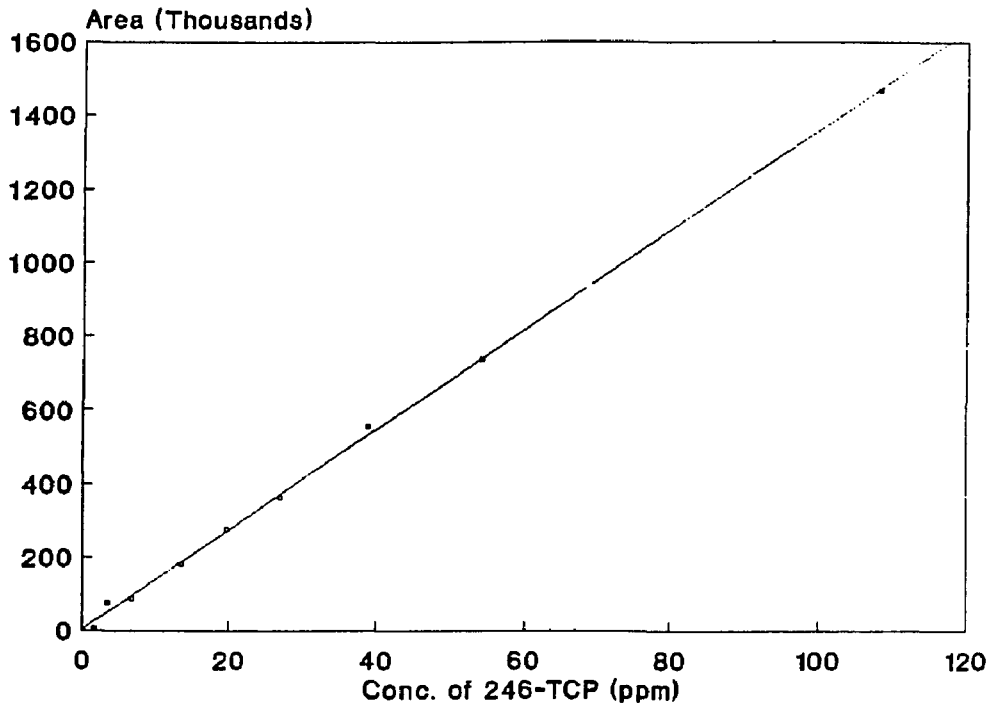
Calibration Curve for Nitrogen as Ammonia



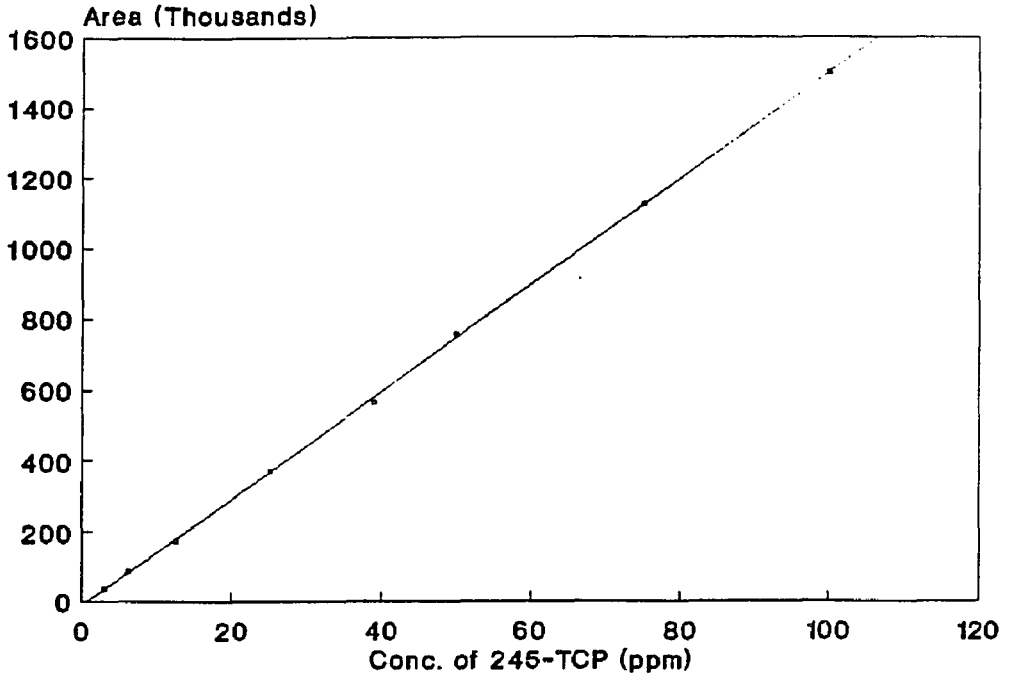
Calibration Curve for Chloride Ion



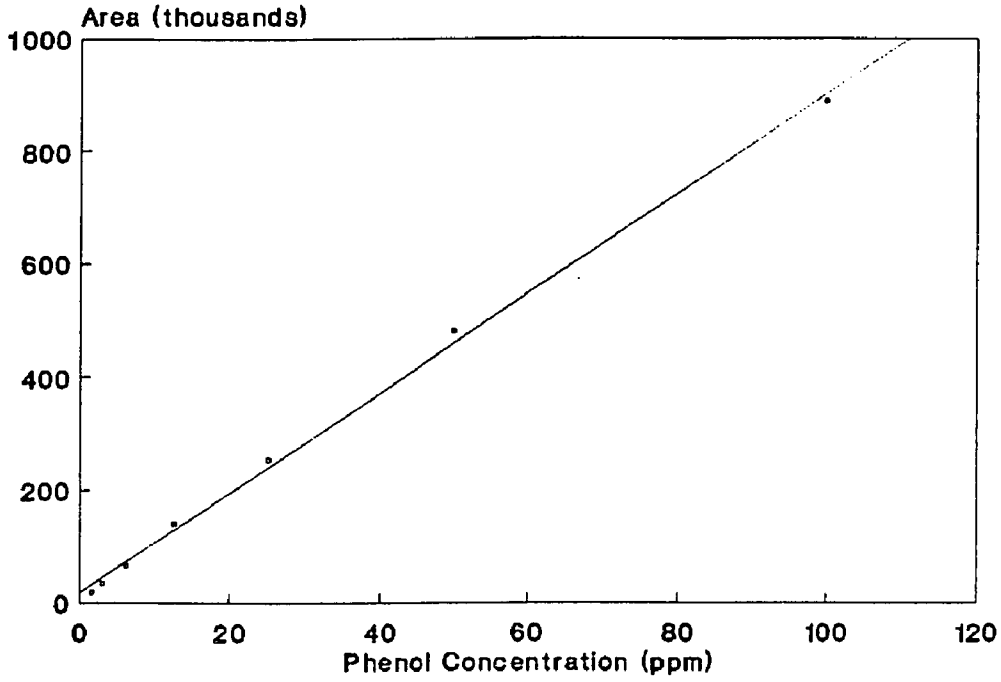
Calibration Curve for Protein



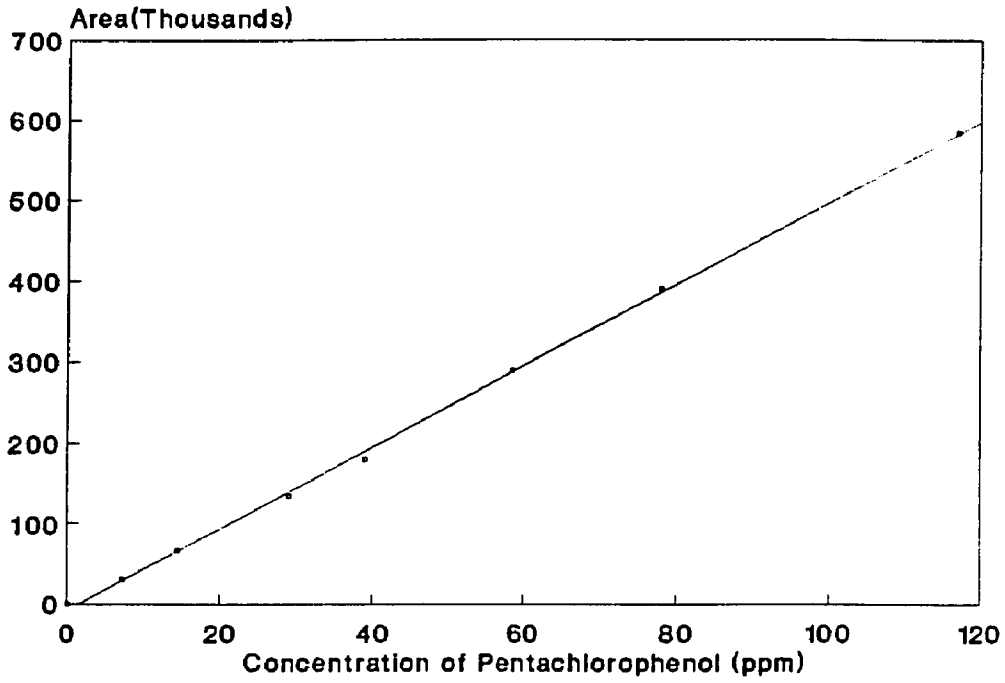
Calibration Curve for 246-TCP



Calibration Curve for 245-TCP



Calibration Curve for Phenol



Calibration Curve for Pentachlorophenol

References

1. Aitken, M.D., R. Venkatadri, and R.Irvine. "Oxidation of Phenolic Pollutants by a Lignin Degrading Enzyme from the White Rot Fungus *Phanerochaete chrysosporium.*" *Water Research.* 4 (1989): 443-450.

2. Ander, P., and K.E. Eriksson. "Influence of Carbohydrates on lignin Degradation by the White Rot Fungus." *Sven.Papperstind.* 18 (1975): 643-652.

3. Arjmand, M., and H. Sandermann. "Mineralization of Chloroaniline/Lignin Conjugates and of Free Chloroanilines by the White Rot Fungus *Phanerochaete chrysosporium.*" *J. of Agricultural Food Chemistry.* 33 (1985): 1055-1060.

4. Asada, Y., M. Miyabe, M. Kikkawa, and M. Kuwahara. "Oxidation of NADH by Peroxidase of a Lignin-degrading Basidiomycete, *Phanerochaete chrysosporium,* and its Involvement in the degradation of a Lignin Model Compound." *Agricultural Biological Chemistry.* 50 (1986): 525-529.

5. Boomingthan, K.; S.B. Dass, and T.A. Randall. "Lignin Peroxidase negative Mutant of the White Rot Basidiomycete *Phanerochaete chrysosporium. J. of Bacteriology.*172 (1990): 260-265.

6. Bugos, R.C., J.B. Sutherland, and J.H. Adler. "Phenolic Compound Utilization by the soft Rot Fungus *Lecythophora hoffmannii.*" *Applied and Environmental Microbiology.* 54 (1988): 1882-1885.

7. Bumpus, J.A., M. Tien, D. Wright, and S.D. Aust. "Oxidation of Persistent Environmental Pollutants by a White Rot Fungus." *Science.*228 (1985): 1434-1436.

8. Bumpus, J.A. "Biodegradation of Polycyclic Aromatic Hydrocarbons by *Phanerochaete chrysosporium." Applied and Environmental Microbiology.* 55 (1989): 154-158.

9. Bumpus, J.A., and S.D. Aust. "Mineralization of Recalcitrant Environmental Pollutants by White Rot Fungus." *Procedings of the National Conference on Hazardous Wastes and Hazardous Materials. Control Research Institute. Silver Spring; MD.* (1987): 146-157.

10. Bumpus, J.A., and S.D. Aust. "Biodegradation of DDT [1,1,1-Trichloro-2,2-Bis(4-Chlorophenyl): Ethane by the White Rot Fungus *Phanerochaete chrysosporium."* 53 (1987): 2001-2008.

11. Buswell, J.A., M. Bruno, and E. Odier. "Lignolytic Enzyme Production by *Phanerochaete chrysosporium* Under Conditions of Nitrogen Sufficiency." *FEMS Microbiology Letters.*25 (1984): 295-299.

12. Crawford, R.L. "Lignin biodegradation and transformation." (1981): *John wiley and sons Inc.* Newyork.

13. Chang, H., T.W. Joyce, T.K. Kirk, and V-B. Huynh. Process of Degrading Chloroorganics by White Rot Fungi." (1985) USA Patent. No: 4,554,075

14. Cheng, K. "Engineering Design of Aerobic- Anaerobic Degradation of Trichlorophenols." Ph.D Dissertation , New Jersey Institute of Technology (1990)

15.Dagley, S. "Catabolism of aromatic Compounds by microorganism." *Advanced Microbiol physiology.* 6 (1971): 1-46.

16. Datta, A., A. Bettermann, and T.K. Kirk. "Identification of a Specific Manganese Peroxide among Ligninolytic Enzymes Secreted by *Phanerochaete chrysosporium* during Wood Decay." *Applied and Environmental Microbiology.* 57 (1991): 1453-1460.

17. Dosoretz, C.G., A.H.C. Chen, and H.E. Grethlein. "Effect of Oxygenation Conditions on Submerged Cultures of *Phanerochaete chrysosporium." Applied Microbiology and Biotechnology.* 34 (1990): 131-137

18. Freudenberg, K. "Lignin -its constituents and Formation from p-hydroxylcinnyl alcohol." *Science* 148 (1965): 595-600

19. Faison, B.D., T.K. Kirk, and R.L. Farrell. "Role of veratryl Alcohol in Regulating Ligninase Activity in *Phanerochaete chrysosporium." Applied and Environmental Microbiology* 52 (1986): 251-254.

20. Faison, B.D., and T.K. Kirk. "Relationship Between Lignin Degrading and Production of Reduced Oxygen Specis by *Phanerochaete chrysosporium." Applied and Environmental Microbiology.* 46 (1988): 1140-1145..

21. Faison, B.D., and T.K. Kirk. "Factors Involved in the Regulation of a Ligninase Activity in *Phanerochaete chrysosp[orium." Applied and Environmental Microbiology.*49 (1985): 299-304.

22. Fenn, B.D., S. Choi, and T.K. Kirk. " Lignolytic activity of *Phanerochaete chrysosp[orium.*: physiology of Suppression by Ammonia and L-Glutamate." *Archives of Microbiology* .130 (1981): 66-71

23. Gold M.H., and K. Valli. "Degradation of 2,4-Dichlorophenol by the Lignin-Degrading Fungus *Phanerochaete chrysosporium." J. of Bacteriology.* 173 (1991): 345-352

24. Gleen, J.K., A.M. Meredith, and M.B.Mayfield. "An Extracellular H_2O_2-requiring Enzyme Preparation Involved in Lignin Biodegradation by the White Rot Basideomycete. "*Biochemical and Biophysical Research Communication.* 114:(1983): 1077-1083.

25. Haggblom, M. "Degradation and Tranformation of Chlorinated Phenolic Compounds by Strains of Rhodococcus and Mycobacterium." Ph.D Dissertation, University of Helsinki, Finland. (1986)

26. Hammel, K.W., and P.J. Tardone. "The oxidative 4-dechlorination of Polychlorinated phenols is Catalyzed by Extracellular Fungal Lignin Peroxidase." *Biochemistry* 27 (1988): 6563-6568.

27. Harvey, P.J., and J.M.Palmer. "Oxidation of Phenolic Compounds by Ligninase." *J.of Bacteriology.*13 (1990): 169-179.

28. Harvey, P.J., H.E. Schoemaker, and J.M. Palmer. " Veratryl Alcohol as a mediator and the role of radical cations in lignin Biodegradation by *Phanerochaete chrysosporium." FEBS Letter.* 195 (1986): 242-246.

29. Harvey, P.J., H.E. Schoemaker, R.M. Bowen, and J. M.Palmer. "Single-electron Tranfer Process and the Reaction Mechanism of Enzyme Degradation of Lignin." *FEBS.* 183 (1985): 13-16.

30. Haq, I. "Reactor Design For Hazardous Waste Treatment Using Immobilized *Phanerochaete chrysosporium."* M.S. thesis. NJIT, New Jersey Institute of Technology. (1990)

31. Huynh, V-B., and R.L.Crawfoprd. "Novel Extracellular Enzymes (Ligninase) of *Phanerochaete chrysosporium." FEMS Microbiology Letter.*28 (1985): 119-123.

32. Jager, A., S. Croan, and T.K. Kirk. "Production of Ligninase and Degradation of Lignin in Agitated Submerged Cultures of *Phanerochaete chrysosporium." Applied and Environmental Microbiology.* 50 (1985): 1274-1278.

33. Janshekar, H., C. Brown, T. Haltmeier, M. Leisola, and A. Fiechter. "Bioalteration of Kraft lignin by *Phanerochaete chrysosporium.*". *Archives of Microbiology.*132 (1982): 14-21.

34. Kelly, R.L., and A.Reddy. "Purification and Charecterization of Glucose Oxidase from Ligninolytic Cultures of *Phanerochaete chrysosporium.*" *J.of Bacteriology.*166 (1986): 269-274.

35. Kelly, R.L., and C.A. Reddy. "Identification of glucose Oxidase Activity as the Primary Source of Hydrogen Peroxide Production in Ligninolytic Cultures of *Phanerochaete chrysosporium." Archives of Microbiology.* 144 (1986): 248-253.

37. Kersten, P.J., and T.K. Kirk. "Involvement of a new Enzyme, glyoxal Oxidase, in Extracellular H_2O_2 Production by *Phanerochaete chrysosporium.*" *J. of Bacteriology.* 169 (1987): 2195-2201.

36. Kirk, T.K., and H.M. Chang., "Decomposition of Lignin by White Rot Fungus Isolation of Heavily Degraded Lignins From Decayed Spurce." *Holzforschung* 28 (1975): 56-64.

38. Kirk, T.K., and M.Tien. "Lignin Degrading Activity of *Phanerochaete chrysosporium* Burds.: Comparison of Cellulose-negative and Other Strains." *Enzyme Microbiol Technology.* 8 (1986): 75-80.

39. Kirk, T.K., E. Schultz, W.J. Connors, L.F. Lorenz, and J.G., Zeikus. "Influence of Culture Parameters on Lignin Metabolism by *Phanerochaete chrysosporium." Archives of Microbiology.* 117 (1986): 277-285.

40. Lamar, R.T, and D.M. Dietrich. "In situ Depletion of Pentachlorophenol from Contaminated Soil by *Phanerochaete chrysosporium* Spp." *Applied and Environmental Microbiology.* 56 (1990): 3093-3100

41. Leatham, G.F., R.L. Crawford, and T.K. Kirk. "Degradation of Phenolic Compounds and Cleavage of Cathecol by *Phanerochaete chrysosporium." Applied and Environmental Microbiology.* 46 (1983):191-197.

42. Lestan, A., A. Strancar, and A.Perdih. "Influence of Some Oils and Surfactants on Lignolytic activity, growth and lipid fatty acids of *Phanerochaete chrysosporium.*" *Applied Microbial Biotechnology.*34 (1990): 426-428.

43. Leisola, M.A., B. Kozulic, F. Meussdoerffer, and A. Fiechter. "Homology among Multiple Extracellular Peroxidases from *Phanerochaete chrysosporium*." *American Society of biological Chemistry*.202 (1987): 419-424.

44. Leisola, M.S.A., and A. Fiechter. "Ligninase Production in Agitated Conditions by *Phanerochaete chrysosporium*." *FEMS Microbiology Letters*.29 (1985) 33-36

45. Lin, J.R., and H.Y. Wang. "Degradation Kinetics of *Phanerochaete chrysosporium*." *Biotechnology and Bioengineering*. 35 (1990): 1125-1134.

46. Lin, J.R., and H.Y. Wang. "Use of Coimmobilized Biological Systems to Degrade Toxic Organic Compounds." *Biotechnology and Bioengineering*. 38 (1990): 273-279.

47. LinKo, Y.Y., M. Leisola, N. Lindholm, J. Troller, J., Linko, and A. Fiechter. "Continuous Production of Lignin Peroxidase by *Phanerochaete chrysosporium*." *J. of Biotechnology*. 4 (1986): 283-291.

48. Liwicki, R., A. Paterson, M.J. Macdonald, and P.Broda. " Phenotypic classes of Phenoloxidase negative mutantsof a lignin Degrading Fungus *Phanerochaete chrysosporium*." *J. of Bacteriology*. (1987): 162 641-644

49. Merrill, W., and E.B. Cowling. " Role of Nitrogen in Wood Detoriation Amounts and Distribution of nitrogen in Tree Steams." *Canadian J. of Botany*, 177 (1966): 1555-1580.

50. Miranda, G.S.C., S. Choi, and T.K. Kirk. "Mycelium Binding and Depolymerization of Synthetic ^{14}C-Labeled Lignin During Decomposition by *Phanerochaete chrysosporium*." *Holzforchung*. 37 (1983): 55-61.

51. Mileski, G.J., J.A. Bumpus, M.A. Jurek, and S.D. Aust. "Biodegradation of Pentachlorophenol by the White Rot Fungus *Phanerochaete chrysosporium*." *Applied and Environmental Microbiology*. 54 (1988): 2885-2889.

52. Molkness, T.A., M. Alic, and M.H. Gold. "Charecterization of Leucine Auxotrophs of the White Rot Basidiomycete *Phanerochaete chrysosporium*." *Applied and Environmental Microbiology*. 51 (1986): 1170-1173.

53. Muheim, A., M.S.A. Leisola, and H.E. Schoemaker. "Aryl-alcohol oxidase and Lignin peroxidase from the White Rot fungus *Bjerkandera adusta*." *J. of Biotechnology*. 13 (1990): 159-167.

54. Orth, A.B., M. Denny, and M.Tien. "Overproduction of Lignin-Degrading Enzymes by an Isolate of *Phanerochaete chrysosporium*." *Applied and Environmental Microbiology*. 57 (1991): 2591-2596

55. Pak, Daewan. "Reactor Design for Hazardous WasteTreatment Using a White Rot Fungus." Ph.D Dissertation. New Jersey Institute of Technology. (1988)

56. Paszczynski, A., V-B. Huynh, and R. Crawford. "Enzymatic activities of an Extracellular Manganese-dependent Peroxidase from *Phanerochaete chrysosporium*." *FEMS Microbiology Letter*.29 (1985): 37-41.

57. Pellienen, J., T.W. Joyce, and H-m.Chang. "Dechlorination of Hihg-molecular-weight Chlorolignin by the White-rot Fungus *p. chrysosporium*." *Tappi Journal*.(1988): 191-194.

58. Reid, I.D., E.E. Chao, and P.S.S. Dawson. "Lignin Degradation by *Phanerochaete chrysosporium* in Agitated Cultures." *Canadian J. of Microbiology*. 31 (1985): 88-90.

59. Reid, I. "Effects of Nitrogen Suppliment on Degradation of Aspen Wood Lignin and Carbohydrates Components by *Phanerochaete chrysosporium*." *Applied and Environmental Microbiology*. 45 (1987): 830-837.

60. Reid, I. "Effects of Nitrogen Sources on Cellulose and Synthetic Lignin Degradation by *Phanerochaete chrysosporium*." *Applied and Environmental Microbiology*. 45 (1987): 838-842.

61. Roels, J.A. "Energetics and Kinetics in Biotechnology." *Elsevier Biomedical Press*. (1983): 30-37.

62. Rochkind, M.L., J.W. Blackburn, and G.S.Sayler. "Microbial Decomposition of Chlorinated Aromatic Compounds" EPA, Cincinnati, Ohio, Contact No 68-03-3074 (1986)

63. Sanglard, D., M.S.A. Leisola, and A. Fiechter. "Role of Extracellular Ligninase in Biodegradation of Benzo(a)pyrene by *Phanerochaete chrysosporium*." *Enzyme Microbiol Technology*. 8 (1986): 209-212.

64. Schoemaker, H.E., P.J. Harvey, R.M. Bowen, and J.M. Palmer. "On the Mechanism of Enzymatic Lignin Breakdown." *FEBS* 183 (1985): 7-12.

65. Tien, M., and S.B.Myer. "Selection and Charecterization of Mutants of *Phanerochaete chrysosporium* Exibiting Lignolytic Activity Under Nutrient-Rich Condition." *Applied and Environmental Microbiology.* 56 (1990): 2540-2544.

66. Tien, M., and T.K.Kirk. "Lignin-degrading Enzyme from *Phanerochaete chrysosporium: Purification, Charecterization, and Catalytic* Properties of a Unique H_2O_2-requiring Oxygenase." *Proc. of National Academy of Science,USA.* 81 (1984): 2280-2284.

67. Toma, M.K., M.P. Ruklisha, J.J. Vangas, M.O. Zeltina, M.P. Leite, N.I. Galinna, U.E. Viesturs, and R.P.Tengerdy. "Inhibition of Microbial Growth and Metabolism by Excess Turbulence." *Biotechnology and Bioengineering.* 38 (1991): 552-556.

68. Ulmer, D.C., M.S.A. Leisola, H.S. Brigitte, and A. Fiecher. "Rapid Degradation of Isolated Lignins by *Phanerochaete chrysosporium." Applied and Environmental Microbiology.* 45 (1983): 1795-18012.

69. Umezaka, T., and T. Higuchi. "Aromatic ring Cleavage of β-O-4 Lignin model dimers without prior demeth(ox)ylation by Lignin Peroxidase." *FEBS Letter* 205 (1986): 293-298.

70. Wood, J.M., R.L. Crawford, E. Munck, J.D. Zimmerman, R.S. Lipscomb, L. Stephens, and W.H.O. Johnson. "Structure and function of dihydrogenesees. One approach to lignin Degradation." *J. of Agricultural Food Chemistry.* 25 (1977): 698-704

71. Yang, H.H., M.J. Effland, and T.K. Kirk. "Factors Influencing Fungal Degradation of Lignin in a Representative lignocellulostic Thermomechanical Pulp." *Biotechnology and Bioengineering.* 22 (1980): 145-148

72. Zitzelsberger, W., W. Ziegler, and P.R. Wallnofer. "Stereospecificity of the Degradation of Veratrylglycerol-β-2,4-Dichlorophenyl-Ether, a Model Compound for Lignin Bound Xenobiotic Residues by *Phanerochaete chrysosporium, cornebacterium equi, and* Photosensitized Riboflabin." *Chemosphere.* 16 (1987): 1137-1142.

73. Zender, R. 1963. " A semimicro estimation technique for measuremens of Sugar." *Clin.Chem.Acta.* 8 (1967): 351-356.