BIOREMEDIATION OF THE ORGANOPHOSPHATE PESTICIDE, COUMAPHOS, USING MICROORGANISMS IMMOBILIZED IN CALCIUM-ALGINATE GEL BEADS

A Dissertation

by

JIYEON HA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Biological & Agricultural Engineering

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Approved by:

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Committee Members,	James R. Wild
	Robin Autenrieth
	Ann Kenimer
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ABSTRACT

Bioremediation of the Organophosphate Pesticide, Coumaphos, Using Microorganisms Immobilized in Calcium-Alginate Gel Beads. (December 2005)

Jiyeon Ha, B.S., Pusan National University, South Korea;

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Chair of Advisory Committee: Dr. Cady R. Engler

Coumaphos is an organophosphate insecticide used predominantly by the US Department of Agriculture, Animal and Plant Health Inspection Services for its tick eradication program. Bioremediation of the hydrolysis products of coumaphos, chlorferon and diethylthiophosphate (DETP), using Ca-alginate immobilized cells was the focus of this research. Consortia of indigenous microorganisms capable of degrading chlorferon and DETP were isolated separately. Since chlorferon inhibited both chlorferon-degrading and DETP-degrading organisms, it was not possible to enrich a consortium of organisms for simultaneous degradation of chlorferon and DETP.

A two-step growth procedure was developed for degradation studies to provide biomass acclimated to the target compound and reaction medium since cells lost their degradation activity during the growth in a rich medium. Without acclimation, approximately a week-long lag period was required before degradation was initiated. Optimum reaction conditions were found for the degradation of chlorferon and DETP using free cells. Reaction kinetics of chlorferon and DETP were determined using

enzyme kinetics because cell growth was not observed during the degradation. Chlorferon degradation followed substrate inhibition kinetics and DETP degradation followed simple Michaelis-Menten kinetics.

A calcium-alginate immobilized cell system was developed, and the optimum bead loadings in the reactor were determined. Degradation rates for immobilized cells were enhanced up to five times that for free cells in untreated cattle dip (UCD) solution. The enhanced degradation of immobilized cells was due to protection of the cells from inhibitory substances present in the UCD solution. In addition, physiological changes of cells caused by Ca-alginate immobilization may have contributed to a slightly increased reaction rate in pure solution.

Diffusion coefficients of chlorferon and DETP into Ca-alginate gel beads were studied to assist in designing and operating bioreactor systems. Diffusion coefficients of chlorferon and DETP increased with increasing agitation speed and decreasing substrate concentration. Increased cell concentration in gel beads caused lower diffusivity. Calcium-alginate gel beads used in this study were not subject to diffusional limitations. Both external and internal mass transfer resistances were negligible, and the degradation rate inside Ca-alginate gel beads was reaction-limited.

To My Parents

With Love

ACKNOWLEDGEMENTS

The time I have spent here at Texas A&M University in pursuit of my doctorate degree will be unforgettable. I would like to express my appreciation to all those who have helped make my time here a great experience. This dissertation could not have been written without their support.

I would like to thank Dr. Cady R. Engler who not only served as my advisor but also encouraged me throughout my academic program. I thank him for giving me an opportunity to work on this challenging project and for his patience and generous support to the end.

I would like to thank my committee members, Dr. James R. Wild, Dr. Robin Autenrieth, and Dr. Ann Kenimer, for their patient guidance throughout this dissertation process. I thank them all for imparting their knowledge onto me.

I would like to thank all other faculty members and staff in the Department of Biological and Agricultural Engineering for their great help throughout my academic program. I am grateful to those who have kindly provided me with technical assistance during my research: Dr. Janet Grimsley and Mr. David Armstrong from the lab of Dr. James R. Wild in the Department of Biochemistry and Biophysics, and Mr. Paulo Fortes Da Silva and Ms. Carmen Gomes in the Department of Biological and Agricultural Engineering. I am also grateful to Bayer Corporation and Dr. Harish Chopade for kindly providing me with the coumaphos and metabolites.

I would like to express my gratitude to my friend, SeungJae Lee, for his help in the completion of my dissertation. I thank him for the time he spent discussing and helping me with computer programming and mathematical calculations. I would like to extend my thanks to my friends who have always supported me and kept my spirits up, especially to SunSoon Kwon, SangWon Lee, SungWon Jung, JuHee Ryu, and HeaSook Choi. They all have enriched my life. I am grateful to my friend, Isabel Yu Espinosa, who has made adapting to a new life overseas a lot easier. I am also grateful to Adriana Z. Diaz who was my only colleague in the lab and is now a good friend.

I am especially grateful to the members of the Pusan National University Alumni Association at Texas A&M University (PNUAggies). They all have given me family comforts, and I shall never forget them or their support.

Finally, I am thankful from the bottom of my heart to my family for their unconditional love and support: my parents, brothers, ChangHoon and MinWoo, sister-in-law, HyungIm, and newborn nephew, JiWoo. I cannot find the words to describe how much I am appreciative of them. I dedicate this dissertation to my parents as an expression of my appreciation and love.

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CHAPTER I
INTRODUCTION

Bioremediation techniques for treatment of hazardous waste have attracted attention over physical-chemical processes because of their environmentally sound and cost effective characteristics. Biological processes generally produce benign end-products, unlike conventional physical-chemical processes which often create other potential pollution hazards, such as emission of toxic byproducts from incineration and leaching of toxic substances from land disposal. By properly establishing and maintaining microbial communities and environmental conditions necessary for biodegradation, most hazardous organic compounds can be biologically detoxified or mineralized.

A waste is considered as hazardous when it poses a substantial present or potential danger to human health or the environment regardless of the form of the waste. In the United States, a waste is called hazardous when it has any of the characteristics of corrosivity, ignitability, reactivity, or toxicity (LaGrega et al., 1994). Nowadays, sources of hazardous wastes are not limited to large chemical companies but include a wide range of industrial, agricultural, and household activities. Amounts produced by various sources vary widely.

This dissertation follows the style of Biotechnology and Bioengineering.

The publication of *Silent Spring* by Rachel Carson in 1962, which emphasized the ecological consequences of pesticide contamination that were widely ignored, began a great public debate on pesticide usage. She reported the hazards of the pesticide DDT (dichloro-diphenyl-trichloro-ethane), the most powerful pesticide in world history. DDT, an organochlorine, was originally synthesized in 1874, but it was in 1939 that DDT was discovered to have insecticide properties by a Swiss scientist, Paul Müller, who was later awarded the Nobel Prize. In 1945, DDT was released for civilian use to become a miracle compound with its capability of killing hundreds of different kinds of insects at once. However, extensive usage of DDT created problems in the environment and in humans because of its toxicity and its bioaccumulation in fatty tissue (Carson, 2002). In 1970, Norway and Sweden banned DDT, and in 1972 the US followed suit. However, DDT is still used in some countries.

Nowadays, the most widely employed pesticides are organophosphates, carbamates, and pyrethroids (Sogorb and Vilanova, 2002). Among these, organophosphate pesticides account for about 50 % of insecticides used in the US. About 60 million pounds of organophosphates are applied to approximately 60 million acres of agricultural crops annually in the US alone. Another 17 million pounds are used annually for non-agricultural purposes (US EPA, 1999). Among the 1241 sites listed on the EPA Superfund final NPL (National Priority List) as of July 1, 2005, 354 sites were reported to have pesticide contamination. However, while these organophosphate pesticides are, in general, highly toxic, they usually are not persistent in the environment and can be rapidly hydrolyzed enzymatically (US EPA, 1999; Rittmann and McCarty,

2001). A wide variety of microorganisms have been found to possess the enzyme catalyzing hydrolysis of organophosphates. This enhances the feasibility of using bioremediation to treat organophosphate compounds. Moreover, application of molecular biology to genetically engineer microorganisms to contain appropriate genes can potentially contribute to making bioremediation more efficient.

Whole cell immobilization has been shown to have remarkable advantages over conventional biological systems using free cells, such as high cell density, avoidance of cell washout even at high dilution rates, easy separation of cells from the reaction system, repeated use of cells, and better protection of cells from harsh environments (Rosevear et al., 1987). With these advantages, immobilization of microorganisms has been applied in many areas including wastewater treatment and remediation of toxic chemicals. Comparing immobilized cell systems with conventional free cell systems, the productivity obtained with immobilized cells is considerably higher. One obvious reason for this is the high cell density maintained in the reaction system (Lee et al., 1996; Rhee et al., 1996). Some research has suggested that this higher productivity results from cellular or genetic modifications induced by immobilization. Evidences indicating that the immobilized cells are much more tolerant to perturbations in the reaction environment and less susceptible to toxic substances make immobilized cell systems particularly attractive for treatment of toxic substances like pesticides (Ramakrishna and Prakasham, 1999; Lee et al., 1994; Lee et al., 1996; Rhee et al., 1996; Manohar et al., 2001; Kim et al., 2002; Jianlong et al., 2002; Dwyer et al., 1986; O'Reilly and Crawford, 1989; Bettmann and Rehm, 1984; Keweloh et al., 1989; Westmeier and Rehm, 1985).

The most commonly used immobilization technique is entrapment of cells within polymeric matrices. Among the various entrapment matrices, maximum attention has been given to alginate gels (Ramakrishna and Prakasham, 1999).

In spite of the advantages, most immobilized cell systems have only been examined at laboratory scale and, hence, are difficult to scale-up (Ramakrishna and Prakasham, 1999; Dervakos and Webb, 1991; Rosevear et al., 1987; Wandrey, 1996). One of the major limitations of cell immobilization is diffusional limitation because nutrients and products have to be transported through external boundary layers and within the matrix. Therefore, mass transfer is one of the major factors affecting the activity and effectiveness of immobilized cell systems (Mavituna, 1986). Research is required to develop proper bioreactor designs to overcome diffusional limitations.

This research was undertaken to study bioremediation of organophosphate pesticides using immobilized microorganisms. The target compound selected was coumaphos, which is an insecticide used predominantly by the US Department of Agriculture, Animal and Plant Health Inspection Services (USDA-APHIS) to eradicate ticks carrying cattle fever at the border between US and Mexico. The hydrolysis of coumaphos was extensively studied in previous research by Kim (1998) using recombinant *E.coli*. Thus, the focus in this research was on degradation of the hydrolysis products, chlorferon and DETP, using indigenous microorganisms isolated from cattle dip solution. Specific objectives included

1) Enrich consortia of microorganisms from waste cattle dip solution capable of biodegrading chlorferon and/or DETP, evaluate degradation kinetics of the consortia,

and establish optimum reaction conditions for degradation using the enriched consortia in freely suspended cell systems.

2) Develop immobilization systems of the enriched consortia for effective degradation of chlorferon and DETP, and determine optimum degradation conditions for the immobilized cells.

3) Determine diffusion characteristics of chlorferon and DETP in the immobilized cell systems to assist in bioreactor design and mathematical modeling of the reaction system and in determining optimum reaction conditions.

CHAPTER II
LITERATURE REVIEW

BIOREMEDIATION

Bioremediation of Hazardous Waste

Ever since the Industrial Revolution, technological development has rapidly enhanced the quality of human life, but at significant risk to the environment and public health. Advances in medical science have benefited human kind with longer lives and a reduced death rate, which has led to fast growth in personal consumption requiring the expansion of industrial production and agricultural supplies. New synthetic products frequently have been introduced for convenience without consideration of unwanted consequences. Until the latter part of the twentieth century, toxic wastes were simply dumped in the environment without considering the effects. In 1962, Rachel Carson published *Silent Spring* raising an alarm about pesticide usage while ignoring ecological consequences. It was the Minamata mercury poisoning disaster in Japan in the late 1960s that raised worldwide awareness of industrial pollution. In the late 1970s, Love Canal incidents gained media attention, which resulted in passage of the Superfund Act (Comprehensive Environmental Response, Compensation, and Liability Act) in 1980 by the US congress. Hazardous waste problems then became the leading environmental

issue (LaGrega et al., 1994). One recent example of a contamination incident that attracted public attention was the oil spill from the Exxon Valdez in 1989, which was the largest oil spill in US waters. Bioremediation technology was applied to treat the oil-contaminated area and the result was successful.

Bioremediation can be defined as a process to detoxify and mineralize pollutants using organisms (Boopathy, 2000; Heitzer and Sayler, 1993). Although phytoremediation using plants has been gaining popularity and animal systems such as pearl oysters have been used, microorganisms are the most widely used mediator for bioremediation. Owing to the characteristics of bioremediation technology, it is environmentally sound and more attractive than conventional treatments, such as incineration, volatilization or immobilization of the pollutants. Pollutants are not completely removed and may be simply transferred into other forms of pollution by conventional treatments (Heitzer and Sayler, 1993). Incineration produces residues. Immobilization only confines pollutants in a certain space, but leakage may occur. Volatilization is a simple mass transfer process to change the phase where pollutants are located and requires a secondary process to actually treat the pollutants. Bioremediation is one of the newer technologies developed as an alternative to these conventional approaches (Boopathy, 2000). It is used more frequently to remediate contamination at hazardous wastes sites because it is less expensive in many cases when compared to conventional techniques and more acceptable to the public (US EPA, 2001).

EPA administers the Superfund program which was established in 1980 to identify and clean up abandoned or uncontrolled hazardous waste sites in the US that

currently or potentially threaten human health or the environment. Under the Superfund program, EPA has established the National Priorities List (NPL), which identifies the worst hazardous waste sites. Since 1988, the use of bioremediation at Superfund sites has increased, with 8 to 12 bioremediation Records of Decision signed each year. At the Superfund remedial action sites, 18 bioremediation projects have been completed and 59 projects are in the operational phase (US EPA, 2001).

For bioremediation technologies to be successful, an interdisciplinary approach involving microbiology, engineering, ecology, geology, and chemistry is required because bioremediation has to be designed to site-specific conditions to have the right microbes in the right place with the right environmental factors for degradation to occur (Boopathy, 2000). Detailed site characterization and treatability studies have to be conducted to provide information to decide if bioremediation is an effective process at a specific site (Boopathy, 2000; Heitzer and Sayler, 1993). Major factors affecting bioremediation are given in Table 2-1 (Boopathy, 2000).

Contaminants potentially suitable for bioremediation are listed in Table 2-2 (Vidali, 2001). Not all contaminants are susceptible to microbial attack. Some contaminants, such as chlorinated organics or highly aromatic hydrocarbons, are degraded either slowly or not at all. This makes it difficult to predict the rate of clean up for a bioremediation site (Vidali, 2001). Some examples of contaminants that currently are impossible to treat by bioremediation are asbestos, asphalt, bitumen, and inorganic acids (Evans and Furlong, 2003).

Table 2-1. Major factors affecting bioremediation (Boopathy, 2000)

Microbial	Growth until critical biomass is reached
	Mutation and horizontal gene transfer
	Enzyme induction
	Enrichment of the capable microbial populations
	Production of toxic metabolites
Environmental	Depletion of preferential substrates
	Lack of nutrients
	Inhibitory environmental conditions
Substrate	Too low concentration of contaminants
	Chemical structure of contaminants
	Toxicity of contaminants
	Solubility of contaminants
Biological aerobic vs. anaerobic process	Oxidation/reduction potential
	Availability of electron acceptors
	Microbial population present in the site
Growth substrate vs. co-metabolism	Type of contaminants
	Concentration
	Alternate carbon source present
	Microbial interaction (competition, succession, and predation)
Physico-chemical bioavailability of pollutants	Equilibrium sorption
	Irreversible sorption
	Incorporation into humic matters
Mass transfer limitation	Oxygen diffusion and solubility
	Diffusion of nutrients
	Solubility/miscibility in/with water

Table 2-2. Some pollutants potentially suitable for bioremediation (Vidali, 2001)

Class of contaminants	Specific examples	More potential sources
Chlorinated solvents	Trichloroethylene	Drycleaners
	Perchloroethylene	Chemical manufacture
Polychlorinated biphenyls	4-Chlorobiphenyl	Electrical manufacturing
	4,4-Dichlorobiphenyl	Power station
		Railway yards
Chlorinated phenol	Pentachlorophenol	Timber treatment Landfills
BTEX	Benzene	Oil production and storage
	Toluene	Gas work sites
	Ethylbenzene	Airports
	Xylene	Paint manufacture
		Port facilities
		Railway yards
		Chemical manufacture
Polyaromatic hydrocarbons	Naphthalene	Oil production and storage
(PAHs)	Antracene	Gas work sites
	Fluorene	Coke plants
	Pyrene	Engine works
	Benzo(a)pyrene	Landfills
		Tar production and storage
		Boiler ash dump sites
		Power stations
Pesticides	Atrazine	Agriculture
	Carbaryl	Timber treatment plants
	Carbofuran	Pesticide manufacture
	Coumaphos	Recreational areas
	Diazinon	Landfills
	Glycophosphate	
	Parathion	
	Propham	
	2,4-D	

Classification of Bioremediation Technologies

A common classification of bioremediation techniques is to divide them into *in situ* and *ex situ* approaches. *In situ* techniques are treatments of contaminants in place with minimal disturbance, while *ex situ* techniques are treatments that remove contaminated materials and move them to the treatment process (Boopathy, 2000; Evans and Furlong, 2003). *In situ* techniques generally are more desirable because they do not involve excavation and transport of contaminated material. This reduces cost as well as possible exposure risks during the processes of excavation and transportation (Vidali, 2001). However, when the contamination is relatively localized within a site close to the surface at medium to high concentration, the *ex situ* approach is generally more suitable. The benefits of *ex situ* techniques are that the treatment conditions are readily optimized, process control is easier to maintain, system monitoring is more accurate and simpler to achieve, and treatment is faster. On the other hand, *in situ* techniques are usually selected when the contamination is widespread at low concentration throughout a site and the treatment time is not restricted since the processes are relatively slow (Evans and Furlong, 2003).

PESTICIDES

Overview

The most widely used pesticides today are organophosphates, carbamates, and pyrethroids (Sogorb and Vilanova, 2002). The basic structures of these pesticides are displayed in Figure 2-1 (Sogorb and Vilanova, 2002). In the past, organochlorine pesticides were commonly used, but due to their persistence in the environment and adverse effects on human health and the environment, most have been banned or taken off the market (e.g. DDT and chlordane). In particular, DDT became a big public concern after the publication of *Silent Spring* by Rachel Carson in 1962.

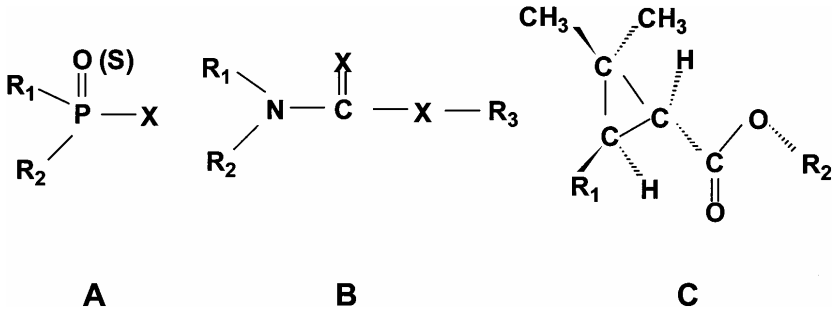


Figure 2-1. Chemical structure of pesticides. A. organophosphates, B. carbamates, C. pyrethroids. The –X group in (A) may be in a wide range of halogen, aliphatic, aromatic or heterocyclic groups and that in (B) may be oxygen or sulphur (Sogorb and Vilanova, 2002)

The toxic effect of organophosphate and carbamate pesticides occurs in the nervous system where the chemicals disrupt the enzyme that regulates acetylcholinesterase, a neurotransmitter. While the phosphorylation of esterases by organophosphates is a non-reversible reaction, carbamylation by carbamates is reversible and less stable (Sogorb and Vilanova, 2002; WHO, 1986; Kuhr and Dorough, 1976). Pyrethroid pesticides are synthetically developed versions of pyrethrin which is a natural pesticide. The pyrethroids are functional toxins that produce adverse effects in a secondary way as a consequence of neuronal hyperexcitability (Sogorb and Vilanova, 2002).

Unlike organochlorine pesticides, organophosphate, carbamate and pyrethroid pesticides are biodegradable. The main detoxification processes are hydrolysis and oxidation, with hydrolysis being the most efficient route for all three types of pesticides. For hydrolysis of organophosphates, phosphotriesterases and carboxylesterases are involved in the reaction, with phosphotriesterases being much more efficient than carboxylesterases. Hydrolysis of organophosphates by phosphotriesterases produces polar metabolites that do not accumulate in fatty tissues and are easily eliminated in urine. Most importantly, the resulting metabolites are less toxic than their parent organophosphates because of their lower phosphorylating power. For detoxification of carbamates and pyrethroids, hydrolysis by carboxylesterases is the most efficient route (Sogorb and Vilanova, 2002). Biodegradability of these pesticides can mitigate public concerns. Traditional methods of pesticide detoxification have relied on landfills and incineration which generate secondary contamination problems due to leaching of

pesticides into surrounding soil and groundwater supplies and production of potentially toxic by-product emissions. Moreover, although incineration is approved by the US EPA, it is a very costly process requiring large amounts of energy (Chen and Mulchandani, 1998). From this point of view, biodegradation is a much safer and economical process for detoxifying pesticides.

According to the EPA Superfund website, as of July 1, 2005, 1241 sites are on the final NPL, and 354 sites on the list were contaminated with pesticides. Pesticides and herbicides account for 27 % of the most common types of contaminants at Superfund sites. Table 2-3 presents performance data for four sites where bioremediation was used to treat the soil contaminated with pesticides and herbicides (US EPA, 2001).

Organophosphate Pesticides

Organophosphate compounds were developed during the early 20th century, and in 1932, it was discovered that humans were subject to the same effects that organophosphates exerted on insects (US EPA, 2005). This was due to their similar chemical structures and modes of action with the nerve gases sarin, soman, and tabun (Ware, 2000). Some of them were poisonous enough to be used as nerve agents during World War II (US EPA, 2005).

Table 2-3. Performance data for bioremediation of soil contaminated with pesticides and herbicides (US EPA, 2001)

Site Name	Technology	Contaminant	Initial Concentration	Final Concentration
Novartis Site, Ontario Canada	Darament™, a composting process developed by the W.R. Grace Company	Metolachlor	84 mg/kg	1 mg/kg
Navajo Indian Reservation Superfund Removal Site, Window Rock, Arizona	Anaerobic slurry-phase bioremediation	Toxaphene	4000 mg/kg	180 mg/kg
Stauffer Chemical Company Site, Tampa, Florida	Xenorem™, a composting process registered by Stauffer Management Company	Chlordane DDD DDE DDT Dieldrin Molinate Toxaphene	47.5 mg/kg 162.5 mg/kg 11.3 mg/kg 88.4 mg/kg 3.1 mg/kg 10.2 mg/kg 469 mg/kg	90% removal[1)] 90% removal[1)] 8.91 mg/kg[2)] 8.91 mg/kg[2)] 0.19 mg/kg[2)] 0.74 mg/kg[2)] 90% removal[1)]
Creotox Chemical Products Superfund Removal Site, Tennessee	Not reported	Chlordane Aldrin BHC Lindane	596 mg/kg Not reported Not reported Not reported	77.3 mg/kg Not reported Not reported Not reported

1) Clean-up goals did not meet: clean-up target concentration of Chlordane – 2.3 mg/kg; DDD – 12.6 mg/kg; Toxaphene – 2.75 mg/kg

2) Cleanup target concentration. Goals met

Organophosphate insecticides became a replacement for organochlorine pesticides, which were found to be persistent in the environment, beginning with their use around the home and garden. Nowadays, organophosphate pesticides such as parathion, methyl parathion, diazinon, coumaphos, and malathion, are the most widely used pesticides in the world. In the US alone, about 60 million pounds of organophosphates are applied to approximately 60 million acres of agricultural crops annually (US EPA, 1999). Another 17 million pounds are used annually for non-agricultural purposes. This amount accounts for nearly half of all insecticides sold in the US (US EPA, 1999). These organophosphate pesticides usually are unstable and not persistent in the environment as they can be rapidly hydrolyzed enzymatically or chemically upon exposure to water (US EPA, 1999; Rittmann and McCarty, 2001). This was the reason they were brought into agricultural use as a replacement for persistent organochlorine pesticides, particularly DDT (Ware, 2000). However, they may cause serious environmental problems because they are, in general, the most toxic to vertebrate animals of all pesticides, even more so than organochlorine insecticides (Ware, 2000). Although hydrolyzed metabolites of organophosphate are generally less toxic than their parent compounds, they are not benign end products. Increasing usage of pesticides on agricultural commodities to increase yields to meet the worldwide demand for food and fiber has raised the potential for environmental contamination by these chemicals (Mateen et al., 1994). Understanding microbial degradation of these pesticides can lead to methods to properly remediate any contamination they may cause (Mateen et al., 1994).

The most significant step in detoxifying organophosphate compounds is hydrolysis since that makes the compounds more vulnerable to further degradation (Kumar et al., 1996). The enzyme responsible for catalyzing this reaction is referred as an esterase or phosphotriesterase. Research has found a wide range of microorganisms possessing the organophosphate hydrolase enzyme (Dave et al., 1993, 1994; Mulbry and Karns, 1989; Mulbry et al., 1986; Mulbry, 2000; Horne et al., 2002; Chaudhry et al., 1988). The most well-known examples of natural isolates able to degrade organophosphates are *Pseudomonas diminuta* MG and *Flavobacterium* ATCC 27551. They have been shown to possess the organophosphate hydrolase (OPH) enzyme. Mulbry (2000) found *Nocardiodes simplex* NRRL B-24074 expressing OPH activity in a microbial consortium he isolated from cattle dip waste for coumaphos degradation. Horne et al. (2002) isolated a *Pseudomonas monteilli* strain having a novel phosphotriesterase activity using coroxon, the oxon analogue of coumaphos, as its sole phosphorus source. In addition, genetically engineered *E.coli* containing the OPH enzyme isolated from *Pseudomonas diminuta* MG or *Flaobacterium* has been successfully used for hydrolyzing organophosphates. The advantage of using recombinant *E.coli* is that they can grow to much higher cell densities in a fermenter than *Pseudomonas diminuta* MG or *Flavobacterium*, so the OPH enzyme can be produced in commercial amounts to enable the development of large scale degradation processes (Chen and Mulchandani, 1998). Table 2-4 shows microorganisms capable of degrading organophosphate pesticides and their metabolites.

Table 2-4. Microorganisms capable of degrading organophosphate pesticides and their metabolites (Mateen et al., 1994)

Compound	Microorganism
Parathion	*Bacillus* strains
	Arthrobacter strains
	Flavobacterium sp. ATCC 27551
	Pseudomonas sp. ATCC 29353
	Pseudomonas spp.
	Pseudomonas strain CTP-01
	Pseudomonas diminuta MG
	Strain SC
Methyl parathion	*Pseudomonas* sp.
	Pseudomonas sp. isolate 50541
	Flavobacterium sp. ATCC27551
	Aufwuchs
p-Nitrophenol	*Flavobacterium* sp.
	Pseudomonas spp. PNP-1, 2, and 3
	Pseudomonas sp. isolate 50445
	Moraxella sp.
	Pseudomonas sp. strain 24
Diethylthiophosphate	*Pseudomonas acidovorans*
Diazinon	*Flavobacterium* sp. ATCC 27551
	Pseudomonas sp. ATCC29353
	Pseudomonas spp.
	Pseudomonas sp.
	Arthrobacter sp. SB3 and SB4
Isofenphos	*Arthrobacter* sp.
	Pseudomonas sp.
Fenetrothion	*Flavobacterium* sp. ATCC 27551
Dichlorovos	*Pseudomonas aeruginosa* sp.
	Pseudomonas sp.
Coumaphos	Isolates B-1, B-2, and B-3
Methidathion	*Bacillus coagulans*

Coumaphos

Coumaphos [0,0-diethyl 0-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate] is an organophosphate insecticide that was originally developed in Germany by G. Schrader (NTP, 1979). It is used principally to control ectoparasites on livestock and poultry (US EPA, 2000). The predominant usage is by the US Department of Agriculture, Animal and Plant Health Inspection Services (USDA-APHIS) for its tick eradication program along the US-Mexico border to prevent the reintroduction of equine and bovine piroplasmosis into the US (Shelton and Somich, 1988; US EPA, 2000). Coumaphos has a relatively low mammalian toxicity in relation to other organophosphate compounds. According to the material safety data sheet (MSDS) from Bayer Corporation, the basic manufacturer of coumaphos and its metabolites, the oral LD_{50} for female rats is 17 mg/kg and that for male rats is > 240 mg/kg. The major effect of exposure to coumaphos is inhibition of the enzyme acetylcholinesterase. Coumaphos is not listed by NTP (National Toxicology Program), IARC (International Agency for Research on Cancer) or regulated as a carcinogen by OSHA (Occupational Safety and Health Administration). US EPA classifies coumaphos as toxicity class II – moderately toxic (EXTOXNET, 1996).

There are 42 vats at APHIS treatment sites each containing approximately 15,000 liters of ‘Co-Ral’ flowable cattle insecticide (42 % coumaphos, 58 % inert ingredients) in which the coumaphos concentration is about 1,500 mg/L (Karns et al., 1995). These vats are used to treat cattle within the quarantine zone in south Texas or imported from

Mexico by dipping them in the vat before they are shipped to other areas in the US. This operation produces approximately 500,000 liters of concentrated insecticide waste annually which presents a considerable waste disposal problem. Initially, the disposal method for this waste was to pump it into evaporation pits or waste lagoons located adjacent to the dip vats. This created contamination problems with high concentrations of coumaphos and its metabolites in the underlying soils because the evaporation pits were mostly unlined (Mulbry et al., 1996). In addition, the hydrophobic nature of coumaphos and one of the metabolites, chlorferon, favor in their adsorption by soil particles.

Research by Mulbry et al. (1996, 1998) demonstrated that microbial consortia in the waste from selected vats could be induced to degrade coumaphos using only proper aeration and pH control. Based on their results, they developed a trickling biofilter system using plastic foam filters suspended in a large fiberglass storage tank to treat 4,000 gallon batches of cattle dip waste solution. Microorganisms growing on the trickling filters were able to remove more than 99 % of the coumaphos from the vat solution in about 15 days. Currently this biofilter system is used at vat sites for treatment of coumaphos waste solution.

Several microorganisms found to express OPH enzyme can be used for coumaphos hydrolysis; however, investigation of degradation of the resulting metabolites, chlorferon and diethylthiophosphate (DETP), has been limited (Shelton and Somich, 1988; Shelton, 1988; Cook et al., 1978; Cook et al., 1980). The degradation pathway for coumaphos is described in Figure 2-2.

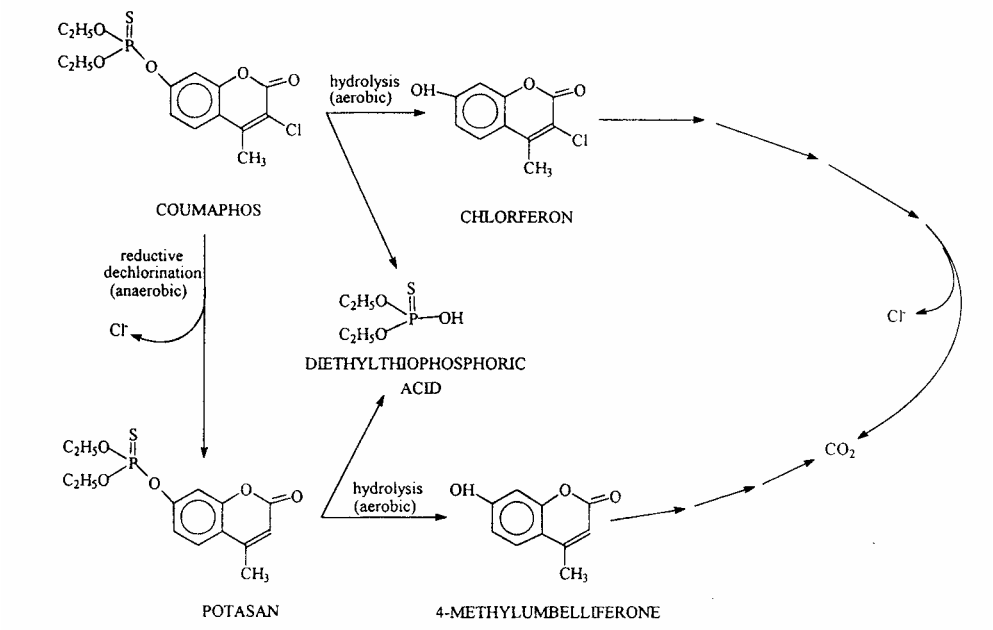


Figure 2-2. Degradation pathway of coumaphos (Mulbry et al., 1996)

Shelton and Somich (1988) isolated three different bacteria, designated as B-1, B-2 and B-3, capable of metabolizing coumaphos. B-1 and B-2 were also found to be capable of metabolizing chlorferon into α-chloro-β-methyl-2,3,4-trihydroxy-*trans*-cinnamic acid (CMTC). In addition, B-1 used chlorferon at low concentration as its carbon source. However, both B-1 and B-2 were inhibited by chlorferon at concentrations greater than 100 mg/L. They concluded that other organisms were responsible for removing inhibitory products resulting from coumaphos metabolism so as to increase chlorferon degradation. Shelton (1988) isolated a consortium of bacteria that mineralized DETP from the cattle dip solution. The consortium used DETP as their carbon and energy source. However, the bacteria present in the consortium at high population densities were not responsible for the initial attack on DETP. He suggested that other bacteria, which were present at low population densities, were responsible for the initial attack on DETP. Cook et al. (1980) found that DETP was used as a sulfur source by *Pseudomonas acidovorans*. They also found an isolate from sewage utilizing DETP as a sole phosphorus source (Cook et al., 1978).

IMMOBILIZATION TECHNOLOGY

Overview

Immobilization is defined as the confinement of biocatalyst within or on a support material. Biocatalysts can be enzymes or whole cells. Methods for

immobilization of enzymes or whole cells are basically same in most cases. However, maintaining a living cell can be more complex than supporting an enzyme in an immobilized system. An advantage of immobilized cells over immobilized enzymes lies in the elimination of enzyme purification processes (Rosevear et al., 1987; Shuler and Kargi, 2002). Immobilized enzymes were introduced in the 1950s for laboratory studies once it was realized that enzymes could be in a water-insoluble form without losing their catalytic activity (Wandrey, 1996; Dervakos and Webb, 1991). Attempts to immobilize whole cells began in the 1970s (Dervakos and Webb, 1991). Research on cell immobilization techniques has been extensive, and numerous immobilization systems have been studied at laboratory scale. However, few immobilized cell systems have been scaled up (Dervakos and Webb, 1991; Rosevear et al., 1987; Wandrey, 1996).

Immobilization of microorganisms has been applied in many areas including wastewater treatment and remediation of toxic chemicals since this technique generally provides several advantages over freely suspended cell systems, such as easy separation of cells from the reaction system, repeated use of immobilized cells, and better protection of cells from harsh environments. Major limitations of immobilization techniques are mass transfer limitation and possible loss of cell activity during the immobilization process (Rosevear et al., 1987; Shuler and Kargi, 2002; Willaert and Baron, 1996). Detailed advantages and limitations are described in Table 2-5 (Rosevear et al., 1987).

Table 2-5. Advantages and limitations of immobilization techniques (summarized from Rosevear et al., 1987)

Advantages	Limitations
Retention of cells	Loss of activity
Repeated or continuous use is possible	Possible inactivation of cells during the immobilization process
Avoidance of cell washout at high dilution rate	
	Mass transfer
	Poor mass transfer causes inefficient use of cells
High concentration of cells	When substrate is nutrient to cells, system longevity can be seriously reduced
Results in higher volumetric reaction rate	Accumulation of degradation product(s) in microenvironment alters kinetics and adversely affect the activity of cells when it is inhibitory
Reduces reactor volume	
Reduces contact time between cells and substrate solution	
Microenvironmental control	Prolonged operation
Mechanical protection inside matrix provides protection from shear damage, toxins and inhibitors	Support materials must be stable during long-term operation
Microenvironmental conditions (i.e. ionic balance, pH gradients, nutrient-product gradients, water activity) can be more favorable for cells resulting in better performance	Leakage of cells
	Uncontrolled proliferation of immobilized cells results in leakage of cells
Separation of cells	
Easy separation of cells from reaction system	
Eliminates expensive cell recovery and recycle processes	

Classification of Immobilized Cell Systems

Immobilized cell systems can be classified based on the physical mechanism causing immobilization as depicted in Figure 2-3 (Kourkoutas et al., 2004): attachment to a surface, entrapment within a porous matrix, containment behind a barrier and self-aggregation. These four classifications have been extensively reviewed by Karel et al. (1985) and Willaert and Baron (1996) as summarized below:

Attachment to a Surface

Surface attachment is an immobilization method in which cells are bound to a surface by various types of interactions, including van der Waals forces, ionic bonds, and covalent interactions. The strength of binding between cells and support material depends on the type of cell and the support. This type of cell immobilization is a quite simple yet popular technique because microorganisms tend to adsorb onto various types of supports in nature. Cells grow on surfaces of support materials, and the thickness of the resulting biofilm can vary from less than a monolayer to several millimeters. Therefore, mass transfer limitation to and within the layers of cells is important. This method is not desirable where cell-free effluent is required because there is no barrier between the cells and the liquid medium. Industrial applications of surface attached cell systems are primarily biofilm systems which are used for wastewater treatment.

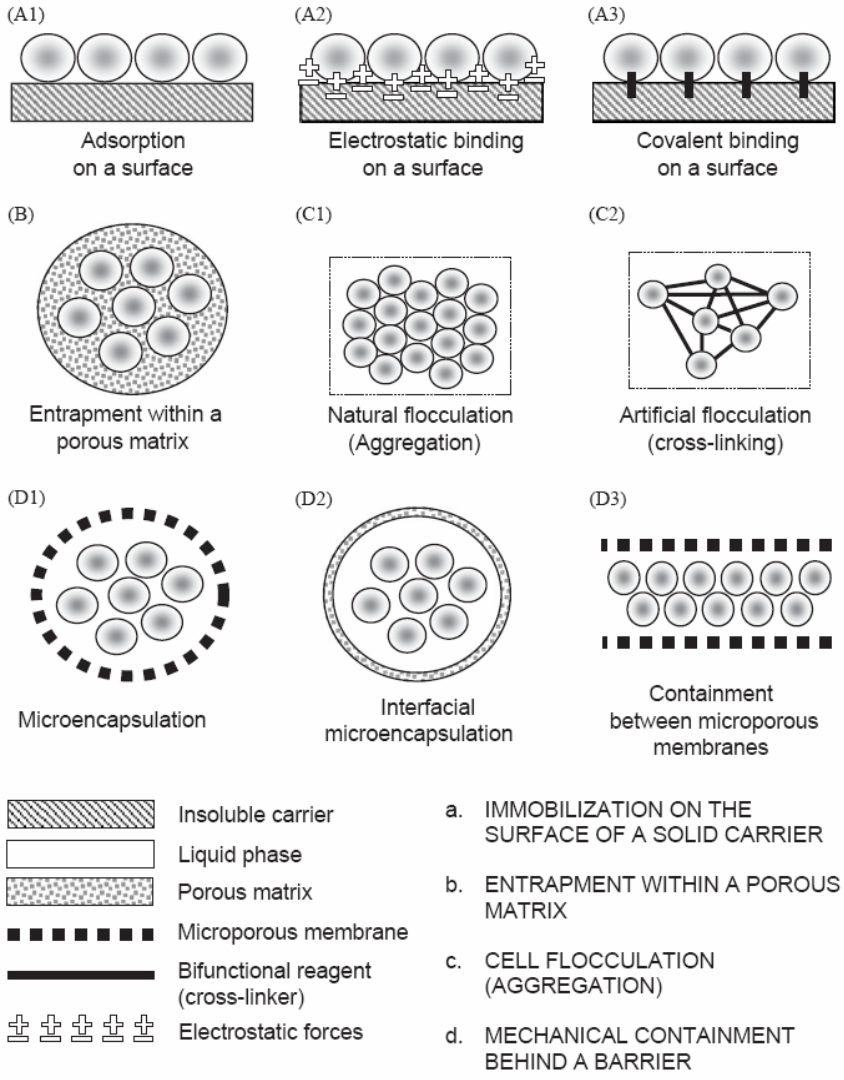


Figure 2-3. Basic methods of cell immobilization (Kourkoutas et al., 2004)

Entrapment within Porous Matrices

Two different approaches are available for entrapment within porous matrices, as illustrated in Figure 2-3. First is gel entrapment which synthesizes the porous matrix *in situ* around the cells being immobilized. The other is to use a preformed porous matrix. Cells are allowed to diffuse into the preformed porous matrix and grow until they are immobilized by the presence of other cells and the matrix. Cells immobilized by these two methods are better protected from fluid shear than surface attached cells.

Gel Entrapment

Due to its simplicity and excellent cell containment, gel entrapment has been used in most research on immobilized cells. Various natural and synthetic compounds can be gelled into matrices to entrap cells with minimal loss of viability. Gels formed from synthetic polymers are generally superior in mechanical strength and longevity. The polymer-cell mixture can be formed in different shapes and sizes. The most common form is small beads approximately 1 to 5 mm in diameter.

Gel entrapment has two important disadvantages compared to other immobilization methods. One is limited mechanical stability. Gel structure can be easily destroyed by cell growth in the gel matrix and carbon dioxide production. The other disadvantage is mass transfer limitation because molecular diffusion is the only process for mass transfer in gel matrices. Diffusion in the matrix itself is fast since the polymer

volume fraction in the matrix is usually very low. The limitation on mass transfer of substrates and products usually occurs in closely packed cell aggregates formed in the matrix.

Preformed Support

In this method, cells are better protected than in the gel entrapment method because the preformed support particles are more resistant to compression and disintegration. Also, high cell viability can be retained. Therefore, this method is more useful for growth-related processes. Unlike the gel immobilization method, the volume fraction of the matrix material is generally significant and high cell packing densities are not achieved. In these systems, cells may be adsorbed onto the preformed support, so the effluent may not be completely free of cells.

Containment behind a Barrier

Containment behind a barrier can be divided into two sub-divisions based on the type of barrier as shown in Figure 2-3. The barrier can be preformed or formed around the cells to be immobilized (miroencapsulation and two phase emulsion). This type of immobilization is especially useful when separation of cells or high molecular weight products from the effluent is required.

Preformed barriers are usually polymeric microfiltration or ultrafiltration membranes. Other types of membranes, such as ceramic or ion exchange membranes, have been used as well. Cells are immobilized behind these membranes by filtration of a cell suspension after growth in a seeded reactor. In the sense of retaining cells behind a semi-permeable wall or membrane, this system is very similar to microencapsulation. The system efficiency can be reduced by mass transfer limitation because both nutrient supply and product removal from the cell mass are achieved by diffusion in most cases.

Two phase emulsion systems immobilize cells in the simple liquid/liquid phase interface between two immiscible fluids. This method can be applied when substrates or products are partitioned separately.

Self Aggregation of Cells

Some cells naturally aggregate or flocculate. Because of the large size of aggregates, cells inside the aggregates are considered to be immobilized. Addition of artificial flocculating agents or cross-linkers enhances aggregation processes of cells that do not naturally flocculate. This type of immobilization has been successfully applied in many full-scale anaerobic wastewater treatment plants to speed up the digestion rate during methanogenesis.

Entrapment of Cells in Hydrogels

Cell immobilization by gel entrapment was utilized in this research. This group of techniques can be further divided in two categories: *in situ* polymerization and non-covalent entrapment (Rosevear et al., 1987). *In situ* polymerization forms gels by polymerization reactions which produce new covalent bonds, while non-covalent entrapment forms gels by weak ionic or adsorptive interactions between gels and biocatalyst. Gels formed by *in situ* polymerization are very stable with well defined properties, but considerable deactivation of the catalyst may occur during the process. Non-covalent entrapment forms gels under milder conditions, but physical properties of gels may be unsatisfactory (Rosevear et al., 1987).

In Situ *Polymerization*

The most extensively used chemical for *in situ* polymerization is polyacrylamide. Although acrylamide gel has good properties as an immobilization matrix, such as its hydrophilic nature, high water content, and high porosity, the acrylamide monomer is toxic. It not only deactivates biocatalysts, causing loss of cell viability, but also is a neurotoxin that can have a cumulative effect. Despite the toxicity, polyacrylamide gels have been used to immobilize a significant number of microbial cells. This method is generally more appropriate for immobilizing enzymes which remain associated with cell

debris because the polymerization reaction rarely damages enzymes (Rosevear et al., 1987).

The most common alternative monomer to acrylamide is hydroxyl ethylmethacrylate (HEMA). This compound is less toxic than acrylamide, but physical properties such as water regain and porosity of the gel are less satisfactory (Rosevear et al., 1987).

Non-covalent Entrapment

For non-covalent entrapment, the biocatalyst is mixed with polymer solution and the mixed suspension is formed into a gel under mild conditions. Thus, the gelation process generally does not adversely affect the activity of the biocatalyst. The majority of useful polymers are hydrophilic polysaccharides and proteins (Rosevear et al., 1987). Some examples of natural polymers are alginate, carrageenan, and agarose. Among them, alginate has been used most successfully for immobilizing a wide range of cells. The synthetic polymer polyvinyl alcohol also is widely used because it is non-toxic and very stable (Leenen et al., 1996b).

Alginate Gels

Alginates have different structures and molecular weights depending on the source, age, and part of the seaweed used and the type of extracting process (Tanaka et

al., 1984). Alginate is copolymer of D-mannuronic-acid and L-guluronic-acid groups produced by seaweeds. Since guluronic acid increases gel strength, alginate containing a high proportion of guluronic acid is recommended for immobilization (Leenen et al., 1996b). Alginate gel is formed by rapid crosslinking of alginate and polyvalent metal ions such as calcium (Ca^{2+}), barium (Ba^{2+}), aluminium (Al^{3+}), and strontium (Sr^{2+}). The strength of the complex increases along the series Ca^{2+}, Sr^{2+}, Ba^{2+} and this affects the physico-chemical properties of the resulting gel beads. Ca-alginate has been the most widely used matrix for whole cell entrapment due to its simplicity of formation and non-toxic characteristics (Leenen et al., 1996a)

The major limitation of using Ca-alginate gel is its sensitivity to chelating agents and non-gelling ions. It can be rapidly dissolved by polyphosphates, citric acid, and EDTA solutions as well as cations such as Na^{+}, Mg^{2+}, and K^{+} thereby releasing the immobilized cells (Leenen et al., 1996b; Ting and Sun, 2000; Paul and Vignais, 1980; Vogelsang and Østgaard, 1996; Cheetham et al., 1979). This makes the Ca-alginate method unsuitable for most field scale applications in wastewater treatment. To increase the physical strength of alginate gels, other divalent cations such as Sr^{2+} and Ba^{2+}, may be used. Although they can be toxic to cells (Rosevear et al., 1987), they have greater capacity for alginate gelation and are unaffected by most chelating agents (Paul and Vignais, 1980; Heimstra et al., 1983). In particular, Ba^{2+} has been found more suitable than Ca^{2+} in many applications because it stabilizes the mechanical and diffusional properties of the gel under conditions when Ca-alginate gel beads would be highly destabilized (Paul and Vignais, 1980; Vogelsang and Østgaard, 1996; Tanaka and Irie,

1988; Leenen et al., 1996b). Another method to strengthen alginate is to mix with other polymers, such as polyvinyl alcohol (PVA) or glutaraldehyde. Mixing with PVA has mutual advantages since PVA is an extremely sticky material causing PVA beads to agglomerate. Addition of alginate can eliminate this problem (Wu and Wisecarver, 1992). Alginate mixed with PVA-SbQ was successfully utilized for domestic wastewater treatment for two years (Vogelsang et al., 1997). Quan et al. (2003) also used the PVA – alginate mixture for immobilizing a mixed culture to treat 2,4-dichlorophenol containing wastewater. Continuous addition of Ca^{2+} and immobilization of $CaCO_3$ in the gel are other methods to increase strength (Vogelsang and Østgaard, 1996). However, increasing the concentration of Ca^{2+} may be undesirable because it triggers many cellular processes, such as encouraging adhesion to surfaces and changes in general metabolism (Rosevear et al., 1987).

Enhanced Degradation for Hazardous Waste Treatment Using Cell Immobilization Technique

Immobilized cells have been successfully used for bioremediation of numerous toxic chemicals. Lee et al. (1996) studied degradation of chlorophenols using free cells and cells immobilized in Ca-alginate gel beads. They found that immobilized cells degraded chlorophenols much faster than free cells and reduced the lag phase for the removal of chlorophenols. They suggested that the enhanced degradation with

immobilized cells resulted from higher cell density in the reactor and greater tolerance of cells to the toxic effects of chlorophenols.

Rhee et al. (1996) examined pyridine degradation using free cells and cells immobilized in Ca-alginate gel beads. The volumetric reaction rate with immobilized cells was six times greater than that for free cells due to the high cell concentration in the immobilized cell system. Furthermore, they could reuse the immobilized cells 16 times without loss of activity.

Manohar et al. (2001) reported that immobilized cells had better tolerance for increasing concentrations of naphthalene and a more rapid naphthalene-degrading rate than free cells. They explained that this increasing degradation by immobilized cells was due to high local cell density in the immobilization matrix and increased cell permeability and membrane stabilization caused by immobilization. However, they did not suggest a clear mechanism for how immobilization provided membrane stability and increasesd cell permeability.

Kim et al. (2002) investigated coumaphos degradation using free cells and cells immobilized in PVA cryogel. Immobilized cells had twice the degradation rate of free cells when treating coumaphos in cattle dip solution, while both free cells and immobilized cells had the same degradation rate when treating pure coumaphos solution. Two possible explanations for enhanced activity only in the cattle dip solution were suggested: Cells may have been protected better from inhibitory factors present in cattle dip solution that reduced the bioavailability of coumaphos and cell permeability may have been increased. Hong et al. (1998) showed an improved transfer rate of

organophosphates across the cell wall and/or membrane which resulted from cryoimmobilization, the same immobilization method used by Kim et al. (2002).

Dwyer et al. (1986) studied the effect of immobilization on a methanogenic consortium used for phenol biodegradation. Cells were immobilized in a long, thin agar strand. Immobilized cells did not have a lag phase that was observed for free cells at a low concentration of phenol. At high phenol concentration (2000 mg/L), free cells were completely inhibited while immobilized cells maintained some activity. The substrate inhibition constant for immobilized cells was twice that of free cells indicating that cells were protected from inhibitory concentrations of phenol inside the immobilization matrix.

O'Reilly and Crawford (1989) immobilized *Flavobacterium* cells (ATCC 39723) in polyurethane foam for biodegradation of pentachlorophenol (PCP). They found that the presence of polyurethane foam decreased the lag phase at higher concentrations of PCP while no differences were detected at low PCP concentrations (< 100 mg/L). Moreover, at 200 mg/L, free cells did not completely mineralize PCP while complete mineralization was achieved by immobilized cells or free cells with cell-free polyurethane foam in the reactor. PCP was reversibly bound to the polyurethane, which reduced the soluble-PCP concentration below the toxic level while allowing all the PCP to be degraded.

Bettmann and Rehm (1984) isolated a *Pseudomonas* sp. for phenol degradation and compared degradation activities in freely suspended and immobilized systems. Cells immobilized in alginate or polyacrylamide-hydrazide (PAAH) degraded phenol up to a

concentration of 0.3 %. On the contrary, 50 % of freely suspended cells were killed at a concentration of 0.2 % in 10 days. Many microcolonies were formed near the outer surface of the gel beads. They presumed that these microcolonies acted as a diffusion barrier for phenol and protected the cells.

Keweloh et al. (1989) studied the tolerance of cells immobilized in Ca-alginate gel matrix to phenol toxicity. They concluded that the tolerance enhancement by immobilization is not specific for phenol degrading organisms, but it is rather a general characteristic of immobilization because they found the same tolerance enhancement with bacteria lacking phenol degrading ability. This phenomenon was also found in another study by Westmeier and Rehm (1985). Immobilized cells exhibited tolerance against more toxic phenol derivatives like chlorinated phenols. Keweloh et al. (1989) said that this enhanced tolerance was the protection effect by significantly developed cell aggregates in the gel matrix. They suggested that external cells of microcolonies in the gel acted as a protective barrier for internal cells and reduced the toxic effect. They also observed that microcolonies formed by bacterial strains immobilized and grown in alginate were surrounded by an envelope-like extracellular structure. They speculated this extracellular structure could inhibit the diffusion of toxic compounds into the colony. Altered cell physiology due to cell-to-cell contact and reduced water activity in microcolonies were proposed to be other possible advantages in the defense against toxic compounds.

Keweloh et al. (1989) compared the tolerance of cells to phenol in three different cases: 1) cells grown in an alginate matrix and released by dissolution, 2) cells grown in

free suspension then immobilized in alginate and dissolved just before phenol addition, and 3) freely grown cells that never had contact with alginate. They found that cells that had been in contact with alginate (cases 1 and 2) exhibited significantly higher phenol tolerance than cells that never had contact with alginate (case 3). They suggested that lipid impurities in the commercial alginate were responsible for this phenomenon (Keweloh et al., 1990; Diefenbach et al., 1992). Keweloh et al. (1990) observed changes in the lipid-to-protein ratio of the membranes in *E.coli* immobilized in Ca-alginate gels. *E.coli* cells in Ca-alginate showed increased tolerance to phenol by uptake of saturated fatty acids from impurities in the alginate and their incorporation into the cell membrane lipids (Keweloh et al., 1991). Diefenbach et al. (1992) found substantial modification of fatty acid patterns in *E.coli* shortly after immobilization in Ca-alginate gels.

MASS TRANSFER IN IMMOBILIZED CELL SYSTEMS

As discussed above, immobilized cells have been shown to enhance reaction rates for biodegradation of various toxic compounds. In many cases, rates are enhanced because different environmental conditions occur inside the matrix (microenvironment) than in the bulk solution (Merchant et al., 1987). These differences between the interior microenvironment and bulk solution illustrate the effects of mass transfer resistance and solute partitioning (Merchant et al., 1987; De Backer et al., 1996; Jovetic et al., 2001). Mass transfer limitations may occur in the external boundary layer (external mass

transfer) or within the matrix (internal mass transfer) in immobilized cell particles. Mass transfer is a major factor affecting the activity and effectiveness of immobilized cell systems (Mavituna, 1986).

Substrate partitioning and mass transfer resistances are quantified by the partition coefficient (K_p), the liquid-solid mass transfer coefficient (k_L), and the effective diffusion coefficient (D_{eff}). In some cases, the diffusion coefficient in bulk solution (D_{aq}) has been used to model the mass transfer phenomena in bioreactors containing immobilized cells when there is a lack of information about the effective diffusion coefficient (Papathanasiou et al., 1988). However, diffusion through the immobilization matrix cannot be characterized by the diffusion coefficient in bulk solution because the pore space is only a fraction of the total volume (the exclusion effect) and impermeable segments of polymer molecules increase the path length for a diffusing substrate (obstruction effect) (Muhr and Blanshard, 1982). Moreover, the pore geometry may be very complex with individual pores frequently changing in size, shape, and direction, and substrate molecules and pores may be of comparable dimensions. The effective diffusion coefficient is a single diffusion coefficient incorporating all these effects (Blanch and Clark, 1996). Thus, knowing actual values for these parameters (K_p, k_L, and D_{eff}) is important for evaluating kinetics of immobilized cell systems and for bioreactor design (Merchant et al., 1987).

In many instances, external mass transfer resistance can be made negligible with proper design of the immobilized cell system because external resistance is related primarily to reactor configuration and flow pattern (Willaert et al., 1996). If cells are

immobilized in porous support particles, increasing the relative velocity of the particle to the bulk solution (Grunwald et al, 1997) or decreasing the particle size (Doran, 1999; Mavituna, 1986) reduces the thickness of liquid-solid film layer resulting in reduced external mass transfer resistance. However, internal mass transfer limitations generally are not negligible and are affected by system parameters, such as particle size, cell loading, flow, substrate concentration and reactor type (Willaert et al., 1996). It is usually assumed that Fickian diffusion is the only transport mechanism in cell-entrapped gels.

Diffusion in Gels

Effective Diffusion Coefficient (D_{eff}) *in Gels*

Effective diffusion coefficients (D_{eff}) can be predicted theoretically or obtained experimentally. Excellent reviews have been written by Westrin and Axelsson (1991) as summarized below.

Assuming that the diffusion coefficient in a gel (D) is independent of position (x) and concentration (S_p) of only one diffusing substrate, the diffusional flux, N, in the x-direction is given by Fick's law:

$$N = -D\frac{\partial S_p}{\partial x} \qquad (2\text{-}1)$$

In equation (2-1), S_p is the substrate concentration in the gel in terms of the amount of substrate per unit volume of gel. The diffusion coefficient (D) used in equation (2-1) must not be confused with the effective diffusion coefficient (D_{eff}); the two are related through porosity (ε):

$$D_{eff} = \varepsilon \cdot D \tag{2-2}$$

Porosity (ε) is the void fraction within which the substrate can diffuse and which depends on the structure and the swelling behavior of the gel. It is approximately the volume fraction not occupied by the polymer (ϕ_p):

$$\varepsilon = 1 - \phi_p \tag{2-3}$$

If the effective diffusion coefficient is used in Fick's law, equation (2-1) takes the following form:

$$N = -D_{eff} \frac{\partial S_v}{\partial x} \tag{2-4}$$

where S_v is the substrate concentration in the liquid occupying the void volume within the gel. Thus, S_p and S_v are related similarly to D and D_{eff}:

$$S_p = \varepsilon \cdot S_v \tag{2-5}$$

The effective diffusion coefficient (D_{eff}) can be estimated from the diffusion coefficient in water, D_{aq}, by two different methods. In one method, D_{eff} is a function of porosity (ε) and tortuosity (τ) where tortuosity represents the obstruction effect explained earlier:

$$D_{eff} = \frac{D_{aq} \cdot \varepsilon}{\tau} \tag{2-6}$$

However, prediction of effective diffusion coefficient with this equation is limited because there is no simple, direct way to measure porosity and tortuosity of gels.

Mackie and Meares (1955) suggested an equation to predict D as a function of polymer volume fraction (ϕ_p) which has been shown to give good agreement with experimental data.

$$\frac{D}{D_{aq}} = \frac{(1-\phi_p)^2}{(1+\phi_p)^2} \tag{2-7}$$

Then, D_{eff} can be predicted by substituting equations (2-2) and (2-3) into equation (2-7).

$$\frac{D_{eff}}{D_{aq}} = \frac{(1-\phi_p)^3}{(1+\phi_p)^2} \tag{2-8}$$

Diffusion characteristics in a gel can be affected by the amount of cells entrapped in the gel and the concentration of polymer used to produce the gel. Numerous models have been developed to describe these effects as reviewed by Muhr and Blanshard (1982) and Westrin and Axelsson (1991).

To measure diffusion coefficients experimentally, several different methods have been reported in the literature. They can generally be divided into two groups: steady-state measurement and non-steady-state measurement. Axelsson and Persson (1988) used steady-state measurement to calculate the effective diffusion coefficient from a diffusional flow established through a gel disc at pseudo-steady-state. For non-steady-state measurement, several approaches have been reported: 1) the time-lag diaphragm cell (Hannoun and Stephanopoulos, 1986), 2) diffusion out of a gel sphere into an infinite solution (Horowitz and Fenichel, 1964), 3) diffusion into a gel sphere from a finite solution (Tanaka et al., 1984; Nguyen and Luong, 1986; De Backer and Baron, 1993; Yankov, 2004), 4) diffusion out of a gel sphere into a finite solution (Papathanasiou and Bijeljic, 1998), and 5) diffusion into a gel slab from a finite solution (Chresand et al., 1988). The third method has been used most frequently in the literature since the partition coefficient can also be obtained from the same experiment.

If diffusion occurs only in the radial direction in a spherical bead, the diffusion equation for a constant diffusion coefficient (D) takes following form:

$$\frac{\partial S_p}{\partial t} = D\left(\frac{\partial^2 S_p}{\partial r_p} + \frac{2}{r_p}\frac{\partial S_p}{\partial r_p}\right) \tag{2-9}$$

where S_p is the substrate concentration in the gel as defined previously, t is time and r_p is the radius of the bead ($0 \le r_p \le R$). Solutions for this equation are given by Crank (1975) for numerous cases of diffusion. Equation (2-10) is the solution for the case of diffusion into a gel sphere from a finite solution (method 3 of the non-steady state measurement techniques). The diffusion coefficient can be calculated by fitting the equation to experimental data.

$$S_p(t) = S_{p,\infty}\left[1 + 6(1+\alpha)\cdot\sum_{n=1}^{\infty}\frac{\exp(-\frac{D\cdot q_n^2\cdot t}{R^2})}{9+9\alpha+q_n^2\alpha^2}\frac{R}{r_p}\frac{\sin\left(\frac{q_n r_p}{R}\right)}{\sin q_n}\right] \qquad (2\text{-}10)$$

where q_n are the positive non-zero roots of:

$$\tan(q_n) = \frac{3q_n}{3+\alpha\cdot q_n} \qquad (2\text{-}11)$$

The value of α is the ratio of the bulk liquid volume (V_b) to the bead volume (V_p). If there is partition factor, K_p, between substrate in the bulk solution and in the gel sphere at equilibrium, α takes following form:

$$\alpha = \frac{V_b}{V_p\cdot K_p} \qquad (2\text{-}12)$$

Diffusion Characteristics

Diffusion in gels is affected by many factors, such as the concentration of immobilized cells, the concentration of diffusing substrate, the types and concentrations of polymer and crosslinking ions, and the size of the gel beads. Diffusion characteristics of various substrates in gels made from polymers such as alginate, carrageenan, and polyvinyl alcohol (PVA) have been studied (Benyahia and Polomarkaki, 2005; Jovetic et al., 2001; Chen et al., 2003; Tanaka et al., 1984; Arnaud and Lacroix, 1991; Merchant et al., 1987).

One of the advantages of immobilized cell systems is the high cell density retained in the reactor, which makes it possible to work at flow rates beyond washout conditions and increase volumetric reaction rates. However, dense cell concentrations in gels may create diffusion hindrance (Westrin and Axelsson, 1991; De Backer and Baron, 1993). Thus, it is expected that the effective diffusion coefficient will decrease with increasing cell concentration in the gel (Chen et al., 2003; Jovetic et al., 2001; De Backer, et al., 1992). Arnaud and Lacroix (1991) found the presence of the bacteria, *S. thermophilus*, did not significantly affect diffusivity, but this was because the cells were calculated to occupy only 0.13 % of the total gel bead volume. Hannoun and Stephanopoulos (1986) reported that diffusivities of glucose and ethanol were not affected by immobilizing a 20 % suspension of dead yeast cells in Ca-alginate. However, since they killed the yeast cells using 50 % ethanol, the permeability of the cell

membrane was increased, which may have allowed glucose and ethanol to diffuse through the cells as well.

Reports on the effect of diffusing substrate concentration on diffusion have been varied. Nguyen and Luong (1986) reported the effective diffusion coefficient decreased in response to an increase in glucose concentration in 3 % *k*-carrageenan gels. However, no significant variation in the effective diffusion coefficient with an increase in the concentration of diffusing substrate has been reported in other papers. Arnaud and Lacroix (1991) studied lactose diffusion in *k*-carrageenan gels finding no difference in diffusivity as lactose concentration increased. Similar results were obtained by Hannoun and Stephanopoulos (1986) with Ca-alginate/glucose and Ca-alginate/ethanol systems. Yankov (2004) also reported no influence of glucose and maltose concentrations on their diffusivities in polyacrylamide gels.

Yankov (2004) reported that the effective diffusion coefficients for glucose and maltose decreased with increasing monomer concentration in polyacrylamide gels. Hannoun and Stephanopoulos (1986) reported similar results for their systems of Ca-alginate/glucose and Ca-alginate/ethanol. In contrast, Tanaka et al. (1984) reported that diffusion for substrates having low molecular weight (glucose, L-tryptophan and α-lactoalbumin) were not affected by increasing the alginate concentration.

Diffusion coefficients reported in the literature show large variations, making it difficult to make comparisons even for the same substrate (see Table 2-6). This is because of considerable variation in the methods used for gel preparation and experimental techniques used.

Table 2-6. Effective diffusion coefficients of various substrates in cell-free gels reported in literature

Substrate	Gel Type	Temp. (ºC)	$D_{eff} \times 10^{10}$ (m^2/s)	References
Glucose	*k*-carrageenan	40	5.71	Chresand et al. (1988)
		40	6.80	Briasco et al. (1990)
		40	9.64	Brito et al. (1990a)
	2 % Ca-alginate	30	6.62	Merchant et al. (1987)
		30	0.30	Ryu et al. (1984)
		30	6.83	Tanaka et al. (1984)
	Polyacrylamide	25	2.57	Adachi et al. (1981)
		30	4.65	Andersson et al. (1997)
		20	3.74	
		10	2.70	
	0.5 % Ca-alginate	30	6.63	Dembczynsk (2000)
	1 % Ca-alginate	30	6.58	

CHAPTER III

BIODEGRADATION OF CHLORFERON BY INDIGENOUS MICROORGANISMS ENRICHED FROM CATTLE DIP SOLUTION

INTRODUCTION

Organophosphate and carbamate pesticides are the most widely used pesticides in the world, treating more than 89 million acres of land each year in the US alone (Smith, 1987). Coumaphos [0,0-diethyl 0-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate] is an organophosphate insecticide used to control pests on livestock and swine bedding. The predominant usage is by the US Department of Agriculture, Animal and Plant Health Inspection Services (USDA-APHIS) for its tick eradication program along the US-Mexico border to prevent the reintroduction of equine and bovine piroplasmosis into the US (Shelton and Karns, 1988; Shelton and Somich, 1988; US EPA, 2000). There are 42 vats at the APHIS sites each containing approximately 15,000 liters of 'Co-Ral' flowable insecticide (42 % coumaphos, 58 % inert ingredients) in which the coumaphos concentration is about 1,500 mg/L (Karns et al., 1995). These vats are used to treat cattle imported from Mexico or coming from the quarantine zone in south Texas. The cattle treating procedure presents a considerable waste disposal problem. Initially, the disposal method for this waste material was to pump the material into evaporation pits or waste lagoons located adjacent to the dip vats.

This created contamination problems with high concentrations of coumaphos and its metaobolites in the underlying soils because the evaporation pits were mostly unlined (Mulbry et al., 1996). In addition, the hydrophobic nature of coumaphos and one of the metabolites, chlorferon, favors its adsorption by soil particles.

Considerable research has shown that organophosphate compounds, including coumaphos, can be hydrolyzed by several microorganisms; however, only a few studies have investigated degradation of the hydrolysis products of coumaphos, chlorferon and diethylthiophosphate (DETP) (Shelton and Somich, 1988; Shelton, 1988; Cook et al., 1978; Cook et al., 1980). Shelton and Somich (1988) isolated bacteria capable of metabolizing coumaphos and also capable of degrading chlorferon. They isolated a degradation product of chlorferon but could not determine if it was the end product. Mulbry et al. (1998) demonstrated that laboratory scale biofilters could effectively reduce the coumaphos concentration using organisms indigenous in the vat solution. Based on their result, vat solutions are currently treated by the biofilter system with treated wastewater then going into concrete evaporation pits. They demonstrated the mineralization of coumaphos by concomitant production of chloride.

The objectives in this chapter were to enrich a consortium of organisms from waste cattle dip solution capable of biodegrading chlorferon, evaluate degradation kinetics of the consortium, and establish optimum reaction conditions for chlorferon degradation using the enriched consortium in a system of freely suspended cells.

MATERIALS AND METHODS

Cattle Dip Vat Solution and Chemicals

Untreated cattle dip (UCD) waste and biofilter rinsate (BR) solutions were obtained from the APHIS dipping vats in Laredo, TX. Analytical grade chlorferon and coumaphos were gifts from Bayer Corporation (Animal Health Division, Merriam, KS). All other chemicals were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO). Chlorferon is highly insoluble in water, and a stock solution was prepared by dissolving it in methanol to a concentration of 5 g/L. Chlorferon solution for degradation studies was produced from pure coumaphos or UCD solution by hydrolysis using *E.coli* genetically engineered to contain the organophosphate hydrolase (OPH) enzyme. Hydrolysis of coumaphos was carried out at the optimum conditions found by Kim (1998). OPH^+ *E.coli* was immobilized in Ca-alginate gel beads to simplify removal from the solution after hydrolysis.

Sample Analysis Method

A HPLC system (Waters, model 2690, Milford, MA) equipped with a PDA detector (Waters, model 996, Milford, MA), an autosampler, and a C-18 column (4.5 mm × 250 mm, Burdick and Jackson, Muskegon, MI) along with a guard column (C-18 4.5 mm × 10 mm, Burdick and Jackson, Muskegon, MI) was used for analysis. The

mobile phase was HPLC grade acetonitrile with 0.75 mM phosphoric acid (80:20 by volume) and the flow rate was 1 ml/min. Absorbance was measured at 320 nm. During the experiments, 0.2 ml samples were collected in 1.5 ml polypropylene micro test tubes and diluted with methanol to achieve appropriate concentrations for HPLC analysis. Tubes were vortexed, and centrifuged at 1,163 × g for 15 min using a microcentrifuge, and the supernatants were filtered using disposable syringe filters (Acrodisk CR13 mm, PTFE, 0.2 μm, Pall Corporation, East Hills, NY). Filtered samples were stored in the refrigerator until analyzed. Cell density was determined by measuring optical density at 590 nm (OD_{590}).

Enrichment of Chlorferon-degrading Organisms

Enrichment was initiated from both UCD and BR solutions. Chlorferon was added to the enrichment cultures either as the analytical grade chemical dissolved in methanol or as hydrolyzed coumaphos in UCD or BR solution. Coumaphos concentrations in UCD and BR solutions were approximately 1500 mg/L and 90 mg/L, respectively. UCD and BR solutions containing chlorferon were incubated at 30 °C in a rotary shaker until degradation of chlorferon was complete. At complete degradation, the solution was used to inoculate (10 % vol/vol) fresh sterilized mineral salt (MS) medium containing chlorferon. MS medium contained $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 5.3 mg, $FeSO_4 \cdot 7H_2O$ 2 mg, $MnSO_4 \cdot 5H_2O$ 0.2 mg, $CuSO_4 \cdot 5H_2O$ 0.4 mg, $ZnSO_4 \cdot 7H_2O$ 0.2 mg, H_3BO_3 30 μg, CuCl 40 μg, $NaMoO_4 \cdot 2H_2O$ 40 μg per liter of 20

mM potassium phosphate buffer solution as described by Brunner et al. (1980). The initial concentration of chlorferon in the enrichment medium was 10 mg/L. When degradation was complete, the concentration of chlorferon was increased stepwise to 20, 50, and 100 mg/L. Several transfers were made to enrich organisms responsible for degradation of chlorferon. The enriched culture solution served as inoculum to grow biomass for degradation studies. Cycloheximide (antibiotic acting on eukaryotes) or ampicillin (antibiotic acting on bacteria) was added to the enrichment culture to determine what types of organisms were responsible for degradation of chlorferon.

Biomass Growth and Acclimation

A two-step growth procedure was developed to produce biomass for degradation studies. The first step was to increase biomass, and the second to acclimate cells to chlorferon and the reaction medium. In the first step, cells were grown in glutamate-yeast extract medium (glutamate 10 g/L, yeast extract 5 g/L, 50 % glycerol 8 ml/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.4 g/L, K_2HPO_4 1.3 g/L, KH_2PO_4 0.4 g/L, pH 7.4) containing chlorferon in a rotary shaking incubator (200 rpm) at 30 °C. Biomass was harvested during the late exponential phase by centrifugation at 4,500 × g at 4 °C for 15 min. The pellet obtained from centrifugation was washed with potassium phosphate buffer (50 mM, pH 7.2) and centrifuged again at the same conditions. The resulting pellet was resuspended into the reaction medium containing 10 mg/L of chlorferon for acclimation and incubated in a rotary shaking incubator (200 rpm) at 30 °C until the

chlorferon was completely degraded. After complete degradation, the biomass was harvested again as described above and used for degradation studies. For all experiments, freshly harvested cells were used.

Media evaluated for biomass acclimation were CHES buffer solution (100 mM, pH 8), potassium phosphate buffer solution (50 mM, pH 8), MS medium (pH 7.5) and MS medium prepared with 20 mM CHES buffer instead of potassium phosphate buffer (MS-CHES, pH 8). All media contained 10 mg/L of chlorferon.

Degradation Studies

Experiments for biodegradation of chlorferon were conducted under batch reactor conditions using a working volume of 50 ml in 250 ml Erlenmeyer flasks with rotary shaking (200 rpm). Temperature was controlled at 30 °C, and experiments were conducted in triplicate. Acclimated biomass was harvested and resuspended in reaction medium containing chlorferon. Samples (0.2 ml) were taken from the bulk phase at intervals during the experiment for determination of chlorferon concentrations. Chlorferon was supplied to the reaction medium as either hydrolyzed pure coumaphos solution or hydrolyzed UCD solution. OD_{590} was measured to observe cell growth during the degradation reactions. Since high cell concentration was used for degradation studies, OD_{590} was measured with diluted samples to be in the linear absorbance range.

Experiments to determine the effects of biomass concentration and reaction pH on chlorferon degradation were carried out with an initial chlorferon concentration of 50

mg/L. For the studies of biomass concentration effects, pH was adjusted initially to 7.5 and biomass concentration was varied from 10 to 140 g/L. For pH effect studies, the initial reaction pH was varied from 6.5 to 8.5 at a biomass concentration of 80 g/L.

The effect of substrate concentration on degradation was studied to estimate kinetic parameters. Chlorferon concentrations tested were in the range of 0 to 150 mg/L with a biomass concentration of 80 g/L and pH of 7.5.

Numerical Calculations

Degradation reaction rates were calculated from slopes of substrate concentration versus time plots using data obtained from each experiment. Kinetic parameters were estimated by determining substrate effects on chlorferon degradation rate. Parameter values were determined by non-linear regression of specific reaction rates using the SigmaPlot software package (SYSTAT Software Inc. Chicago, IL).

RESULTS AND DISCUSSION

Enrichment of Chlorferon-degrading Organisms

Chlorferon-degrading organisms were present in both BR and UCD solutions. The BR solution had a chlorferon concentration of approximately 50 mg/L after

hydrolysis, and degradation of chlorferon occurred promptly in this solution. However, the concentration of chlorferon in hydrolyzed UCD solution was approximately 870 mg/L which inhibited degradation. Therefore, UCD solutions had to be diluted to a concentration less than 50 mg/L to initiate degradation; nevertheless, it was not initiated as promptly as in the BR solution.

For both BR and UCD solutions, when chlorferon degradation was complete, the culture solution was added to fresh sterilized MS medium (10 % vol/vol) containing 10 mg/L chlorferon. Figure 3-1 shows chlorferon degradation during cell enrichment. The initial degradation rate after the first transfer was slow, giving a reaction rate of 0.25 mg/L·h. Subsequent degradation rates were faster with chlorferon concentrations increased stepwise up to 100 mg/L, for which the reaction rate was 1.59 mg/L·h. However, with a chlorferon concentration of 150 mg/L, degradation stopped completely. With chlorferon concentration above 100 mg/L, OD_{590} was unreliable due to sticky extracellular compounds the cells produced.

Several sequential transfers were made to selectively enrich chlorferon-degrading organisms. For each transfer, the initial chlorferon concentration was 10 mg/L and additional chlorferon was added in stepwise increments (see Figure 3-1) up to 100 mg/L. When degradation of the 100 mg/L addition was complete, the culture was used to inoculuate fresh medium. Whenever the culture was transferred to fresh medium, the initial chlorferon concentration had to start from 10 mg/L to prevent inhibition.

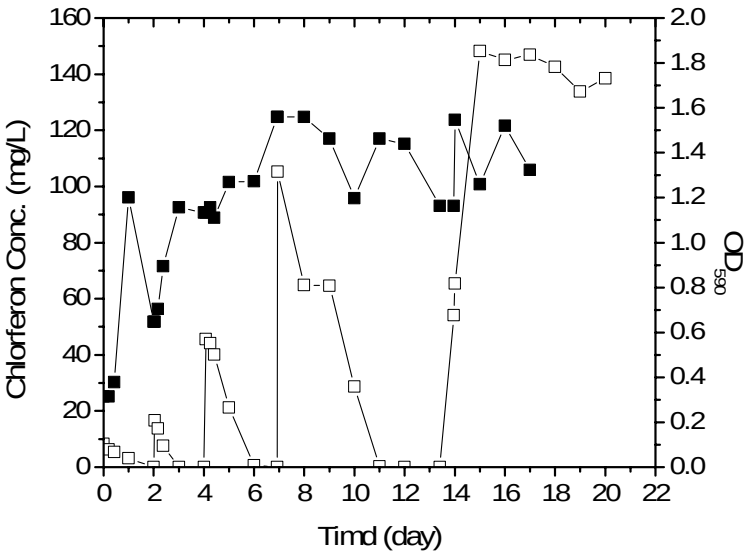


Figure 3-1. Chlorferon degradation and cell growth during enrichment of chlorferon-degrading organisms. Reaction conditions: reaction in MS medium; temperature 30 °C; agitation speed 200 rpm in a rotary shaking incubator (□ chlorferon concentration; ■ OD_{590})

Cell growth during the enrichment was monitored by OD_{590}. Increases in OD_{590} were clearly observed during the enrichment as shown in Figure 3-1, but it was not clear if chlorferon served as the carbon source for biomass growth. Since chlorferon dissolved in methanol was used as the stock solution to prepare the medium, methanol may have served as the carbon source. The chlorferon stock solution was prepared at a concentration of 5000 mg/L, and to achieve an initial chlorferon concentration of 10 mg/L, the methanol concentration was 1584 mg/L. To determine if chlorferon without methanol could support growth, chlorferon was suspended in water, sonicated for 20 minutes and added to the enriched culture of chlorferon-degrading organisms. As shown in Figure 3-1, addition of chlorferon stock solution to give a chlorferon concentration of 10 mg/L caused cells to grow to almost 10 times their initial concentration based on OD_{590}. However, OD_{590} did not change during complete degradation of chlorferon suspended in water as shown in Figure 3-2. After the chlorferon was degraded, methanol at a concentration of 1584 mg/L was added and OD_{590} increased rapidly by a magnitude approximately the same as obtained with the chlorferon stock solution. Thus, it appeared that chlorferon did not support growth of chlorferon-degrading organisms.

Adding yeast extract, also a carbon source, to the reaction medium slowed chlorferon degradation but OD_{590} increased to twice that in MS medium. Figure 3-3 shows the results of cell enrichment in MS medium containing 200 mg/L of yeast extract (MS200YE). Thus, it was concluded that chlorferon-degrading organisms preferentially used methanol and yeast extract as their carbon source, and chlorferon degradation could be slowed when readily degradable carbon is abundant.

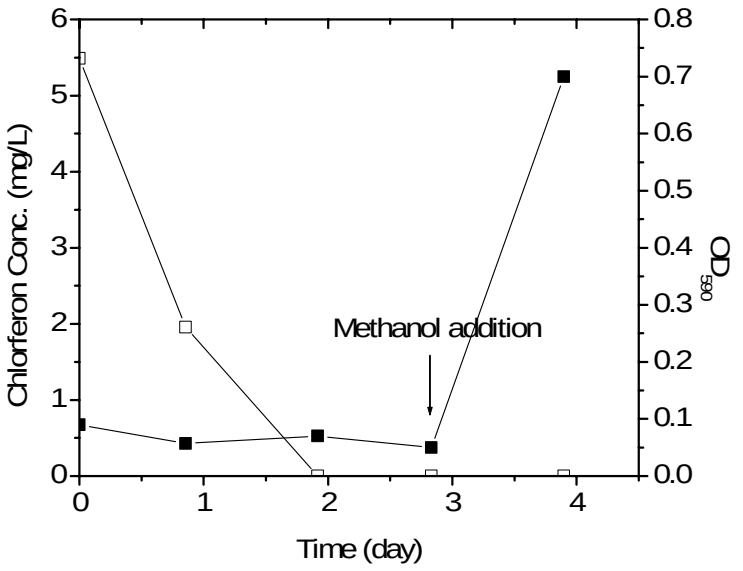


Figure 3-2. Chlorferon degradation and cell concentration profile represented by OD_{590} during enrichment with chlorferon supplied as pure chemical well-suspended in water by sonication. Reaction conditions: reaction in MS medium; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system (□ chlorferon concentration; ■ OD_{590})

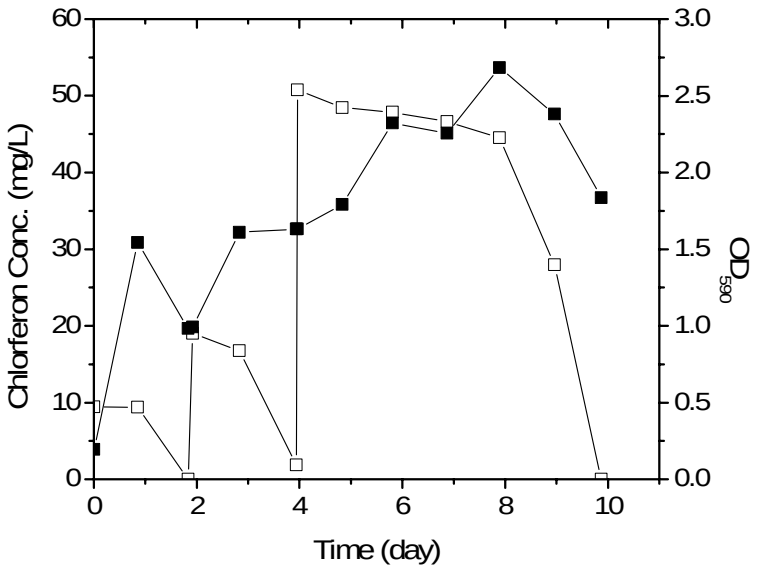


Figure 3-3. Chlorferon degradation and cell growth during enrichment of chlorferon-degrading organisms in MS200YE medium. Reaction conditions: temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. (□ chlorferon concentration; ■ OD_{590})

Shelton and Somich (1988) isolated two species of bacteria from the UCD solution that were capable of degrading chlorferon. They reported that addition of chlorferon to cells in the late log phase of growth resulted in prompt degradation of chlorferon which was metabolized to α-chloro-β-methyl-2,3,4-trihydroxy-*trans*-cinnamic acid (CMTC) and also partly incorporated into biomass. Isolates from the enriched cultures in this study were evaluated for chlorferon degradation, but no single isolate showed this activity. Chlorferon degradation in this study was achieved by a consortium of organisms. HPLC analysis showed the appearance of a new peak as chlorferon degraded. It was believed to be a metabolic product of chlorferon, but we could not identify it as CMTC. The peak disappeared quickly following complete degradation of chlorferon, indicating it was further degraded by the consortium.

To identify microorganisms responsible for degradation of chlorferon, cycloheximide or ampicillin was added to the enrichment culture at a concentration of 50 mg/L. Neither cycloheximide nor ampicillin had a noticeable effect on chlorferon degradation activity of the enrichment culture as shown in Figure 3-4. Cycloheximide inhibits protein synthesis in eukaryotic cells while ampicillin is a type of penicillin which inhibits the synthesis of bacterial cell wall (peptidoglycan). Microscopic observation and Gram-staining showed that the culture was mostly Gram-negative rod type bacteria. Since the Gram-negative cell wall is rich in lipid and contains less peptidoglycan, the chlorferon-degrading organisms were thought to be bacteria which were not affected significantly by the ampicillin.

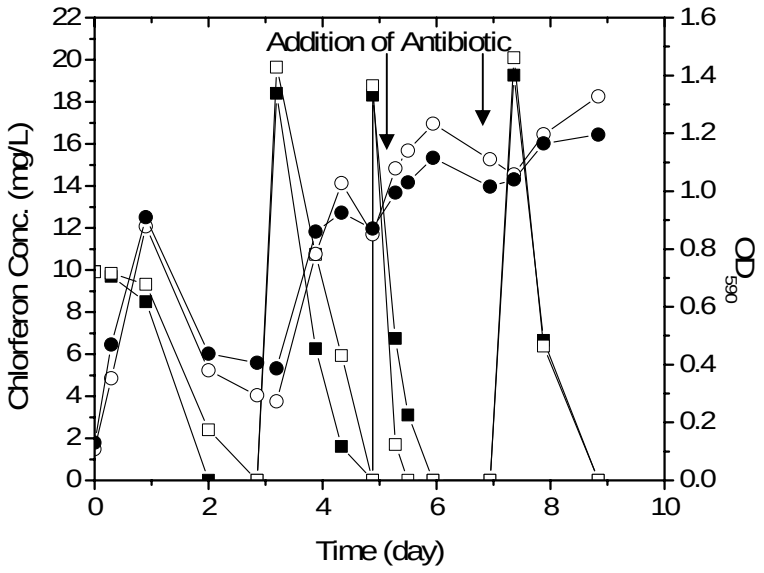


Figure 3-4. Influence of antibiotic on chlorferon degradation during the enrichment of chlorferon-degrading organisms. Reaction conditions: reaction in MS medium; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. (Chlorferon concentration: ■ addition of ampicillim; □ addition of cycloheximide; OD_{590}: ● addition of ampicillin; ○ addition of cycloheximide)

Biomass Acclimation Studies

Chlorferon-degrading organisms were grown following the two-step growth procedure. For the acclimation step, the fastest chlorferon degradation was achieved in MS medium. Chlorferon in MS medium was degraded in approximately 3 – 4 days when it was supplied as hydrolyzed pure coumaphos solution. In potassium phosphate buffer solution, no degradation occurred so no further studies were conducted with that medium.

A two-step growth procedure was initially developed to provide biomass acclimated to the target compound for degradation studies since cells could lose their ability to degrade the target compound during growth in a rich medium. There was a lag time of approximately 5 to 8 days when biomass was resuspended in the degradation reaction medium without acclimation depending on the initial concentration of chlorferon. Lower concentrations of chlorferon resulted in shorter lag periods (Figure 3-5). This lag phase was eliminated only when the acclimation medium and degradation reaction medium were the same. When the reaction medium was different from the acclimation medium, there was still a lag phase. However, acclimation to the medium without chlorferon did not eliminate the lag phase (Figure 3-6). Therefore, it was concluded that the cells needed to be acclimated to both the target compound and the reaction medium.

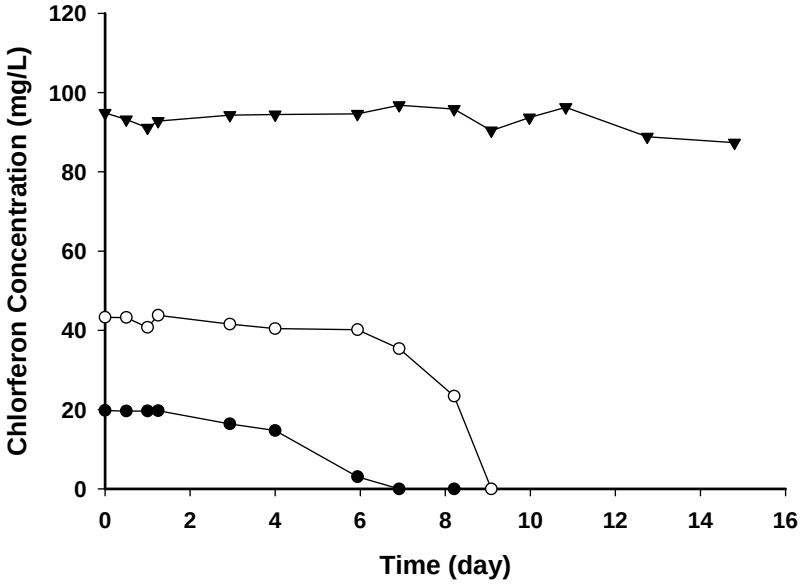


Figure 3-5. Lag period before degradation depending on the initial chlorferon concentration without acclimation. Reaction conditions: reaction in MS medium (pH 7.5); biomass concentration 40 g/L; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system

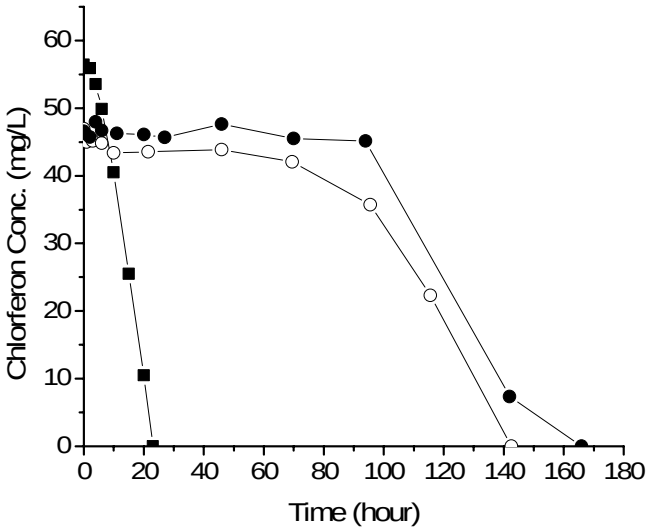


Figure 3-6. Effects of cell acclimation on chlorferon-degradaing activity in various reaction media. Reaction conditions: biomass concentration 40 g/L; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system (• degradation in CHES buffer solution (100 mM, pH 8) using cells acclimated in MS medium containing chlorferon (pH 7.5); ○ degradation in MS medium (pH 7.5) using cells acclimated in MS medium without chlorferon; ■ degradation in MS medium using cells acclimated in MS medium containing chlorferon)

Degradation Studies

Degradation studies were conducted in MS medium with chlorferon supplied either as hydrolyzed pure coumaphos solution or as hydrolyzed UCD solution. When chlorferon was supplied as hydrolyzed UCD solution, biomass acclimation was completed in two days. This was faster than with hydrolyzed pure coumaphos solution, although the degradation rates were about the same in both cases. When hydrolyzed UCD solution was used to supply chlorferon for degradation studies, native organisms were added as well. Control samples without biomass addition were incubated to determine the effect of native organisms. No degradation occurred in the control samples when incubated for the same amount of time as the samples with biomass added. This was expected based on the enrichment studies in which degradation of chlorferon in UCD solution took almost a month to be initiated and occurred only at a low concentration of chlorferon.

Optical density was monitored to determine cell growth during degradation. Hydrolyzed pure coumaphos solution was added to supply chlorferon for this experiment to avoid the influence of contaminants such as dirt found in the UCD solution. No change in OD_{590} was observed during the degradation (Figure 3-7). In addition, colony forming units (CFU) were counted on both nutrient agar plates and MS medium agar plates containing chlorferon before and after the degradation reaction. The number of CFUs did not increase during the degradation. The reason for this non-growing behavior

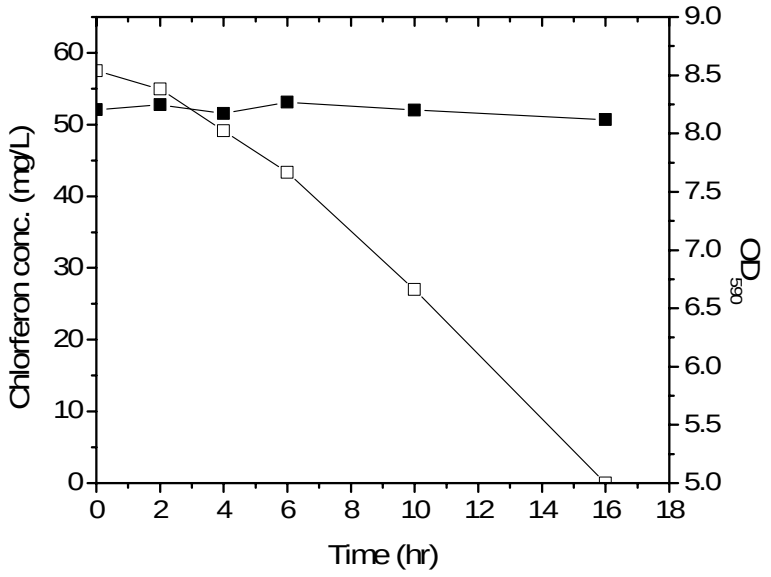


Figure 3-7. The profile of cell concentration represented by OD_{590} during chlorferon degradation. Reaction conditions: reaction in MS medium (pH 7.5); cell concentration 80 g/L; agitation speed 200 rpm in a rotary shaking system; temperature 30 °C (■ OD_{590}; □ chlorferon concentration)

might be attributed to carbon limiting conditions in the reaction medium. Since coumaphos stock solution was prepared in methanol, the reaction medium prepared with hydrolyzed pure coumaphos solution contained methanol as well. Assuming that methanol in the coumaphos stock solution was not volatilized during the hydrolysis, the amount of methanol added to the chlorferon degradation medium to achieve 50 mg/L of chlorferon concentration was 0.4 g. Based on thermodynamics, cell mass produced using methanol was estimated to be 0.3 g in dry weight following the procedure of Rittmann and McCarty (2001) using energy-transfer efficiency of 0.6. Assuming the water content is 80 % in wet cell wight, the maximum increase of 0.3 g dry cell weight results in the increase of 29.6 g/L in wet cell concentration. This should be noticeable in the degradation reaction medium. A similar methanol concentration was achieved during enrichment with a chlorferon concentration of 50 mg/L (See Figure 3-1) which allowed the cell growth to be measurable. The immeasurable cell growth in the degradation medium could be explained by that most of the methanol in hydrolyzed coumaphos solution was volatilized during hydrolysis. Another possible explanation is cessation of growth due to contact inhibition. It has been reported that bacterial cells stop growing in dense suspensions (Pirt, 1975; Rainina et al., 1994).

Hydrolyzed pure coumaphos solution also contains DETP, the other hydrolysis products, which can serve as a carbon source as well. However, the concentration of DETP did not decrease as chlorferon was degraded, indicating that DETP was not used as a carbon source.

Effect of Biomass Concentration on Chlorferon Degradation

Specific reaction rates and volumetric reaction rates were evaluated for various biomass (wet weight) concentrations in the degradation reactor. Results are shown in Figure 3-8. The optimum biomass concentration for chlorferon degradation was found to be 80 g/L. Both the specific reaction rate and volumetric reaction rate were at their maximum value at this biomass concentration, giving a specific reaction rate of 0.034 mg/g-biomass·h and a volumetric reaction rate of 2.76 mg/L·h. The volumetric reaction rate increased proportionally with biomass concentration up to 80 g/L and then it remained constant at concentrations above the optimum.

Effect of Reaction pH on Chlorferon Degradation

The effect of pH on chlorferon degradation was tested in the range of pH 6.5 to 8.5. Because pH did not change much during the degradation reaction, it was adjusted initially and no further adjustments were made. In the range tested, no effect of pH was observed. All degradation rates within the range tested were in close proximity based on a 95 % confidence interval (Figure 3-9). For further studies, pH 7.5, the middle of the tested range, was selected as the operating pH.

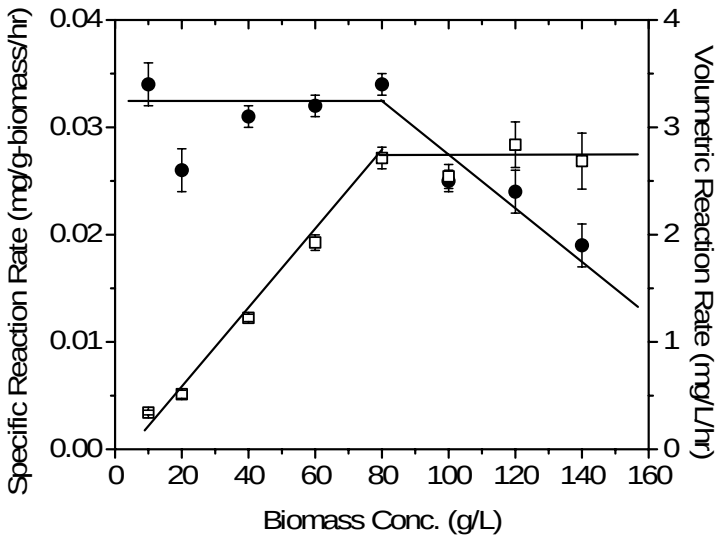


Figure 3-8. Effects of biomass concentration on chlorferon degradation. Reaction conditions: reaction in MS medium with chlorferon supplied as hydrolyzed UCD solution; initial concentration of chlorferon 50 mg/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system (● Specific reaction rate; □ Volumetric reaction rate; mean ± standard error)

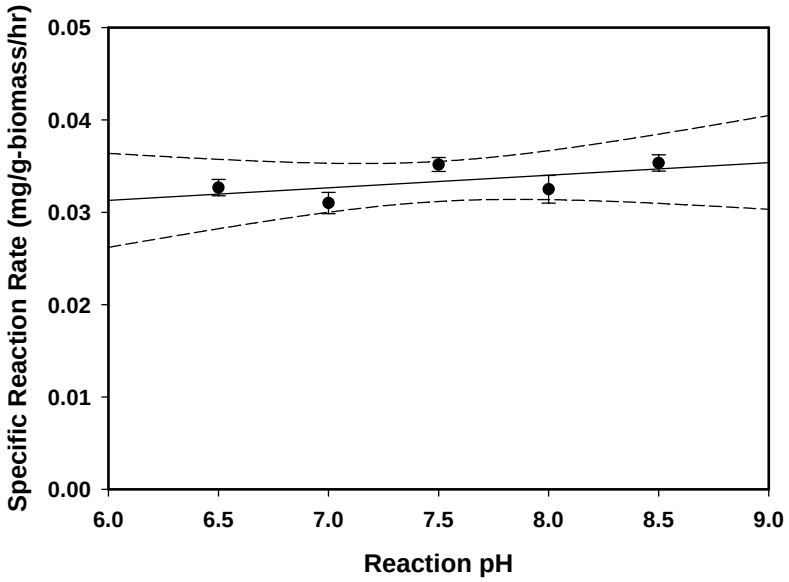


Figure 3-9. Effects of initial reaction pH on chlorferon degradation. Reaction conditions: reaction in MS medium with chlorferon supplied as hydrolyzed UCD solution; biomass concentration 80 g/L; initial chlorferon concentration 50 mg/L; temperature 30 °C; agitation speed 200 rpm (● experimental data ± standard error; — linear regression of data; --- 95 % confidence interval)

Effect of Substrate and Chlorferon Degradation Kinetics

The chlorferon degradation reaction followed substrate inhibition kinetics within the range of substrate values tested (Figure 3-10) as described by the following expression:

$$V = \frac{V_m \cdot S}{K_m + S + \frac{S^2}{K_{Si}}} \qquad (3\text{-}1)$$

where S is the substrate concentration, V_m is the maximum reaction rate, K_m is the substrate half-saturation constant, and K_{Si} is the substrate inhibition constant. Substrate inhibition kinetics for an enzymatic reaction were selected to describe chlorferon degradation because no cell growth was observed during the reaction. Estimated kinetic parameters are presented in Table 3-1.

It was expected that chlorferon degradation followed substrate inhibition kinetics from the enrichment studies. During enrichment of chlorferon degrading organisms, chlorferon degradation was completely stopped at a concentration of 150 mg/L (Figure 3-1). For the degradation studies, however, degradation of chlorferon at 150 mg/L was slow but eventually completed, most likely due to the high cell concentration.

Table 3-1. Kinetic parameters for chlorferon degradation

V_m (mg/g-biomass· h)	K_m (mg/L)	K_{Si} (mg/L)
0.06 ± 0.01	20.6 ± 7.3	117.6 ± 44.8

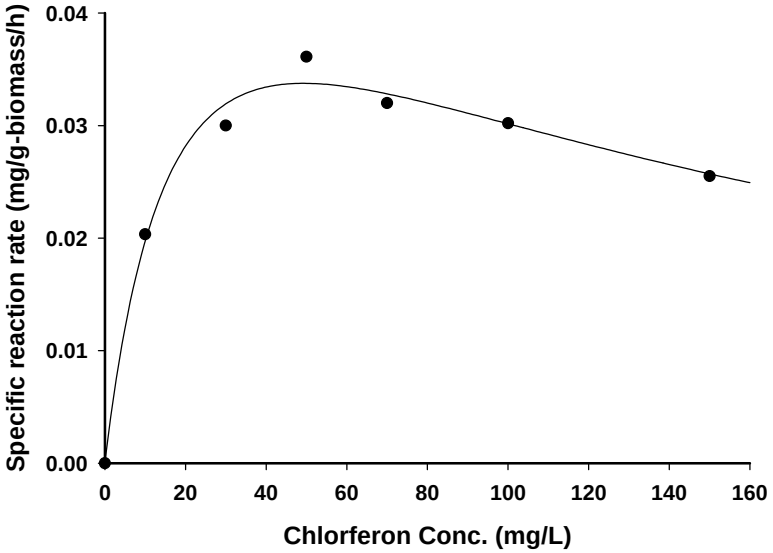


Figure 3-10. Effects of chlorferon concentration on the degradation. Reaction conditions: reaction in MS medium with chlorferon supplied as in hydrolyzed UCD solution; biomass concentration 80 g/L; reaction pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. Symbol represents specific reaction rate determined by experiments. The line represents the best fit of the experimental data using the substrate inhibition model

CHAPTER IV
BIODEGRADATION OF DIETHYL THIOPHOSPHATE (DETP) BY INDIGENOUS MICROORGANISMS ENRICHED FROM CATTLE DIP SOLUTION

INTRODUCTION

Diethylthiophosphate (DETP) is a product of the hydrolysis of coumaphos [0,0-diethyl 0-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate], an organophosphate pesticide. Coumaphos is used predominantly by the US Department of Agriculture, Animal and Plant Health Inspection Services (USDA-APHIS) for its tick eradication program along the US-Mexico border (Shelton and Karns, 1988; Shelton and Somich, 1988). Initially, the disposal method for waste solution from the dipping vats was to pump the material into evaporation pits or waste lagoons located adjacent to the vats. This created contamination problems with high concentrations of coumaphos and its metaobolites in the underlying soils because the evaporation pits were mostly unlined (Mulbry et al., 1996). Currently, vat solutions are treated by biofilters developed by Mulbry et al. (1998) with the treated wastewater then going into concrete evaporation pits.

Organophosphate pesticides are the most widely used pesticides in the world. They usually are not persistent in the environment and can be rapidly hydrolyzed

enzymatically or chemically upon exposure to water (US EPA, 1999; Rittmann and McCarty, 2001). However, they may cause serious environmental problems because they are, in general, highly toxic and hydrolysis does not mean complete mineralization. Considerable research has been conducted to develop solutions to problems that may be caused by organophosphate compounds. It has been shown that coumaphos can be hydrolyzed by several microorganisms; however, only a few studies have investigated degradation of the resulting metabolites, chlorferon and DETP (Shelton and Somich, 1988; Shelton, 1988; Cook et al., 1978; Cook et al., 1980). Shelton (1988) studied an enriched consortium obtained from cattle dip solution that was able to use DETP as a carbon and energy source in the presence of excess sulfate and phosphate. He found that none of the strains present at high population densities in the enriched consortium were capable of metabolizing DETP. He suggested that other bacteria, which were present at low population densities, were responsible for the initial attack on DETP. Cook et al. (1980) isolated *Pseudomonas acidovorans* from sewage and found that it was capable of using DETP as the sole sulfur source. They also found an isolate capable of using DETP as the sole phosphorus source (Cook et al., 1978), but those organisms required other carbon sources.

The objectives of this work were to enrich a consortium of organisms from cattle dip solution capable of biodegrading DETP, evaluate degradation kinetics of the consortium, and establish reaction conditions for DETP degradation using the enriched consortium.

MATERIALS AND METHODS

Cattle Dip Vat Solution and Chemicals

Untreated cattle dip waste (UCD) solution was obtained from the APHIS dipping vats in Laredo, TX. DETP is not available commercially; instead, DETP-potassium salt (DETP-K) was obtained from Sigma-Aldrich (St. Louis, MO) and used as a standard for DETP. Analytical grade chlorferon was a gift from Bayer Corporation (Animal Health Division, Merriam, KS). All other chemicals were of reagent grade and obtained from Sigma-Aldrich (St. Louis, MO).

Sample Analysis Method

A HPLC system (Waters, model 2690, Milford, MA) equipped with a PDA detector (Waters, model 996, Milford, MA), an autosampler, and a C-8 column (4.5 mm × 250 mm, Burdick and Jackson, Muskegon, MI) along with a guard column (C-18, 4.5 mm × 10 mm, Burdick and Jackson, Muskegon, MI) was used for analysis. The mobile phase was acetonitrile with 0.75 mM phosphoric acid (80:20 by volume) and the flow rate was 1 ml/min. Absorbance was measured at 210 nm. During the experiments, 0.2 ml samples were collected in 1.5 ml polypropylene micro test tubes and diluted to achieve appropriate concentrations for HPLC analysis. Tubes were vortexed and centrifuged at 1,163 × g for 15 min using a microcentrifuge, and the supernatants were filtered using

disposable syringe filters (Acrodisk CR 13 mm PTFE, 0.2 μm, Pall Corporation, East Hills, NY). Filtered samples were stored in the refrigerator until analyzed. Cell density was determined by measuring optical density at 590 nm (OD_{590}).

Enrichment of DETP-degrading Organisms

It was assumed that native organisms present in the UCD solution would be capable of degrading DETP. Thus, enrichment was initiated from that solution. DETP was added to 50 ml of UCD solution to give a concentration of 100 mg/L and the solution was incubated at 30 °C in a rotary shaker. When DETP was completely degraded, the solution was used to inoculate (10 % vol/vol) sterilized fresh mineral salt yeast extract (MSYE) medium containing DETP. MSYE medium was mineral salt (MS) medium containing 10 mg/L of yeast extract as described by Shelton (1988). MS medium was the same as used for chlorferon degradation. The initial DETP concentration in the enrichment medium was 100 mg/L. When degradation was complete, additional DETP was added in steps to give concentrations of 200, 500, and 1,000 mg/L. Several transfers were made followed by stepwise addition of DETP to enrich organisms responsible for degradation. Cycloheximide (antibiotic acting on eukaryotes) or ampicillin (antibiotic acting on bacteria) was added to the enrichment culture to determine what types of organisms were responsible for degradation of DETP.

Inhibition Studies

Inhibition studies were conducted during enrichment to determine effects of DETP and chlorferon. DETP was added to the enrichment medium at concentrations up to 1,000 mg/L. Chlorferon was added at a concentration of 50 mg/L along with DETP after the culture had successfully degraded DETP of 100 mg/L.

Biomass Growth and Acclimation

A two-step procedure was developed to produce biomass for degradation studies. The enriched culture solution served as inoculum. The first step was to rapidly increase biomass, and the second step was to acclimate cells to DETP and the degradation reaction medium. In the first step, cells were grown in glutamate-yeast extract medium (glutamate 10 g/L, yeast extract 5 g/L, 50 % glycerol 8 ml/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.4 g/L, K_2HPO_4 1.3 g/L, KH_2PO_4 0.4 g/L, pH 7.4) containing 100 mg/L of DETP in a rotary shaking incubator (200 rpm) at 30 °C. Biomass was harvested during the late exponential phase by centrifugation at 4,500 × g at 4 °C for 15 min. The pellet was washed with potassium phosphate buffer (50 mM, pH 7.2) and centrifuged again at the same conditions; the resulting pellet was resuspended into the reaction medium containing 100 mg/L of DETP for acclimation and incubated in a rotary shaking incubator (200 rpm) at 30 °C until DETP was completely degraded. After complete

degradation, biomass was harvested again as described above and used for degradation studies. For all experiments, freshly harvested cells were used.

Degradation Studies

Experiments for biodegradation of DETP were conducted under batch reactor conditions using a working volume of 50 ml in 250 ml Erlenmeyer flasks with rotary shaking (200 rpm). Biomass harvested after the acclimation step was resuspended in reaction medium containing DETP. Temperature was controlled at 30 °C, and experiments were conducted in triplicate. Samples (0.2 mL) were taken from the bulk phase at intervals during the experiment to determine DETP concentration. Cell growth during the degradation reaction was observed by measuring OD_{590}.

Several media were evaluated for both the biomass acclimation step and the degradation reaction process including CHES buffer solution (100 mM, pH 8), potassium phosphate buffer solution (50 mM, pH 8), MSYE (pH 7.5) and MSYE prepared with 20 mM of CHES buffer instead of potassium phosphate buffer (MSYE-CHES, pH 8). All media contained 100 mg/L of DETP when used for acclimation. DETP concentrations for degradation studies were 500 mg/L or 1,000 mg/L. Medium pH was adjusted initially to 7.5 using 1N KOH.

Effects of biomass concentration and reaction pH were studied in MSYE medium which was the optimum found in the experiments described above. DETP concentration for these studies was 500 mg/L. For the studies of biomass concentration effect, biomass

concentration was varied from 10 to 100 g/L and medium pH was adjusted initially to 7.5. The pH effect on DETP degradation was studied because DETP degradation produced acidic component(s). The pH of the reaction medium was adjusted initially to 6.5, 7, 7.5, 8, or 8.5 and monitored during the time course of the reaction. The optimum pH was selected, and initial adjustment of pH was compared to controlling pH at the optimum value during the reaction. To control pH, 1N KOH solution was used every two hours when samples were taken. The biomass concentration used for this study was 60 g/L.

Substrate concentration effects on degradation were studied to estimate kinetic parameters. DETP concentration was varied from 0 to 2,000 mg/L. Biomass concentration and reaction pH for this study were 60 g/L and 7.5, respectively.

Numerical Calculations

Degradation reaction rates were calculated from the slope of substrate concentration versus time plots using data obtained from each experiment. Kinetic parameters were estimated by determining substrate effects on DETP degradation. Parameter values were determined by non-linear regression of specific reaction rates using the SigmaPlot software package (SYSTAT Software Inc. Chicago, IL).

RESULTS AND DISCUSSION

Enrichment of DETP-degrading Organisms

DETP-degrading organisms were present in the UCD solution. Unlike the slow chlorferon degradation in UCD solution, DETP degradation occurred promptly by native organisms in UCD solution. As DETP was degraded, pinkish flocs were formed in the medium during enrichment, making it impossible to measure OD_{590}. Other than the flocs, the medium was clear. After the flocs were formed, the medium was centrifuged and both clear supernatant and flocs were transferred separately into fresh MSYE medium containing DETP. Both transfers resulted in degradation of DETP. When clear supernatant was transferred into fresh medium, it took approximately 2 – 3 days to start forming flocs. OD_{590} could not be measured, but the growth of flocs was clearly observed. Although the enrichment medium contained yeast extract, the concentration was low (10 mg/L). Also, no other carbon sources were added to the medium during the enrichment. Thus, it was thought that the cells used DETP as their carbon and energy source. The medium pH was controlled around 7.5 during enrichment by addition of 1N KOH because degradation of DETP produced acidic product(s) causing the pH to drop. Figure 4-1 shows DETP concentrations during cell enrichment.

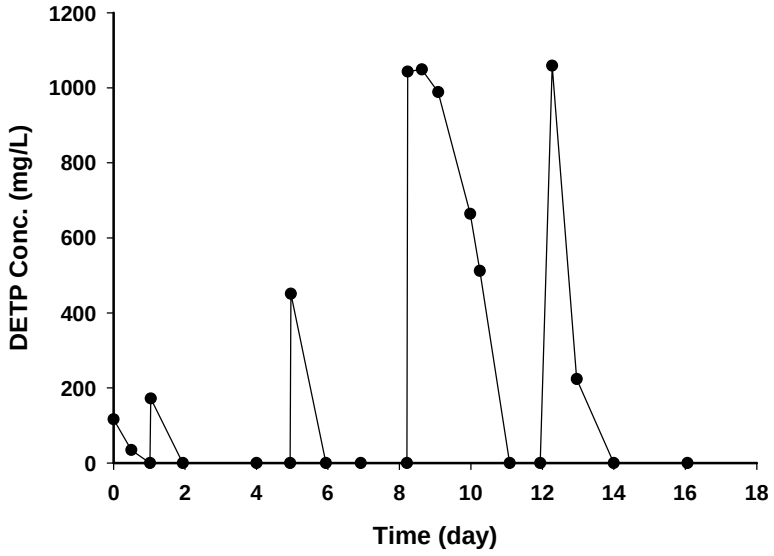


Figure 4-1. DETP degradation during enrichment of DETP-degrading organisms. Reaction conditions: reaction in MSYE medium; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system

Shelton (1988) developed an enriched consortium from UCD solution that was able to use DETP as a carbon and energy source. He found that the ethyl group of DETP was used as the carbon source. The consortium was a bacterial culture that consisted of six morphologically distinct bacteria at high population densities, showing a flocculating nature of growth. However, none of the strains was capable of degrading DETP either individually or collectively. He suggested that initial attack on DETP was made by other bacteria present at low population densities. The mixed culture enriched in this research was considered to be similar based on the flocculating nature of growth and use of the same enrichment medium. No single bacterium capable of degrading DETP was isolated in this study. A single organism capable of degrading DETP was isolated by Cook et al. (1978, 1980). They found *Pseudomonas acidovorans* from sewage that utilized DETP as the sole sulfur source (Cook et al., 1980) and another isolate that used DETP as the sole phosphorus source (Cook et al., 1978). Both organisms required other carbon sources.

Cell enrichment experiments were conducted with either cycloheximide or ampicillin added to the enrichment medium. Addition of cycloheximide during the enrichment had no effect on degradation ability of the culture while addition of ampicillin stopped DETP degradation completely. Since ampicillin is an antibiotic acting on bacteria, it is clear that DETP degradation is achieved by bacterial metabolism. The results are shown in Figure 4-2. Under microscopic examination with Gram-staining, the bacteria were mostly Gram-negative rod types.

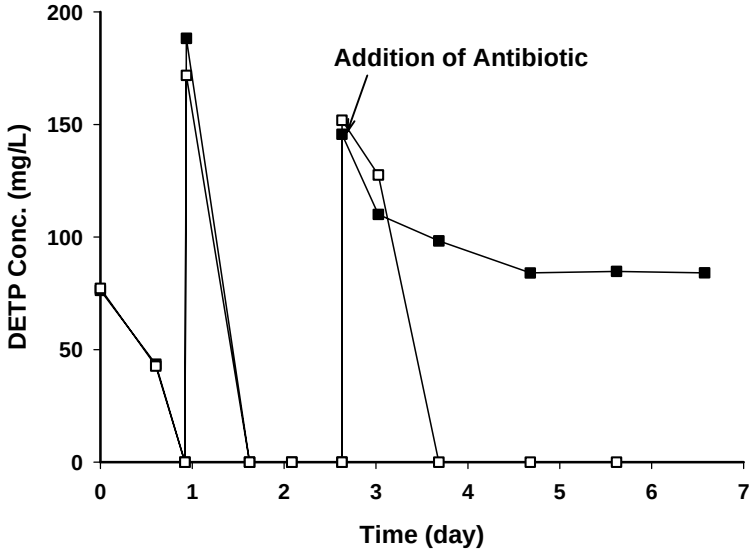


Figure 4-2. Influence of antibiotics on DETP degradation during cell enrichment. Reaction conditions: reaction in MSYE medium; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system (■ addition of ampicillin; □ addition of cycloheximide)

Inhibition Studies

DETP did not appear to be inhibitory to the bacteria responsible for its degradation up to concentrations of 1,000 mg/L, the maximum tested. Successive degradation batches were conducted with 100, 200, 500 and 1,000 mg/L, and the corresponding degradation rates were 4.88, 8.11, 19.29, and 3.55 mg/L·h. The degradation rate slowed when DETP was added at 1,000 mg/L; however, the bacteria acclimated rapidly to the high concentration. The degradation rate increased to 44.08 mg/L·h when 1,000 mg/L of DETP was added a second time (Figure 4-1). Higher concentrations were not tested because the maximum DETP concentration from coumaphos hydrolysis in UCD solution is approximately 630 mg/L (average coumaphos concentration in the vat solution is approximately 1,500 mg/L).

Chlorferon, the other hydrolysis product of coumaphos, was found to inhibit the DETP-degrading bacteria completely at a concentration of 50 mg/L as shown in Figure 4-3. The chlorferon was added along with DETP after the culture had successfully degraded 100 mg/L of DETP.

Since chlorferon-degrading organisms were successfully enriched from UCD solution as described in Chapter III, an attempt was made to enrich the two consortia together for simultaneous degradation of chlorferon and DETP. With this mixed consortium, DETP degradation could be achieved only when chlorferon concentration was low and after chlorferon was completely degraded (Figure 4-4(a)). Increasing chlorferon concentration to 120 mg/L stopped DETP degradation (Figure 4-4(b)).

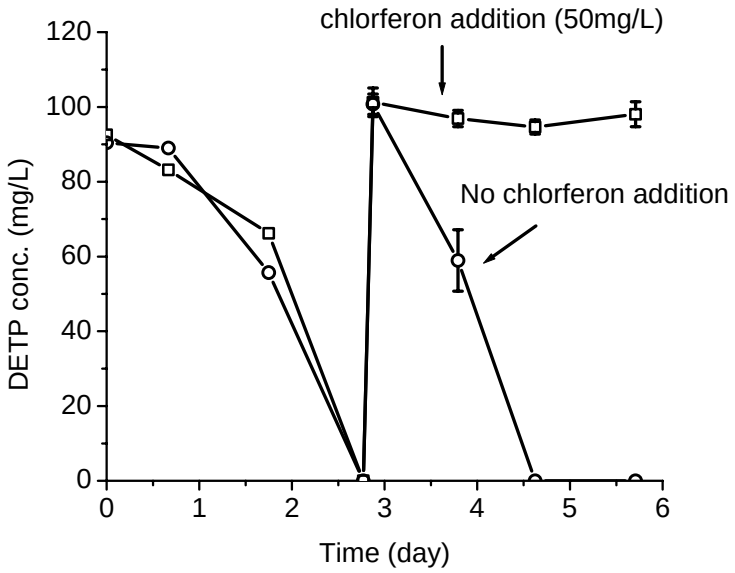
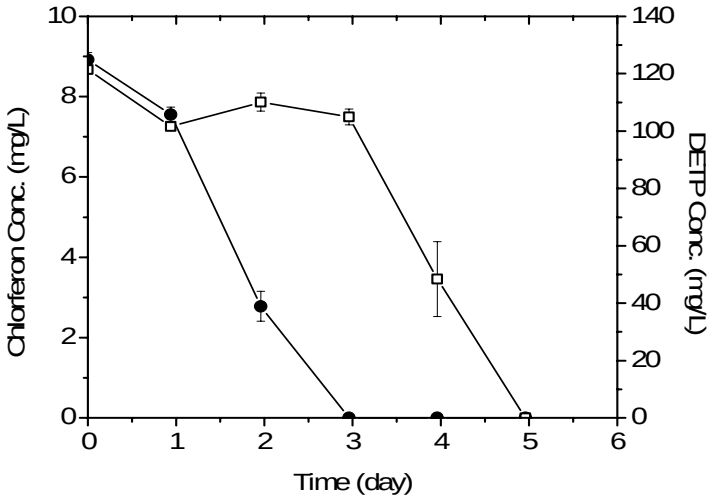
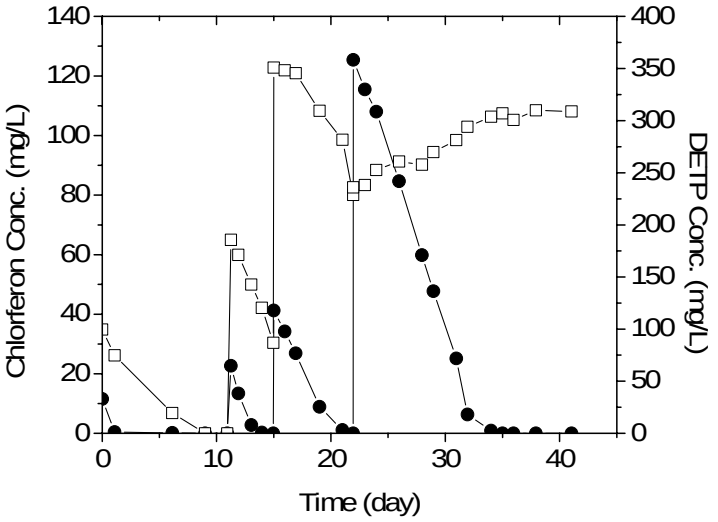


Figure 4-3. Chlorferon inhibition of the DETP-degrading organisms during cell enrichment. Reaction conditions: reaction in MSYE medium; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. (○ DETP concentration during enrichment without chlorferon addition; □ DETP concentration during enrichment with 50 mg/L of chlorferon addition along with DETP on day 3; mean ± standard error)



(a)



(b)

Figure 4-4. Chlorferon inhibition of the DETP-degrading organisms during enrichment of organisms for simultaneous degradation of chlorferon and DETP in MSYE medium. Reaction conditions: temperature 30 °C; agitation speed 200 rpm in a rotary shaking system (● chlorferon concentration, □ DETP concentration)

Degradation Studies Using Freely Suspended Cells

Selection of Reaction Medium and Biomass Acclimation

A two-step growth procedure for degradation studies was developed to provide large quantities of biomass acclimated to the target compound since cells lost some of their ability to degrade the target compound during growth in a rich medium. The same phenomenon was found in the chlorferon degradation studies. Figure 4-5 shows DETP degradation results without cell acclimation. A week-long lag period occurred before degradation was initiated.

For the acclimation step, degradation of 100 mg/L DETP was evaluated in various media. The fastest acclimation occurred in MSYE medium with a lag phase of approximately 3 days and complete degradation in less than one day. Degradation in CHES buffer took approximately 5 days to complete. No degradation occurred in either potassium phosphate buffer or MSYE-CHES medium, so no further studies were conducted with those media.

CHES buffer solution (100 mM, pH 8) and MSYE medium were evaluated for the reaction medium. The degradation rate was faster when the acclimation medium and reaction medium were the same, which was the same result found in the chlorferon degradation studies. When biomass acclimated in CHES buffer was resuspended in CHES buffer solution containing 1,000 mg/L DETP, there was no lag phase. The degradation rate was slow, initially giving a specific reaction rate of 1.19 mg/g-cell·h

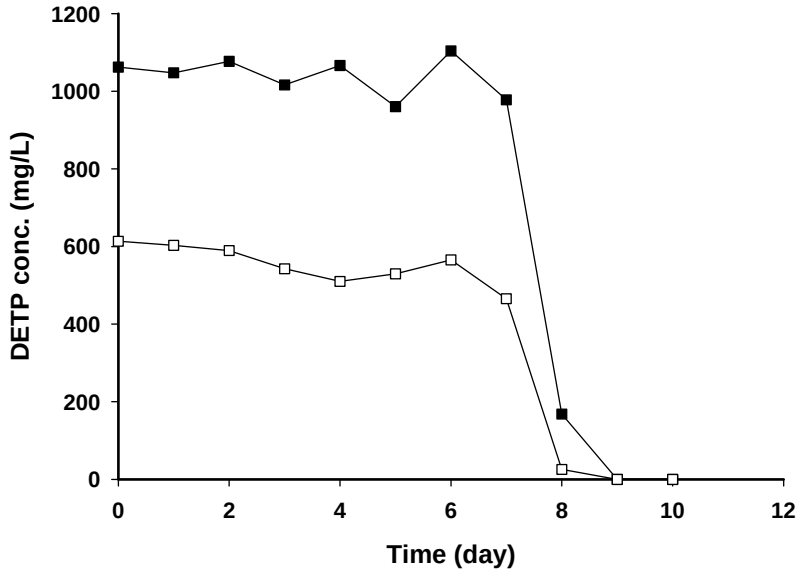


Figure 4-5. DETP degradation without acclimation at different initial concentrations. Reaction conditions: biomass concentration 30 g/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system

during the first 20 hours but increasing to 5.32 mg/g-biomass·h after 20 hours. When biomass acclimated in MSYE medium was resuspended in CHES buffer solution, there was an apparent lag phase lasting approximately 70 hours with an initial DETP concentration of 1,000 mg/L. After the lag phase, the specific reaction rate was 0.87 mg/g-biomass·h (Figure 4-6). When biomass acclimated in MSYE medium was resuspended in MSYE as the reaction medium, there was no lag phase and degradation of 500 mg/L was completed in less than 10 hours (Figure 4-7). Thus, cells required acclimation to both their target compound and the reaction medium. Based on these results, MSYE was selected as both the acclimation and degradation medium.

Effect of Biomass Concentration on DETP Degradation

Specific reaction rates and volumetric reaction rates were evaluated for various biomass (wet weight) concentrations in the degradation reactor. Results are shown in Figure 4-8. The optimum biomass concentration for DETP degradation was found to be 60 g/L, giving the maximum value of both specific and volumetric reaction rates. Both the specific reaction rate and volumetric reaction rate were at their maximum value at this biomass concentration, giving a specific reaction rate of 1.23 mg/g-biomass·h and a volumetric reaction rate of 73.84 mg/L.

OD_{590} was monitored to determine biomass growth during the degradation. No change in OD_{590} was observed during the degradation reaction (See Figure 4-7). This was most likely because only limited amounts of carbon source were available to support

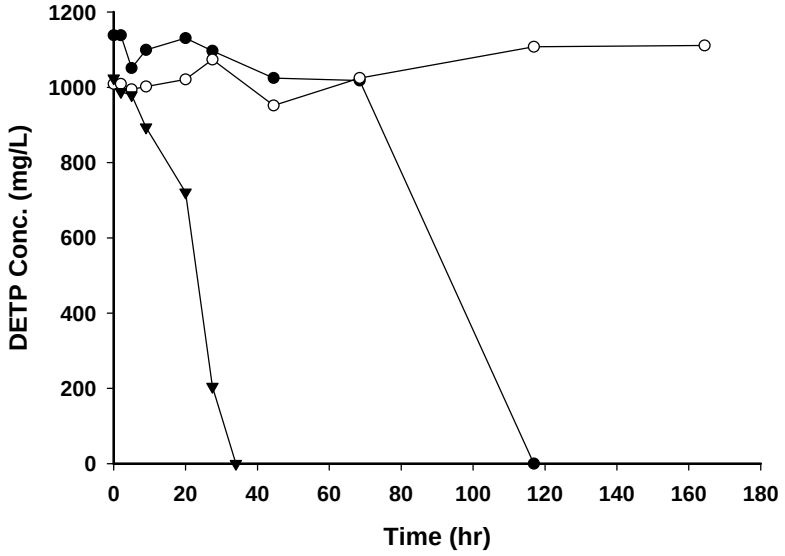


Figure 4-6. DETP degradation after acclimation for various combinations of media. Reaction conditions: biomass concentration 60 g/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. (● acclimation in MSYE and degradation in CHES; ○ acclimation in MSYE and degradation in potassium phosphate buffer solution; ▼ acclimation in CHES and degradation in CHES)

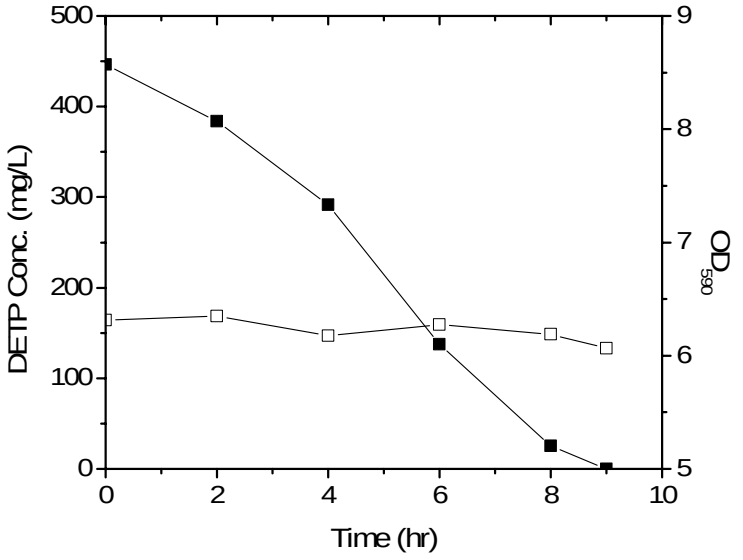


Figure 4-7. DETP degradation and cell concentration profile in MSYE medium using cells acclimated in MSYE medium. Reaction conditions: biomass concentration 60 g/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system (■ DETP concentration; □ OD_{590})

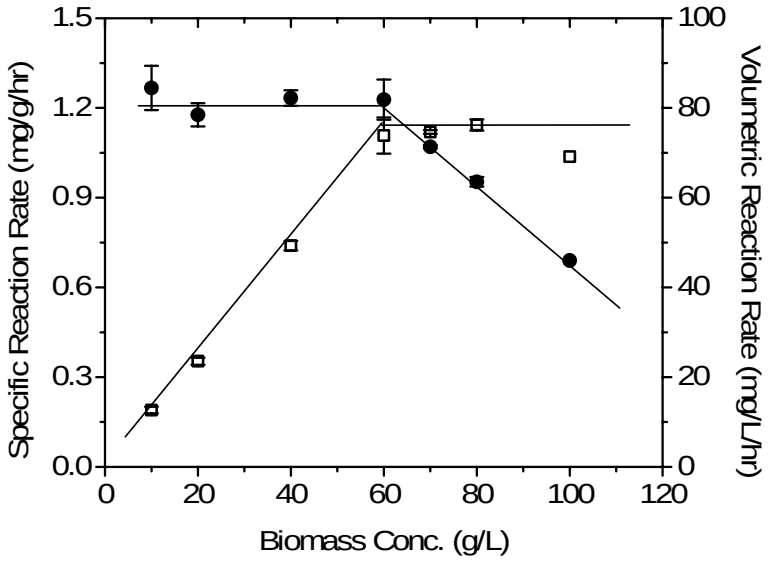


Figure 4-8. Effects of biomass concentration on DETP degradation. Reaction conditions: reaction in MSYE medium with DETP; initial concentration of DETP 500 mg/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. (□ volumetric reaction rate; ● specific reaction rate; mean ± standard error)

cell growth (500 mg/L of DETP and 10 mg/L of yeast extract). At such a low concentration, carbon was probably used for cell maintenance. Based on thermodynamics, assuming all the DETP was used for cell synthesis, cell mass produced was estimated to be only 0.02 g in dry weight. If the water content in wet cell weight is 80 %, the maximum increase in cell concentration is 2 g/L. This small increase would not be noticeable with such a high cell density in the degradation medium. Another possible explanation is cessation of cell growth due to contact inhibition. It has been reported that bacterial cells stop growing in dense suspensions (Pirt, 1975; Rainina et al., 1994).

Effect of pH on DETP Degradation

During DETP degradation, the pH of the reaction medium decreased indicating that acidic products were formed. This is consistent with the pH fluctuation and concomitant increase in sulfate concentration during DETP degradation reported by Shelton (1988). The specific reaction rate for DETP degradation was highest with an initial pH of 7.5 giving a specific reaction rate of 1.23 mg/g-biomass·h. With an initial pH of 8, the rate was only 2 % less (Figure 4-9). Thus, pH 7.5 to 8 was selected as the operating pH range. When the pH was maintained at 7.5 during the reaction, the reaction rate decreased 22 % from the rate with only an initial adjustment to 7.5.

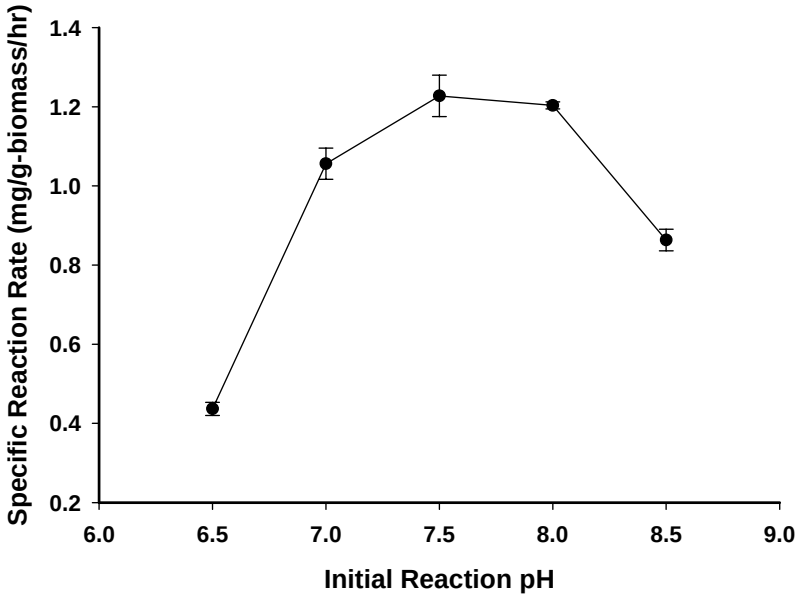


Figure 4-9. Effects of initial reaction pH on DETP degradation. Reaction conditions: reaction in MSYE medium; biomass concentration 60 g/L; initial concentration of DETP 500 mg/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system

Effect of Substrate and Estimation of Kinetic Parameters

The DETP degradation reaction followed simple Michaelis-Menten kinetics (Figure 4-10) described as in equation (4-1):

$$V = \frac{V_m \cdot S}{K_m + S} \tag{4-1}$$

where V_m is the maximum reaction rate and K_m is the substrate half-saturation constant. Kinetics for an enzymatic reaction were selected to describe DETP degradation because no cell growth was observed during the reaction. V_m was estimated as 1.52 ± 0.1 mg/g-biomass·h and K_m was estimated to be 610 ± 106 mg/L.

From coumaphos hydrolysis in the UCD solution (coumaphos concentration approximately 1,500 mg/L), resultant concentrations of chlorferon and DETP are theoretically 870 and 630 mg/L, respectively. DETP degradation followed Michaelis-Menten kinetics up to a concentration of 2,000 mg/L with a K_m value of approximately 610 mg/L. However, chlorferon degradation followed substrate inhibition kinetics with an inhibition constant of 118 mg/L (Chapter III). Thus, degradation of chlorferon would be the governing process for overall coumaphos degradation. It is necessary to either maintain chlorferon at a low concentration for simultaneous degradation of chlorferon and DETP or completely degrade chlorferon before commencing DETP degradation in a separate degradation process.

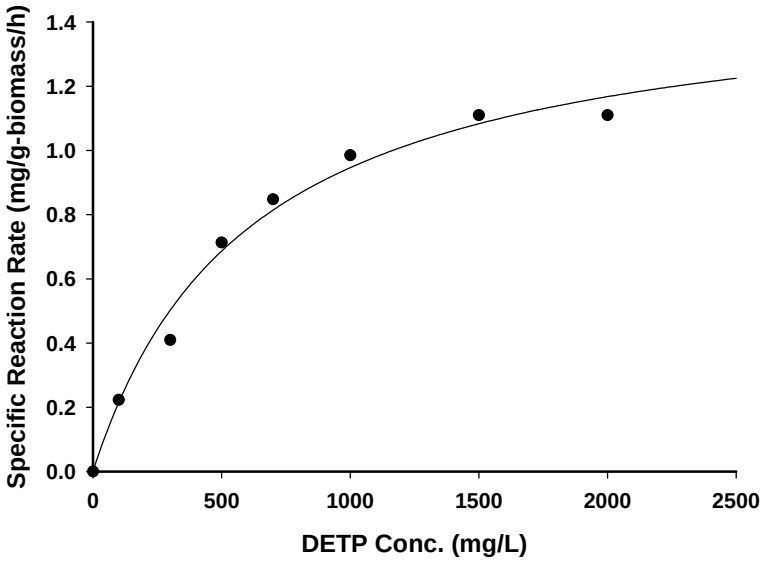


Figure 4-10. Effects of DETP concentration on the degradation. Reaction conditions: reaction in MSYE medium; biomass concentration 60 g/L; initial pH 7.5; temperature 30 °C; agitation speed 200 rpm in a rotary shaking system. Symbol represents experimental data of specific reaction rate. Line represents the best fit of the experimental data using the kinetic models

CHAPTER V
BIODEGRADATION OF COUMAPHOS, CHLORFERON, AND DETP USING CELLS IMMOBILIZED IN CALCIUM-ALGINATE GEL BEADS

INTRODUCTION

In previous chapters, enrichment of microorganisms responsible for degradation of chlorferon and DETP and their use in freely suspended cell systems were described. This chapter describes immobilization of the enriched cultures in Ca-alginate and degradation of chlorferon and DETP by the immobilized cultures.

Immobilization of microorganisms offers several advantages over freely suspended cells in bioreaction systems, including easy separation of the cells from reaction systems, repeated use of cells, and avoidance of cell washout during continuous fermentation at high dilution rates (Willaert and Baron, 1996). Additionally, higher volumetric reaction rates are obtained with immobilized cells as a result of a high local cell concentration (Lee et al., 1994; Lee et al., 1996; Rhee et al., 1996; Manohar et al., 2001; Luong and Tseng, 1984) or altered cell permeability allowing better transfer of substrate into the cell (Manohar et al., 2001; Kim et al, 2002). Immobilized cells have been reported to have enhanced tolerance to toxic compounds (Lee et al., 1996; Dwyer et al., 1986; Bettmann and Rehm, 1984; Keweloh et al., 1989; Westmeier and Rehm, 1985).

Keweloh et al. (1989) suggested that altered cell physiology due to cell-to-cell contact, interactions between cells and immobilizing material, and reduced water activity in microcolonies increased the tolerance of immobilized cells to toxic compounds.

Calcium-alginate cross-linking is one of the most commonly used immobilization methods because the immobilization procedure is simple, relatively mild and does not have toxic effects on the cells, although this natural polysaccharide material is susceptible to biodegradation and has low mechanical stability (Leenen et al., 1996a). Calcium-alginate gels are sensitive to chelating agents. They can be rapidly dissolved by polyphosphates (2 %), citric acid (0.1M) and EDTA (0.1M), releasing the immobilized cells (Rosevear et al., 1987; Leenen et al., 1996b; Ting and Sun, 2000; Paul and Vignais, 1980). This makes the Ca-alginate method unsuitable for most field scale applications in wastewater treatment. However, alginate can be strengthened by mixing with other polymers, such as polyvinyl alcohol (PVA). Vogelsang et al. (1997) mixed alginate with PVA-SbQ and successfully utilized the gel for domestic wastewater treatment over a two-year period. Quan et al. (2003) also used PVA to strengthen alginate.

Coumaphos degradation was previously investigated by Kim (1998) using recombinant *E. coli* as freely suspended cells and immobilized in PVA cryogel. The degradation rate with immobilized *E.coli* was twice that of free cells using waste cattle dip solution as substrate. The same recombinant *E. coli* strain was immobilized in Ca-alginate in the present study to compare with the previous method of immobilization.

MATERIALS AND METHODS

Cattle Dip Vat Solution and Chemicals.

Untreated cattle dip (UCD) waste solution was obtained from the APHIS dipping vats in Laredo, TX. Analytical grade chlorferon and coumaphos were gifts from Bayer Corporation (Animal Health Division, Merriam, KS). Chlorferon and coumaphos are highly insoluble in water, and stock solutions were prepared by dissolving in methanol to concentrations of 5 g/L and 10 g/L, respectively. Chlorferon solution for degradation studies was also produced by hydrolyzing coumaphos in UCD solution using genetically engineered *E.coli* containing the organophosphate hydrolase (OPH) enzyme. Hydrolysis of coumaphos was conducted at the optimum conditions found by Kim (1998). OPH^{+} *E.coli* was immobilized in Ca-alginate beads to simplify removal from the solution after hydrolysis. The potassium salt of DETP (DETP-K) was obtained from Sigma-Aldrich (St. Louis, MO) and used as a standard for DETP. All other chemicals, including sodium alginate (65 – 70 % guluronic acid and 25 – 35 % mannuronic acid), were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO).

Sample Analysis Method

A HPLC system (Waters, model 2690, Milford, MA) equipped with a PDA detector (Waters, model 996, Milford, MA), an autosampler, and a guard column (C-18,

4.5 mm × 10 mm, Burdick and Jackson, Muskegon, MI) was used for analysis. Coumaphos and chlorferon were analyzed using a C-18 column (4.5 mm × 250 mm, Burdick and Jackson, Muskegon, MI), and a C-8 column (4.5 mm × 250 mm, Burdick and Jackson, Muskegon, MI) was used for DETP analysis. The mobile phase was HPLC grade acetonitrile with 0.75 mM phosphoric acid (80:20 by volume) at a flow rate of 1 ml/min. Absorbance was measured at 320 nm for coumaphos and chlorferon and 210 nm for DETP. During the experiments, 0.2 ml samples were collected in 1.5 ml polypropylene micro test tubes and diluted with methanol to achieve appropriate concentrations for HPLC analysis. Tubes were vortexed and centrifuged at 1,163 × g for 15 min using a microcentrifuge, and the supernatants were filtered using disposable syringe filters (Acrodisk CR13 mm, PTFE, 0.2 μm, Pall Corporation, East Hills, NY). Filtered samples were stored in the refrigerator until analyzed.

Microorganisms and Cultivation

Microorganisms for Degradation of Chlorferon and DETP

Bacterial consortia responsible for degrading either chlorferon or DETP were enriched from UCD solution, as described in Chapters III and IV, and the enriched culture solutions served as inocula to produce biomass. A two-step growth procedure was developed: the first step to increase biomass and the second to acclimate cells to the target compound and reaction medium. In the first step, cells were grown in glutamate-

yeast extract medium (glutamate 10 g/L, yeast extract 5 g/L, 50 % glycerol 8 ml/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.4 g/L, K_2HPO_4 1.3 g/L, KH_2PO_4 0.4 g/L, pH 7.4) containing the target compound in a rotary shaking incubator (200 rpm) at 30 °C. Biomass was harvested during the late exponential phase by centrifugation at 4,500 × g at 4 °C for 15 min. The pellet obtained from centrifugation was washed with potassium phosphate buffer (50 mM, pH 7.2) and centrifuged again at the same conditions. The resulting pellet was resuspended in reaction medium containing the target compound, 10 mg/L of chlorferon or 100 mg/L of DETP, for acclimation and incubated in a rotary shaking incubator (200 rpm) at 30 °C until the target compound was completely degraded. After complete degradation, biomass was harvested again as described above and immobilized for degradation studies. For all experiments, freshly harvested cells were used.

OPH$^+$ E. coli *for Coumaphos Hydrolysis*

The recombinant *E. coli* strain used in this study has been previously described by Dave et al. (1993). Standard recombinant DNA techniques were employed in the construction of plasmid and phage vectors of *E. coli* (Maniatis et al., 1982). A BamH1 restriction fragment containing a deletion of the 2-29 *opd* open reading frame with an altered Shine-Delgarno sequence was introduced into pUC19 (Miller, 1992). The resulting *opd* gene was transformed into *E. coli* strain DH5α [*supE44, δlacU169(ϕ80 lacZδM15), hsdR17, recA1, endA1, gryA96, thi-1, relA1*], which was made competent for

transformation by the $CaCl_2$ method (Sambrook et al., 1989). The recombinant *E. coli* strain containing OPH enzyme was obtained from Dr. James R. Wild, Department of Biochemistry and Biophysics, Texas A&M University.

To produce biomass for coumaphos degradation, an isolated colony of OPH^+ *E. coli* was cultured overnight in a 5 ml tube of LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.0) supplemented with 50 mg/L ampicillin. The cultured solution was transferred to 1 L of TB medium (Terrific Broth: tryptone 12 g/L, yeast extract 24 g/L, and 50 % glycerol 8 ml in 900 ml DI water; KH_2PO_4 2.31 g and K_2HPO_4 12.54 g in 100 ml DI water; autoclave each solution for 20 minutes, cool down to room temperature then mix together) supplemented with 50 mg/L ampicillin and 1 mM $CoCl_2$. The culture was incubated for 37 hours in a rotary shaking incubator (200 rpm) at 30 °C. Additional ampicillin (50 mg/L) was added into the growth at 12 hours. Biomass was washed and harvested as described above. Freshly harvested cells were used for all experiments.

Cell Immobilization Method

Biomass for immobilization was prepared separately for each target compound as described above and acclimated before immobilization. The harvested biomass was resuspended in 2 % sodium alginate solution to a final cell concentration of 80 g/L for chlorferon-degrading organisms, 60 g/L for DETP-degrading organisms and 70 g/L for OPH^+ *E. coli*. Sodium alginate solution was prepared in sterilized DI water. The

alginate-cell suspensions were then added dropwise to well-stirred, sterilized 2 % $CaCl_2$ solutions using a syringe. Calcium-alginate gel beads having a spherical shape were formed instantly. Bead size was approximately 0.165 – 0.17 cm in radius. Beads were left in $CaCl_2$ solution with gentle stirring for 4 hours to allow them to harden. They were then washed with DI water. The procedure is summarized in Figure 5-1. Before use for degradation studies, bead surfaces were blotted dry with paper towels.

Reaction System

Degradation studies using immobilized cells were performed in a 1.25 L BioFlo II fermentor system (New Brunswick Scientific Co., Inc., Edison, NJ) with a working volume of 0.3 L. Temperature was maintained at 30 ºC and reaction pH was adjusted to the optimum determined for freely suspended cells. The reaction medium was agitated at 200 rpm by a flat bladed turbine impeller (radius of 3.25 cm). Aeration was not provided to the system.

Degradation of Chlorferon and DETP Using Immobilized Cells

Experiments were conducted to determine optimum bead loading using loadings of 10, 20, 30, 40, and 50 % (wt beads/total vol.). Bead loading greater than 50 % could not be evaluated because higher concentrations were not completely suspended in the liquid medium.

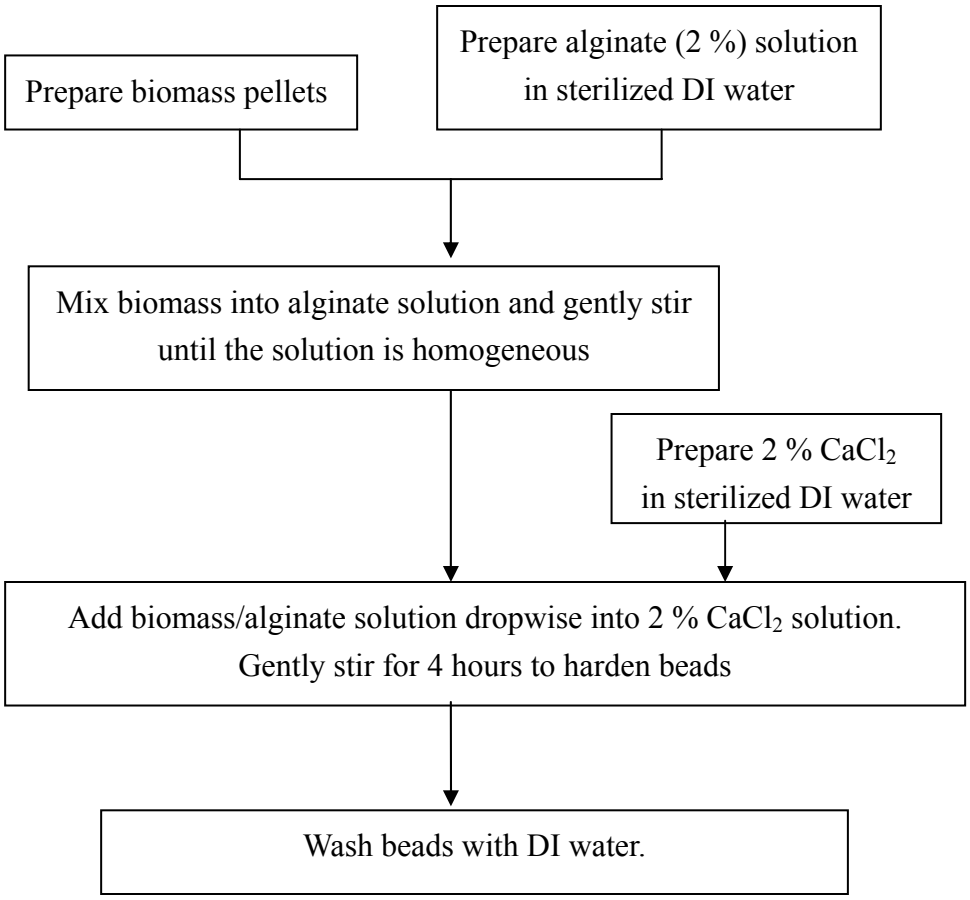


Figure 5-1. Procedure for cell immobilization in Ca-alginate beads

The reaction medium for chlorferon degradation was hydrolyzed UCD solution diluted with MS medium to provide a final chlorferon concentration of 50 mg/L. For DETP degradation studies, MSYE medium with DETP-K at an initial concentration of 250 mg/L was used. The initial pH was adjusted to 7.5 for both media. Cell concentrations in the beads were 80 g/L for chlorferon-degrading organisms and 60 g/L for DETP-degrading organisms.

Hydrolysis of Coumaphos Using Immobilized Cells

Coumaphos hydrolysis studies using Ca-alginate immobilized cells were conducted to compare with the PVA cryogel method used by Kim (1998). Experiments using freely suspended cells were performed under batch reactor conditions in 250 ml Erlenmeyer flasks with a working volume of 50 ml. Experiments were operated at optimum conditions found by Kim (1998). Biomass concentration was 70 g/L and initial pH was adjusted to 8. Temperature was controlled at 30 ºC. The reaction medium was either UCD solution or pure coumaphos dissolved in methanol added to CHES buffer solution (100 mM, pH 8). Initial coumaphos concentrations for experiments in pure solution and UCD solution were 500 mg/L and 1,800 mg/L, respectively. Experiments for degradation of coumaphos using immobilized cells were conducted in the BioFlo II fermentor system described above. Cell concentration in the beads was 70 g/L, and 20 % bead loading (wt beads/total vol.) was used. All other conditions were the same as for free cells.

Estimation of Initial Cell Concentration in the Immobilized Cell System

The experimentally determined optimum cell concentrations in freely suspended cell systems were used as the concentrations of cells within the Ca-alginate gel beads. Initial cell concentrations in the immobilized cell reactor were estimated using the following equation:

$$X_i = \frac{L_p \times M_p}{V_w} \tag{5-1}$$

where X_i = initial cell concentration in immobilized cell system

M_p = mass of total beads in working volume (g)

V_w = total working volume (L)

L_p = initial cell concentration per g-beads (g-biomass/g-bead)

The initial cell concentration per g-bead was estimated using the following equation:

$$L_p = \frac{X_f \cdot \varepsilon_{pe} \cdot V_p}{m_{pe} + X_f \cdot \varepsilon_{pe} \cdot V_p} \tag{5-2}$$

where X_f = optimum cell concentration for freely suspended cells (g/L)

ε_{pe} = porosity of cell-free beads

V_p = bead volume (L)

m_{pe} = mass of cell-free beads (g)

RESULTS AND DISCUSSION

Degradation of Chlorferon Using Immobilized Cells

Optimum Bead Loading

The optimum bead loading for chlorferon degradation was determined to be 20 % (200 g-beads/L) (Figure. 5-2). The volumetric reaction rate was the highest at a bead loading of 30 %. However, a bead loading of 20 % was selected because the specific reaction rate at 30 % bead loading was significantly lower than at 20 % bead loading.

Comparison of Immobilized Cells and Free Cells

The volumetric reaction rate for chlorferon degradation at the optimum bead loading was 2.45 mg/L·h. At a 20 % bead loading, the biomass concentration of immobilized chlorferon-degrading organisms was 15.06 g-biomass/L in the total working volume. From studies using freely suspended cells (Chapter III), the volumetric reaction rate of chlorferon increased linearly with increasing biomass concentration up to the optimum concentration (80 g/L). The volumetric reaction rate for a biomass concentration of 15.06 g/L was estimated to be 0.49 mg/L·h (Figure 3-8) indicating that

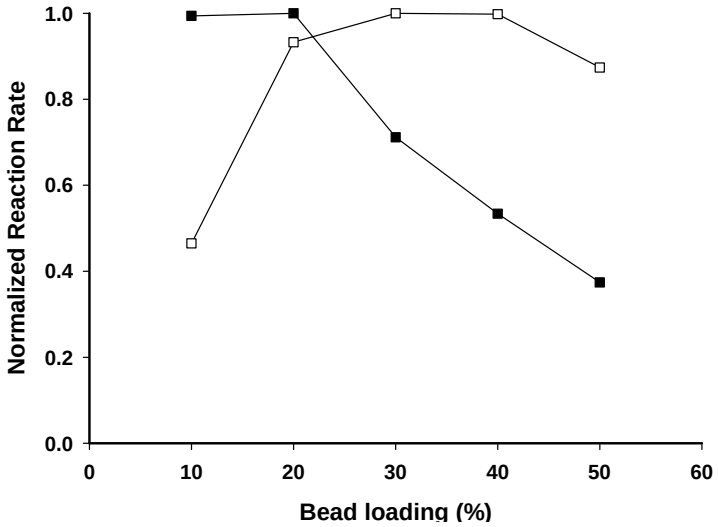


Figure 5-2. Effects of bead loading on chlorferon degradation in terms of normalized reaction rates. Reaction conditions: reaction in hydrolyzed UCD solution + MS medium; initial chlorferon concentration of 50 mg/L; initial pH 7.5; cell concentration in the bead 80 g/L (■ normalized specific reaction rate, □ normalized volumetric reaction rate)

the volumetric reaction rate of chlorferon degradation was almost five times higher with immobilized cells (Figure 5-3).

Similar results were found in comparing specific reaction rates of free cells and immobilized cells. The beads were prepared using the optimum concentration determined for the freely suspended cells as the concentration within the beads. Comparing specific reaction rates of freely suspended cells at the optimum cell concentration and immobilized cells showed that cell performance was better in the immobilized cell system (Figure 5-3). The specific reaction rate with freely suspended cells was 0.034 mg/g-biomass·h, which was only 20 % of that with immobilized cells, 0.163 mg/g-biomass·h.

Degradation of DETP Using Immobilized Cells

Optimum Bead Loading

The optimum bead loading for DETP degradation was determined to be 30 % (300 g-beads/L) based on the volumetric reaction rate (Figure 5-4). The specific reaction rate was decreasing but the volumetric reaction rate was increasing up to a bead loading 30 % then remained constant for higher loadings.

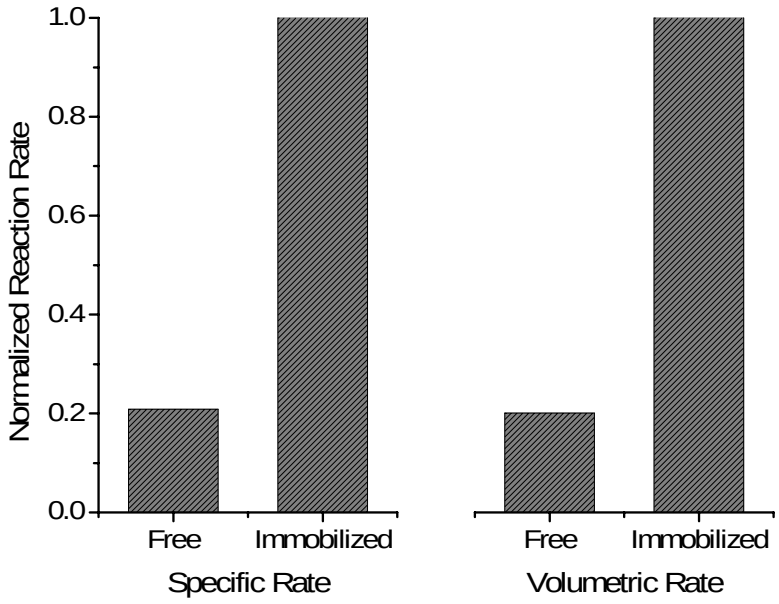


Figure 5-3. Comparison of chlorferon degradation rate by freely suspended cells and immobilized cells in terms of the normalized reaction rate. Reaction conditions: reaction in hydrolyzed UCD solution + MS medium; initial chlorferon concentration 50 mg/L; bead loading of 20 % in the immobilized cell system; cell concentration of 80 g/L in the beads and in the freely suspended cell system

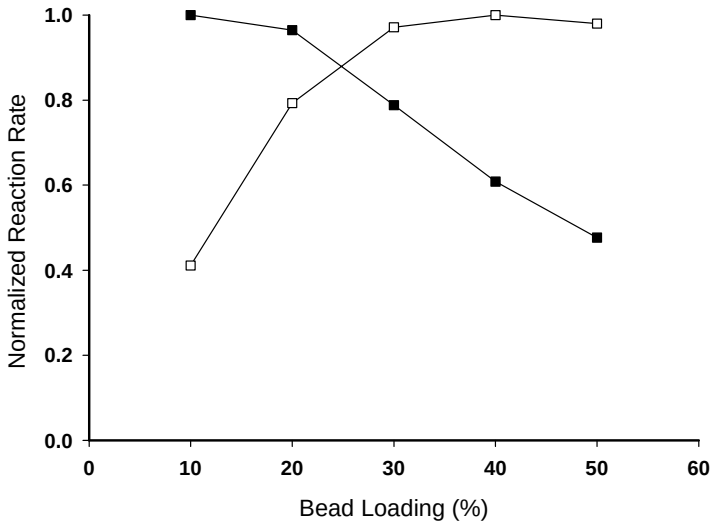


Figure 5-4. Effects of bead loading on DETP degradation in terms of normalized reaction rates. Reaction conditions: reaction in MSYE medium; initial DETP concentration of 250 mg/L; initial pH 7.5; cell concentration in the bead 60 g/L (■ normalized specific reaction rate, □ normalized volumetric reaction rate)

Comparison of Immobilized Cells and Free Cells

The concentration of DETP-degrading organisms in the total working volume was estimated as 15.71 g-biomass/L at the optimum bead loading (30 %). Studies using freely suspended cells showed that the volumetric reaction rate of DETP degradation increased linearly with increasing biomass concentration up to the optimum concentration of 60 g/L. The volumetric reaction rate for a biomass concentration of 15.71 g/L was estimated to be 19.29 mg/L·h (Figure 4-8). The volumetric reaction rate for DETP degradation at 30 % bead loading was 22.65 mg/L·h (Figure 5-5) which is slightly higher.

Comparing specific reaction rates of freely suspended cells at the optimum cell concentration (60 g/L) and immobilized cells showed the same result. The specific reaction rate for freely suspended cells was 1.23 mg/g-biomass·h, which was 85 % of that for immobilized cells at 30 % bead loading, 1.44 mg/g-biomass·h (Figure 5-5).

Hydrolysis of Coumaphos Using Free Cells and Immobilized Cells

In previous research, hydrolysis of coumaphos by OPH^+ *E.coli* cells in free suspension and immobilized in PVA cryogel was investigated (Kim, 1998; Kim et al., 2002). In this research, similar studies were conducted to evaluate effects of immobilization of OPH^+ *E.coli* cells in Ca-alginate gels on coumaphos hydrolysis.

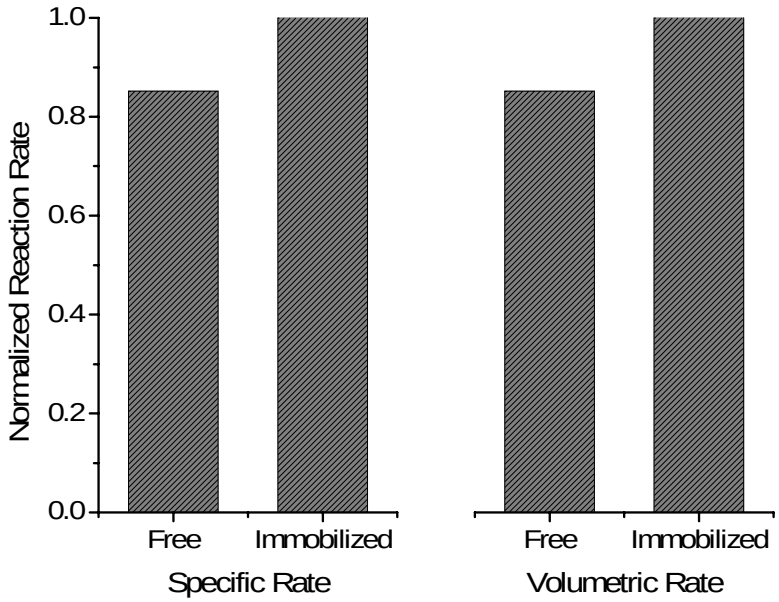


Figure 5-5. Comparison of DETP degradation rate by freely suspended cells and immobilized cells in terms of the normalized reaction rate. Reaction conditions: reaction in MSYE medium; initial DETP concentration of 250 mg/L; bead loading of 30 % in the immobilized cell system; cell concentration of 60 g/L in the beads and in the freely suspended cell system

Figure 5-6 shows the experimental results of coumaphos hydrolysis using free cells and immobilized cells with pure coumaphos solution and UCD solution as substrates. With pure coumaphos solution as substrate, the specific reaction rate with freely suspended cells (1.85 mg/g-biomass·h) was 90 % of the rate with immobilized cells (2.06 mg/g-biomass·h). However, the specific reaction rate for free cells with UCD solution as substrate was only 1.24 mg/g-biomass·h or 67 % of the rate with pure coumaphos; whereas, the rate for immobilized cells with UCD was 3.16 mg/g-biomass·h or 35 % greater than with pure coumaphos. With UCD substrate, the rate for free cells was only approximately 40 % of that for immobilized cells. These results agree with those for the studies of chlorferon and DETP degradation. Chlorferon degradation studies were conducted in diluted UCD solution and showed five times higher degradation rate with immobilized cells than with free cells (Figure 5-3). DETP degradation studies conducted in pure solution showed only a 15 % difference in degradation rate between immobilized cells and free cells (Figure 5-5). Moreover, the results obtained in this study agree with the previous research for coumaphos hydolysis by Kim et al. (2002). With UCD solution as substrate, the hydrolysis rate obtained by Kim et al. (2002) with OPH^+ *E.coli* immobilized in PVA cryogel was twice that of freely suspended cells. In this study, the coumaphos hydrolysis rate using OPH^+ *E.coli* immobilized in Ca-alginate with UCD solution as substrate was approximately 2.5 times higher than with free cells.

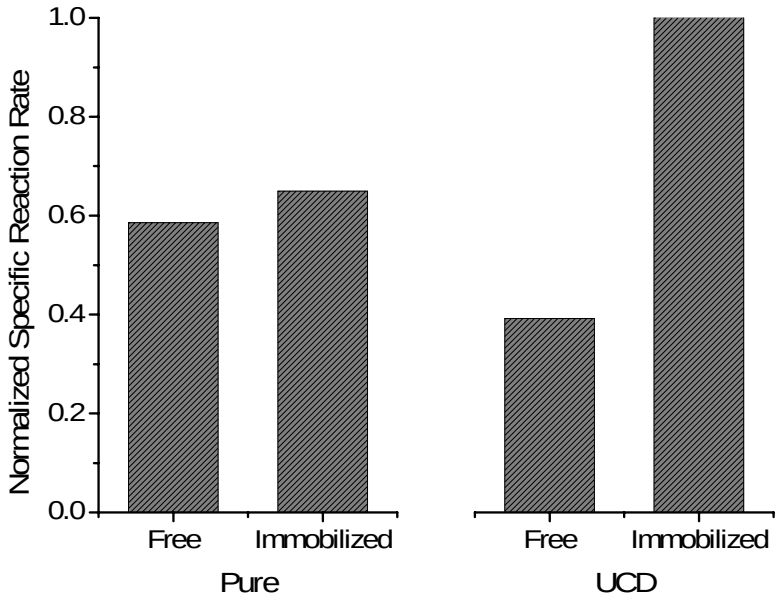


Figure 5-6. Normalized specific reaction rates of coumaphos hydrolysis by freely suspended cells and immobilized cells in pure coumaphos solution and UCD solution. Reaction conditions: initial coumaphos concentration of 500 mg/L in pure coumaphos solution and 1,800 mg/L in UCD solution; bead loading of 20 % in the immobilized cell system; cell concentration of 70 g/L in the beads and in the freely suspended cell system

Although the same OPH^+ *E. coli* strain and the optimum conditions determined by Kim et al. (2002) were used in this study, degradation rates obtained in this study were much lower. The specific reaction rate in UCD solution at an initial coumaphos concentration of 1800 mg/L with freely suspended cells reported by Kim et al. (2002) was approximately 7.18 mg/g-biomass·h, almost six times higher than that obtained in this study (1.24 mg/g-biomass·h). Similar results were found for immobilized cells. The specific reaction rate in UCD solution with immobilized cells reported by Kim et al. (2002) was 14.25 mg/g-biomass·h, approximately five times higher than that obtained in this study (3.16 mg/g-biomass·h). Different batches of UCD solution were used for the two sets of experiments, so different contaminants were likely present in the UCD solutions. However, the rate difference is higher when using pure coumaphos as substrate. The specific reaction rate in pure coumaphos solution with free cells reported by Kim et al. (2002) was 14.57 mg/g-biomass·h, which is almost eight times higher than that obtained in this study (1.85 mg/g-biomass·h). Although the coumaphos concentration at which the rate was obtained was not specified, it was assumed to be 500 mg/L which is similar to the concentration used in this study. It appears that the OPH^+ *E. coli* cells used in the current work had lower activity, possibly as the result of instability in the recombinant genes. In addition, there may have been some differences in experimental technique and conditions.

However, a slower reaction rate for coumaphos hydrolysis might be a benefit to an overall degradation process. Since chlorferon has an inhibitory effect on both chlorferon-degrading organisms and DETP-degrading organisms, slower release of

chlorferon could help control its concentration below the inhibitory level. The maximum chlorferon degradation rate was 0.034 mg/g-biomass·h at concentration of 50 mg/L. Thus, to match the coumaphos hydrolysis rate with the chlorferon degradation rate, the hydrolysis rate should be maintained even lower than that obtained in this study.

Enhanced Degradation with Immobilized Cells

Enhanced volumetric reaction rates for immobilized cells have been reported for many studies, mostly attributed to high local cell concentrations in immobilized cell systems (Lee et al., 1994; Lee et al., 1996; Rhee et al., 1996; Manohar et al., 2001; Luong and Tseng, 1984). However, in this study, volumetric reaction rates of free and immobilized cells were compared at the same cell concentration. The possibility of partition effects was ruled out because the partition coefficients for both chlorferon and DETP into Ca-alginate beads were close to one which will be discussed in Chapter VI.

A possible explanation for enhanced rates with immobilized cells is protection of the cells from unknown inhibitory factors present in UCD solution. Kim et al. (2002) reported that coumaphos degradation was enhanced by cells immobilized in PVA cryogels either because the cells were protected from inhibitory factors present in the UCD solution or because of increased cell permeability resulting from cryoimmobilization. The presence of inhibitory factors in UCD solution was the most likely reason because in pure coumaphos solution the same degradation rate was observed with both freely suspended cells and immobilized cells, while in UCD solution,

the degradation rate with immobilized cells was twice that of freely suspended cells, which was confirmed in the current study. The rate with cells immobilized in Ca-alginate was approximately 2.5 times higher than with free cells. Chlorferon degradation in hydrolyzed UCD solution mixed with MS medium was five times higher with immobilized cells than with free cells. Therefore, the same conclusion could be drawn in this study that cells inside the beads were protected from inhibitory factors present in the UCD solution. It has been reported that immobilizing material and the external cells of microcolonies in the gel bead act as a barrier to protect internal cells (Dwyer et al., 1986; Bettmann and Rehm, 1984; Keweloh et al., 1989).

The slightly higher degradation rates of both coumaphos and DETP with pure substrate solutions suggest that other factors such as alteration of cell physiology and increased cell permeability may also be affecting the immobilized cells used in this study. Physiological alteration upon Ca-alginate immobilization resulting in an enhanced reaction rate has been reported in the literature. Smith et al. (1993) found that calcium was responsible for physiological changes in cells which enhanced production of 1,2-epoxypropane from propene by *Mycobacterium* sp. E3. Vieira et al. (1990) reported that calcium stimulated alcoholic fermentation by yeast cells. Brito et al. (1990b) found a similar calcium effect on exopolysaccharide production by *Pseudomonas elodea*. Keweloh et al. (1991) and Diefenbach et al. (1992) suggested that lipid impurities in the commercial alginate were responsible for the physiological changes of cells and their enhanced tolerance to toxic compounds. *E.coli* cells immobilized in Ca-alginate showed increased tolerance to phenol by uptake of saturated fatty acids from impurities in the

alginate and incorporating them into cell membrane lipids (Keweloh et al., 1991). Santos-Rosa and Galván (1989) reported that Ca-alginate immobilization increased cell membrane permeability as a consequence of cell-matrix interactions.

Although conclusive explanations for increased rates with immobilized cells were not developed in this work, it is clear that immobilization did enhance degradation of all three compounds studied. In addition, it does not appear that Ca-alginate immobilization caused any undesirable changes in the cells.

Repeated Operations of Immobilized Cells

One important advantage of immobilized cell systems is extended use of the biocatalyst for repeated batches. However, based on the preliminary studies, Ca-alginate beads started weakening within three days in UCD solution and completely dissolved in two weeks. The beads used for studies of DETP degradation or chlorferon degradation were stable over a longer period than in UCD solution because those studies were conducted in MSYE medium or UCD solution diluted with MS medium.

It is well known that a Ca-alginate gel is sensitive to chelating agents, and it can be rapidly dissolved by polyphosphates, citric acid or EDTA (Rosevear et al., 1987; Leenen et al., 1996b; Ting and Sun, 2000; Paul and Vignais, 1980). The stability of Ca-alginate gels may also be reduced by competition with non-gelling ions such as Na^+ (Vogelsang and Østgaard 1996). UCD solution contained approximately 5000 mg/L of TP-PO_4^{3-} and 450 mg/L of Na^+. Attempts were made to strengthen the alginate beads by

mixing with polyvinyl alcohol (PVA) or using Ba^{2+} instead of Ca^{2+} as an alternative gel hardening ion. However, none of the modifications improved stability in UCD solution.

Experiments conducted for repeated operations of immobilized cells showed increasing degradation rates with successive batches (Figures 5-7 and 5-8). DETP degradation using Ca-alginate immobilized cells was repeated for five consecutive batches in MSYE medium. The reaction medium was completely replaced for each batch. For chlorferon degradation, three consecutive batches were repeated in UCD solution diluted with MS medium with complete replacement of reaction medium for each batch. At completion of the third batch, beads started weakening so no further batches could be run. The increase in degradation rates for successive batches indicates that cells became better adapted to the reaction conditions as batches were repeated. In both cases, there was a short initial lag period for the first batch which did not occur with later batches. Swollen beads were not observed after completion of the fifth batch for DETP degradation and third batch for chlorferon degradation, indicating that cell growth in the gel beads was minimal. It is not likely cell growth could have occurred since none was observed with freely suspended cells. Since the reaction medium was completely replaced at the completion of each batch, growth of released cells was not likely either. Although no clear explanation can be made for this without further studies, increased cell membrane permeability caused by Ca-alginate immobilization (Santos-Rosa and Galván, 1989) or increased enzyme production might be other possibilities.

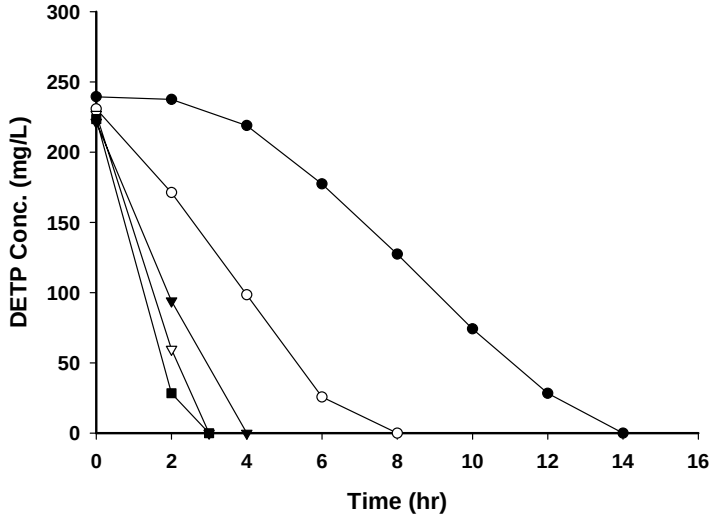


Figure 5-7. Repeated use of Ca-alginate immobilized cells for DETP degradation. Reaction conditions: reaction in MSYE medium; bead loading 30 %; cell concentration 60 g/L in the bead. (● 1st batch; ○ 2nd batch; ▼ 3rd batch; ∇ 4th batch; ■ 5th batch)

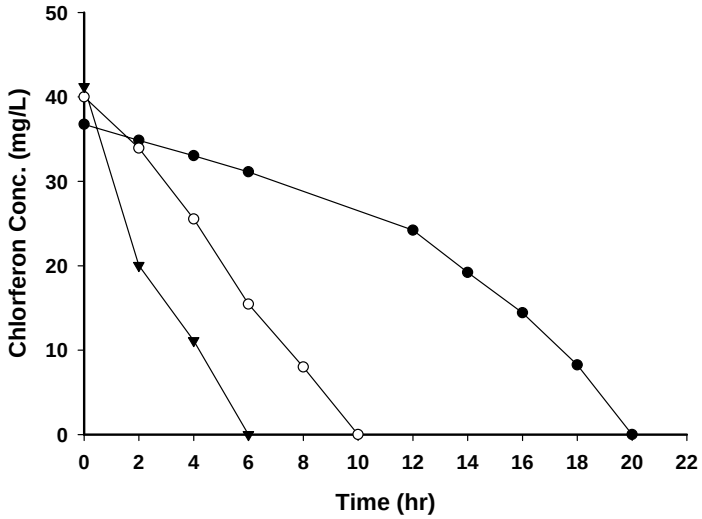


Figure 5-8. Repeated use of Ca-alginate immobilized cells for chlorferon degradation. Reaction conditions: reaction in UCD + MS medium; bead loading 20 %; cell concentration 80 g/L in the bead. (● 1st batch; ○ 2nd batch; ▼ 3rd batch)

CHAPTER VI

DETERMINATION OF DIFFUSION COEFFICIENTS AND DIFFUSION CHARACTERISTICS OF CHLORFERON AND DETP IN CALCIUM-ALGINATE GEL BEADS

INTRODUCTION

Cell immobilization techniques have been successfully applied for numerous biotechnological processes because of advantages, such as easy separation of cells from the reaction system, repeated use of immobilized cells, and better protection of cells from harsh environments. However, mass transfer is one of the major factors affecting activity of immobilized cells since nutrients and products have to be transported through external boundary layers (external mass transfer) and within the matrix (internal mass transfer) (Merchant et al., 1987; De Backer et al., 1996; Jovetic et al., 2001). Thus, the effectiveness of immobilized cell systems can be reduced by mass transfer limitations (Mavituna, 1986). With proper design of the immobilized cell reactor configuration and flow pattern, external mass transfer resistance can be made negligible (Willaert et al., 1996). Simply increasing the relative velocity of the gel particle to the bulk solution (Grunwald et al, 1997) or decreasing the particle size (Doran, 1999; Mavituna, 1986) reduces external mass transfer resistance. However, internal mass transfer resistance, which is affected by system design parameters such as particle size, cell loading, flow,

substrate concentration and reactor type, generally is not negligible (Willaert et al., 1996). Studies of internal mass transfer are essential for better understanding, operation, and modeling of immobilized cell systems.

The bulk diffusivity in water (D_{aq}) has been used in some cases to model mass transfer in cell-containing particles due to lack of information about effective diffusivity (Papathanasiou et al., 1988). However, diffusion through the immobilization matrix cannot be characterized by the bulk diffusivity because the pore space is only a fraction (ε) of the total volume, which is referred to as the exclusion effect, and the impermeable segments of polymer molecules increase the path length for a diffusing substrate, which is referred to as the obstruction effect (Muhr and Blanshard, 1982). The effective diffusivity is a single parameter incorporating all these effects (Blanch and Clark, 1996). Information about effective diffusivity is essential for describing the kinetics of immobilized cell reactions (Merchant et al., 1987).

Several experimental methods for measuring diffusion coefficients have been reported in the literature. For diffusion in gels, only the substrate concentration in the bulk solution can be measured effectively. Crank's equation (equation (2-10), Crank, 1975), which describes the change of substrate concentration in the bulk solution with time, has been used to calculate diffusion coefficients by fitting the equation to experimental data (Yankov, 2004; Dembczynski and Jankowski, 2000; Jovetic et al., 2001; Arnaud and Lacroix, 1991; Merchant et al., 1987; Tanaka et al., 1984; De Backer and Baron, 1993; Papathanasiou and Bijeljic, 1998). However, since diffusion in gels is affected by many factors, such as the concentrations of immobilized cells, diffusing

substrate, and polymer and crosslinking ions, diffusion coefficients reported in the literature have high variability even for the same substrate.

In this chapter, diffusion characteristics of chlorferon and DETP in Ca-alginate gel beads were determined. In addition, effects of agitation speed of the bulk solution, the concentration of diffusing substrate, and the concentration of cells immobilized in the beads were investigated.

THEORETICAL BACKGROUND

A method to determine the diffusion coefficient of substrate in a spherical porous particle was reported by Crank (1975). A spherical bead free of substrate is suspended in a well-stirred solution having a uniform initial concentration of substrate ($S_{b,0}$). The bead is allowed to equilibrate and the substrate concentration in the bulk solution is measured. The diffusion coefficient is estimated from the change in substrate concentration in the bulk solution using the following assumptions:

1. The structure inside the bead is homogeneous.
2. Fickian diffusion is the only mechanism of substrate transport within the bead; there is no convection inside the bead.
3. The diffusion coefficient in the bead is constant.
4. The concentration of substrate in the bulk solution is uniform.
5. External mass transfer resistance is negligible, thus the substrate concentration at the bead surface ($r_p = R$) is equal to the substrate concentration in the bulk liquid.

If diffusion is only in the radial direction in a spherical bead, the diffusion equation for a constant diffusion coefficient (D) is

$$\frac{\partial S_p}{\partial t} = D\left(\frac{\partial^2 S_p}{\partial r_p} + \frac{2}{r_p}\frac{\partial S_p}{\partial r_p}\right) \tag{2-9}$$

The concentration of substrate within the sphere (S_p) at time t is given by Crank (1975) as:

$$S_p(t) = S_{p,\infty}\left[1 + 6(1+\alpha)\cdot\sum_{n=1}^{\infty}\frac{\exp(-\frac{D\cdot q_n^2\cdot t}{R^2})}{9+9\alpha+q_n^2\alpha^2}\frac{R}{r_p}\frac{\sin\left(\frac{q_n r_p}{R}\right)}{\sin q_n}\right] \tag{2-10}$$

where $S_{p,\infty}$ is the substrate concentration in the bead after infinite time and q_n are the positive non-zero roots of following equation:

$$\tan(q_n) = \frac{3q_n}{3+\alpha\cdot q_n} \tag{2-11}$$

The value of α is the ratio of the bulk liquid volume (V_b) to the bead volume (V_p):

$$\alpha = \frac{V_b}{V_p} \tag{6-1}$$

Originally, equation (2-10) was derived for an isolated sphere; however, it has been shown that it can be applied to multiple spheres (Nguyen and Luong, 1986; De Backer and Baron, 1993; Arnaud and Lacroix, 1991). For a system containing multiple spheres, V_p is the total bead volume. In addition, the partition coefficient (K_p) for substrate between the gel beads and bulk solution must be considered. Taking all these factors into account, α is defined for multiple spheres as follows:

$$\alpha = \frac{V_b}{V_p \cdot K_p} \tag{2-12}$$

In equation (2-10), $S_{p,\infty}$ can be determined by mass balance:

$$S_{p,\infty} \cdot V_p + S_{b,\infty} \cdot V_b = S_{p,0} \cdot V_p + S_{b,0} \cdot V_b \tag{6-2}$$

where V_p = total bead volume

V_b = volume of the bulk solution excluding bead volume

S_b = substrate concentration in the bulk solution

Initially, gel beads are free of substrate ($S_{p,0}$ = 0). Using the definition of the partition coefficient, equation (6-2) can be rearranged as follows:

$$S_{p,\infty} = S_{b,0} \times \frac{V_b}{V_p + \frac{V_b}{K_p}} = \frac{S_{b,0} \cdot V_b \cdot K_p}{V_p \cdot K_p + V_b} = S_{b,0} \cdot K_p \cdot \frac{\alpha}{1+\alpha} \tag{6-3}$$

Thus, equation (2-10) can be rewritten as follows:

$$S_p(t) = \frac{S_{b,0} \cdot K_p \cdot \alpha}{1+\alpha}\left[1 + 6(1+\alpha) \cdot \sum_{n=1}^{\infty} \frac{\exp(-\frac{D \cdot q_n^2 \cdot t}{R^2})}{9 + 9\alpha + q_n^2 \alpha^2} \frac{R}{r_p} \frac{\sin\left(\frac{q_n r_p}{R}\right)}{\sin q_n}\right] \quad (6\text{-}4)$$

Using assumption 5, the concentration at the particle surface ($r_p = R$) becomes

$$S_p(t) = K_p \cdot S_b(t) \quad (6\text{-}5)$$

Then, the substrate concentration profile in the bulk solution can be predicted by the following equation:

$$S_b(t) = \frac{\alpha \cdot S_{b,0}}{1+\alpha}\left[1 + 6(1+\alpha) \cdot \sum_{n=1}^{\infty} \frac{\exp(-\frac{D \cdot q_n^2 \cdot t}{R^2})}{9 + 9\alpha + q_n^2 \alpha^2}\right] \quad (6\text{-}6)$$

Diffusion coefficients were estimated by measuring concentration changes in the surrounding bulk solution and fitting equation (6-6) to the experimental data. This is a non-steady-state measurement yielding the molecular diffusion coefficient, D. Since D_{eff} and D are related through porosity (ε), D_{eff} can be calculated from

$$D_{eff} = \varepsilon \cdot D \qquad (2\text{-}2)$$

MATERIALS AND METHODS

Cattle Dip Solutions and Chemicals

Untreated cattle dip (UCD) waste solution was obtained from the APHIS dipping vats in Laredo, Texas. Chlorferon solution was produced from UCD solution or pure coumaphos solution by hydrolysis of coumaphos using genetically engineered *E.coli* containing organophosphate hydrolase (OPH) enzyme as described in Chapter V. Analytical grade chlorferon was a gift from Bayer Corporation (Animal Health Division, Merriam, KS). DETP-potassium salt (DETP-K) and sodium alginate consisting of 65 – 70 % guluronic acid and 25 – 35 % mannuronic acid were obtained from Sigma-Aldrich (St. Louis, MO).

Sample Analysis Method

A HPLC system (Waters, model 2690, Milford, MA) equipped with a PDA detector (Waters, model 996, Milford, MA), an autosampler, and a guard column (C-18, 4.5 mm × 10 mm, Burdick and Jackson, Muskegon, MI) was used for analysis. Chlorferon and DETP were analyzed using a C-18 column and a C-8 column (4.5 mm × 250 mm, Burdick and Jackson, Muskegon, MI), respectively. The mobile phase was

HPLC grade acetonitrile with 0.75 mM phosphoric acid (80:20 by volume) at a flow rate of 1 ml/min. Absorbance was measured at 320 nm for chlorferon and 210 nm for DETP. During the experiments, 0.2 ml samples were collected in 1.5 ml polypropylene micro test tubes and diluted with methanol to achieve appropriate concentrations for HPLC analysis. Tubes were vortexed, and centrifuged at 1,163 × g for 15 min using a microcentrifuge, and the supernatants were filtered using disposable syringe filters (Acrodisk CR13 mm, PTFE, 0.2 μm, Pall Corporation, East Hills, NY). Filtered samples were stored in the refrigerator until analyzed.

Preparation for Microorganisms and Ca-alginate Gel Beads

Microorganisms were prepared and immobilized in Ca-alginate gel beads as described in Chapter V. Alginate concentration in the beads was 2 % for all diffusion experiments. Cells were immobilized without acclimation to their target compound and reaction medium to minimize degradation during diffusion studies. Cell-free beads were also prepared.

Characterization of the Ca-alginate Beads

Bead density was measured by displacement analysis. The mass (wet wt.) of approximately 30 beads was measured and the beads were placed in a measured volume of water in a graduated cylinder. The increase in volume after bead addition was

measured, and density was calculated from the mass and volume. Beads were blotted dry with paper towels before measuring the mass. Average density was determined from seven experiments.

Bead diameters were measured directly using a ruler, and the average for 60 beads was calculated. This was compared with the size calculated from the average volume determined using the displacement method described above.

Porosity of beads was measured using the procedure of Kim (1998):

1) Equilibrate beads in a solution containing a known concentration of substrate by agitating the solution vigorously (200 rpm) on a magnetic stirrer until a constant substrate concentration (S_c) is achieved in the bulk phase

2) Collect beads using a strainer and blot dry the surface with paper towels

3) Equilibrate the beads in another flask containing a specific volume (V_b) of substrate solution with a different concentration (S_b) by agitating vigorously (200 rpm) until the bulk concentration is constant (S)

Porosity is calculated using the following equation:

$$\varepsilon_p = \frac{3V_b(S - S_b)}{4\pi r_p^3 N(S_c - S)} = \frac{V_b(S - S_b)}{V_p(S_c - S)} \tag{6-7}$$

where ε_p = porosity of bead

N = number of beads

V_p = total bead volume

Determination of Partition Coefficient

The equilibrium partition coefficient, K_p, of substrate in Ca-alginate gel beads was determined by mass balance of the substrate, following the method of Arnaud and Lacroix (1991). The mass balance of substrate is

$$V_{b,0} \cdot S_{b,0} = V_p \cdot S_{p,eq} + V_b \cdot S_{b,eq} + V \cdot \sum_{j=1}^{i} S_{bj} \qquad (6\text{-}8)$$

where $V_{b,0}$ = initial volume of the bulk solution excluding bead volume

V_p = total bead volume

V_b = volume of the bulk solution excluding bead volume and sampling volume

V = sampling volume

$S_{b,0}$ = initial substrate concentration in the bulk solution

$S_{p,eq}$ = substrate concentration in the beads at equilibrium

$S_{b,eq}$ = substrate concentration in the bulk solution at equilibrium

S_{bj} = substrate concentration in the bulk solution at sampling time

At equilibrium, K_p is defined as:

$$K_p = \frac{S_{p,eq}}{S_{b,eq}} \qquad (6\text{-}9)$$

Combining equations (6-8) and (6-9) and rearranging gives

$$K_p = \frac{V_{b,0} \cdot S_{b,0} - V_b \cdot S_{b,eq} - V \cdot \sum_{j=1}^{i} S_{bj}}{V_p \cdot S_{b,eq}} \qquad (6\text{-}10)$$

Partition coefficients can be calculated from the same experimental data used to estimate diffusion coefficients.

Estimation of Diffusion Coefficient

Experimental Setup

Experiments to determine diffusion coefficients were performed in a 1.25 L BioFlo II fermentor system (New Brunswick Scientific Co., Inc., Edison, NJ) with 0.3 L of bulk solution and 60 g beads. Bead surfaces were blotted dry with paper towels prior to measuring their mass. Beads were then added to the bulk solution containing substrate (chlorferon or DETP) and agitated by a flat bladed turbine impeller (3.25 cm radius) at 200 rpm unless specified otherwise. Temperature was controlled at 30 ºC (the temperature used for degradation studies). Samples were taken from the bulk phase at varying intervals over a one-hour period to determine substrate concentration as a function of time. Sample analysis was conducted using a HPLC system as described above.

Measurement of Diffusion Coefficient

Experiments to determine the diffusion coefficients of chlorferon and DETP in Ca-alginate gel beads were conducted at the same conditions used for the degradation studies of each compound, except that cells were immobilized before acclimation to their target compound and reaction medium to minimize degradation during the diffusion experiments. Since the cells had approximately a week-long lag period without acclimation to the target compound and reaction medium as shown in Chapters III and IV, it was assumed that no degradation would occur during the one-hour diffusion experiments. A set of control samples confirmed this assumption.

To study diffusion characteristics of chlorferon and DETP in Ca-alginate gel beads, a series of experiments with variations in bulk solution (UCD, MS/MSYE medium), agitation speeds (100 – 300 rpm) and substrate concentration (50 – 500 mg/L for DETP, 50 – 600 mg/L for chlorferon) were carried out using cell-free beads. Effects of immobilized cell concentration were studied using beads containing cells at various concentrations (0 – 100 g-wet cell/L-gel).

Numerical Calculation

Nonlinear regression analysis was performed for each experiment to calculate the diffusion coefficient (D). The equation (6-6) was solved with three parameters, α, D and S_b, using Compaq Visual FORTRAN 6.6 (Compaq Computer Corporation). S_b was

measured experimentally and the value of α was calculated using equation (2-12). Theoretical predictions made by varying D in equation (6-6) were then fitted to the substrate concentration profile of each experiment. Percent deviation was calculated between experimental data and predicted values using equation (6-11):

$$Percent\ \ deviation = \frac{\sum\left[\frac{(S_b)_{\exp} - (S_b)_{pred}}{(S_b)_{pred}}\right]^2}{N-1} \times 100 \qquad (6\text{-}11)$$

where $(S_b)_{exp}$ and $(S_b)_{pred}$ are the experimental and predicted values, respectively, of substrate concentration in the bulk solution and N is the number of experimental points considered. The value of D giving the lowest percent deviation was determined as the best fit.

The q_n values were determined from equation (2-11) using the Secant method (Sharma, 2004) and only the initial ten terms were used for calculations. Using more than 10 terms did not make a noticeable difference in D values. Arnaud and Lacroix (1991) reported that adding more than the first six terms had no significant difference in diffusivity values. All other parameters were determined from experiments.

Viscosity of Bulk Solution

Viscosities of bulk solutions, including UCD solution and MS/MSYE medium were measured using a viscometer (Brookfield Rheometer Model DV-III, Torque HV

with UL adapter, Brookfield Engineering Laboratory, Stoughton, MA). For data acquisition, Rheocalc. V.1.1 software (Brookfield Engineering Laboratory, Stoughton, MA) was used. Temperature was maintained at 30 °C during the measurement using a thermostatically controlled water bath.

Estimation of External Mass Transfer Resistance

One of the assumptions applied to determine the diffusion coefficient was that the external mass transfer resistance was negligible. In order to justify this assumption, the Biot number (*Bi*) was calculated. Generally, the effect of external mass transfer resistance is negligible when the Biot number is greater than 100 (Blanch and Clark, 1996). The Biot number is defined as the relative magnitude of external and internal diffusional resistances as shown in equation (6-12):

$$Bi = \frac{k_L \cdot r_p}{D_{eff}} \tag{6-12}$$

where k_L is the liquid-solid mass transfer coefficient. The value of k_L can be estimated using

$$k_L = \frac{D_{aq} \cdot N_{sh}}{2r_p} \tag{6-13}$$

where D_{aq} = diffusion coefficient in bulk solution (m^2/s)

N_{sh} = Sherwood number (dimensionless) $N_{sh} = 2 + 0.6 N_{sc}^{1/3} N_{\mathrm{Re}}^{1/2}$ (6-14)

N_{sc} = Schmidt number (dimensionless) $N_{sc} = \dfrac{\mu_b}{\rho_b \cdot D_{aq}}$ (6-15)

N_R = Reynolds number (dimensionless) $N_{\mathrm{Re}} = \dfrac{v_s \cdot 2 r_p \cdot \rho_b}{\mu_b}$ (6-16)

μ_b = viscosity of bulk solution (kg/m·s)

ρ_b = density of bulk solution (kg/m^3)

v_s = slip velocity (m/s)

Values for k_L were determined following the procedure recommended by Harriott (1962). The diffusion coefficient in bulk solution (D_{aq}) was calculated using the Wilke-Chang correlation (Geankoplis, 1993) as follows:

$$D_{aq} = 1.173 \times 10^{-16} (\varphi_b M_b)^{\frac{1}{2}} \times \frac{T}{\mu_b V_{mo}^{0.6}} \qquad (6\text{-}17)$$

where φ_b = association parameter of the bulk solution

M_b = Molecular weight of bulk solution

T = Temperature in K

V_{mo} = Molar volume of substrate at the boiling point

RESULTS AND DISCUSSION

Characteristics of Ca-alginate Gel Beads

The Ca-alginate gel beads were observed to be spherical. Table 6-1 shows densities, bead radii and porosities determined for the beads. Bead radii were determined by both direct measurement and the displacement method. Values obtained from both methods were in good agreement. Bead radii obtained from displacement method were used throughout the calculations. The average radii of cell-free beads and beads containing DETP-degrading organisms were the same, 0.165 cm, but beads containing chlorferon-degrading organisms were slightly larger at 0.171 cm.

The porosity of cell-free Ca-alginate beads was determined to be 0.96. In addition, bead water content was measured to be 0.965 which was almost the same as the porosity. Bead water content was determined by measuring the difference in mass of wet beads and dried beads. When measuring the mass of wet beads, bead surfaces were blotted dry with paper towels. The mass of dried beads was measured after drying the beads overnight at 120 °C.

Measured values for the porosity of Ca-alginate gel beads have not been reported in the literature but have been assumed to be 0.99 (Papathanasiou and Bijeljic, 1998; Monbouquette et al., 1989). Papathanasiou and Bijeljic (1998) made the assumption based on their observation that the size of Ca-alginate beads shrank to the extent of being invisible to the naked eye when the beads were allowed to dry.

Table 6-1. Characteristics of Ca-alginate gel beads

	Density (ρ_p, g/cm^3)	Radius (r_p, cm)		Porosity (ε)
		Direct Measurement	Displacement Method	
Cell-free beads	1.0485 ± 0.0405	0.159 ± 0.008	0.165 ± 0.003	0.96
Beads-chlorferon[1)]	1.0782 ± 0.0265	0.172 ± 0.011	0.171 ± 0.003	0.89
Beads-DETP[2)]	1.1521 ± 0.0766	0.161 ± 0.010	0.165 ± 0.007	0.91

[1)] Beads containing chlorferon-degrading organisms of 80 g/L

[2)] Beads containing DETP-degrading organisms of 60 g/L

Estimation of External Mass Transfer Resistance

Biot numbers were calculated to determine the extent of external mass transfer resistance for the diffusion of chlorferon and DETP. Values of the parameters used for the calculation and resultant Biot numbers are given in Table 6-2. For chlorferon, parameter values were for hydrolyzed UCD solution mixed with MS medium. For DETP, parameter values were for MSYE medium. Agitation speed and temperature were 200 rpm and 30 °C, respectively. Calculated Biot numbers were greater than 100 indicating that external mass transfer was not limiting for the diffusion of both chlorferon and DETP. Thus, the assumption of negligible external mass transfer resistance was valid for an agitation speed of 200 rpm.

Table 6-2. Parameters used for Biot number calculation

Parameter	Units	Value	
		Chlorferon (H.UCD[1)]+MS)	DETP (MSYE)
T	°C	30.0	30.0
μ_b[2)]	$\times 10^3$ kg/m·s	3.03	2.87
ρ_b[2)]	kg/m^3	999.7	998.3
ρ_p[2)]	kg/m^3	1078.2	1152.1
r_p[2)]	$\times 10^3$ m	1.71	1.65
D_{aq}[3)]	$\times 10^{10}$ m^2/s	1.98	2.36
N_{Re}[4)]	dimensionless	38.21	72.77
N_{Sc}[4)]	dimensionless	15314	12166
N_{Sh}[4)]	dimensionless	94.10	119.72
k_L[4)]	$\times 10^5$ m/s	0.82	1.29
D_{eff}[2)]	$\times 10^{11}$ m^2/s	2.96	4.40
Bi[5)]	dimensionless	472.41	482.58

[1)] H.UCD is hydrolyzed UCD solution

[2)] Determined experimentally.

[3)] Calculated from Wilke-Chang correlation (equation (6-17), Geankoplis, 1993).

[4)] Calculated using the procedure recommended by Harriott (1962).

[5)] Calculated from the equation (6-12)

Biot numbers and external film thicknesses were calculated for various agitation speeds (10 – 400 rpm) for chlorferon and DETP as shown in Figure 6-1. Biot numbers were greater than 100 at all agitation speeds indicating that external mass transfer can be ignored at speeds as low as 10 rpm. However, external film thickness decreased significantly with increasing agitation speed from 10 to 100 rpm and it was almost constant above 100 rpm. Therefore, it seems more reasonable to maintain agitation speed above 100 rpm to ensure negligible external mass transfer resistance.

Determination of Partition Coefficient

Partition coefficients for chlorferon and DETP in cell-free Ca-alginate gel beads were determined as 0.958 (± 0.044) and 0.991 (± 0.023), respectively. For cell-containing beads, the partition coefficient for chlorferon decreased to 0.923 (± 0.02) while that for DETP increased slightly to 1.081 (± 0.046). However, since all partition coefficients are close to unity, partition effects are considered to be negligible.

Determination of Diffusion Coefficients

Experiments for the diffusion of chlorferon and DETP in Ca-alginate gel beads were carried out in hydrolyzed UCD solution diluted with MS medium and MSYE medium, respectively (the reaction media for degradation studies). Figure 6-2 shows the diffusion profiles of chlorferon and DETP into cell-free Ca-alginate gel beads.

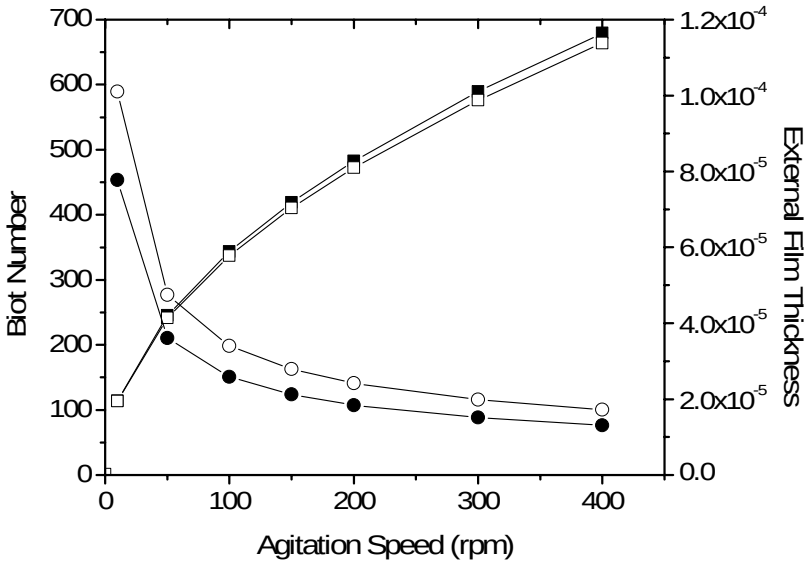
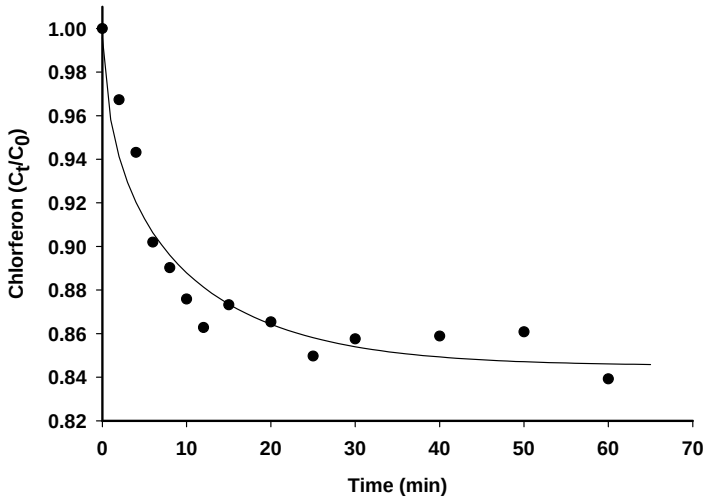
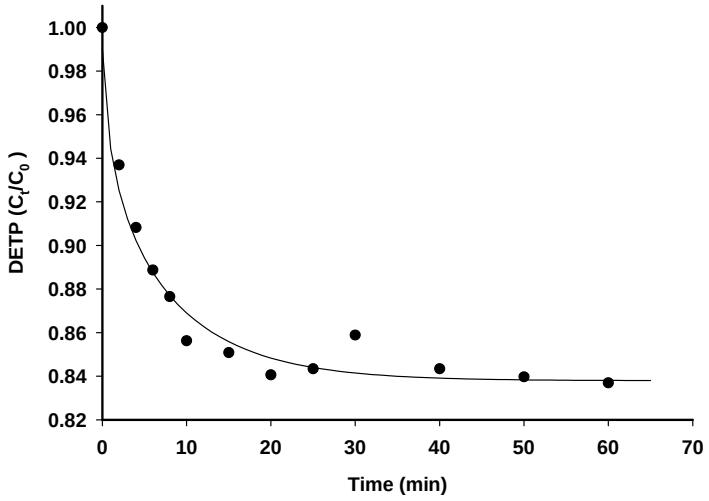


Figure 6-1. Calculated Biot number and external film thickness of Ca-alginate gel beads at various agitation speed (■ Biot number calculated for DETP; □ Biot number calculated for chlorferon; ● External film thickness calculated for DETP; ○ External film thickness calculated for chlorferon)



(a)



(b)

Figure 6-2. The diffusion of chlorferon and DETP in cell-free Ca-alginate gel beads. Symbols represent normalized substrate concentrations in the bulk solution obtained from experiments. Lines represent predicted diffusion curves from the best fit of equation (6-6) with experimental data. Reaction conditions: temperature 30 °C; (a) bulk solution: hydrolyzed UCD solution + MS medium; initial chlorferon concentration of 80 mg/L; (b) bulk solution: MSYE medium; initial DETP concentration of 50 mg/L

Diffusion coefficients of chlorferon and DETP into cell-free Ca-alginate gel beads were determined to be 3.08 (± 0.23) × 10^{-11} m^2/s and 4.58 (± 0.12) × 10^{-11} m^2/s, respectively. Diffusion coefficients of chlorferon and DETP in cell-containing Ca-alginate gel beads were 2.7 (± 0.11) × 10^{-11} and 4.28 (± 0.12) × 10^{-11} m^2/s, respectively, which were lower than the corresponding values in cell-free gel beads. Cell concentrations in the beads were 80 g/L for chlorferon and 60 g/L for DETP, the cell concentrations for degradation studies.

Since no previous studies on diffusion of chlorferon or DETP in any matrices were available, no comparison could be made to verify the diffusion coefficients determined in this study for chlorferon and DETP. Many diffusion studies have been done with simple sugar compounds, such as glucose, lactose, or maltose. For these compounds, the reported diffusion coefficients in cell-free Ca-alginate gel beads were about 90 % of the corresponding bulk diffusivities because Ca-alginate has a very high porosity (Axelsson and Persson, 1988; Tanaka et al., 1984; Hannoun and Stephanopoulos, 1986; Merchant et al., 1987; Dembczynski and Jankowski, 2000). In this study, the diffusion coefficients for chlorferon and DETP were only about 10 – 20 % of the corresponding bulk diffusion coefficients.

One possible factor causing lower diffusion coefficients in the gel matrix is that the molecular radii of chlorferon and DETP were approximately the same magnitude as the pore size of the Ca-alginate gel beads. Molecular radius can be estimated from the diffusion coefficient in a non-viscose solution using the Stokes-Einstein equation (Blanch and Clark, 1996):

$$D_{aq} = \frac{k \cdot T}{6\pi \cdot r_s \cdot \mu_b} \tag{6-18}$$

where k is the Boltzmann constant ($1.3806503 \times 10^{-23}$ $m^2 \cdot kg/s^2 \cdot K$), T is the absolute temperature, r_s is the solute spherical radius (m). Using D_{aq} values estimated using the Wilke-Chang correlation (equation 6-17), molecular radii of chlorferon and DETP at 30 °C were estimated to be 3.67×10^{-10} and 3.25×10^{-10} m, respectively. The molecular radius of glucose at 25 °C is reported to be 3.54×10^{-10} m (Arnaud and Lacroix, 1991) which is close to the radii found for chlorferon and DETP. Effective diffusion coefficients for glucose in Ca-alginate gel beads reported in the literature are very near the bulk diffusion coefficient as mentioned above, suggesting the pore size of Ca-alginate gel beads is much larger than the molecular radius of glucose. Although different gel preparation methods produce different gel characteristics causing variability in effective diffusion coefficients for the same substrate (Arnaud and Lacroix, 1991), it does not appear that the pore size of the Ca-alginate used in this study was small enough to hinder diffusion of chlorferon and DETP.

In equation (2-6), D_{aq} and D_{eff} are related through porosity and tortuosity. Porosity determined for cell-free gel beads was 0.96. It is possible that the tortuosity of the Ca-alginate gel beads used in this study was high resulting in lower diffusion coefficients. Another possible explanation for this is that D_{aq} might be overestimated. D_{aq} values used in this study for chlorferon and DETP were calculated from the Wilke-Chang correlation while D_{aq} for glucose reported in the literature was an experimentally measured value.

Effect of Bulk Solution Composition on Diffusion

Effects of bulk solution composition on diffusion were determined by measuring diffusion coefficients for chlorferon and DETP into cell-free Ca-alginate gel beads in various bulk solutions including UCD solution, hydrolyzed UCD + MS medium, MSYE medium, and DI water. Diffusion coefficients for chlorferon and DETP into Ca-alginate beads determined in UCD solution and MSYE medium were 2 – 3 times lower than in DI water (Table 6-3). This difference was primarily the result of differences in viscosities of the bulk solutions (μ_b). Diffusivity is related to T/μ_b where T and μ_b are the operating temperature and the viscosity of bulk liquid, respectively (Ranz and Marshall, 1952; Nguyen and Luong, 1986; Chresand et al., 1988). Brown and Johnsen (1981) reported a linear relation between log$D$ and inverse viscosity of water. Since the operating temperature for this experiment was constant at 30 °C, the differences found in diffusion coefficients were inversely related to viscosities of the bulk solutions.

Table 6-3 shows the diffusion coefficients for chlorferon and DETP in various bulk solutions and viscosities of those solutions. Viscosities of MSYE medium and UCD solution at 30 °C were 3.5 - 4 times higher than that of DI water. Due to differences in viscosity, bulk diffusivities (D_{aq}) in DI water were calculated to be approximately four times greater than those in UCD solution and MS/MSYE medium. Since the association parameter (φ_b) and molecular weight of bulk solution (M_b) were not known for MSYE medium and UCD solution, the values for water were used.

Table 6-3. Viscosities of various bulk solutions and diffusion coefficients for chlorferon and DETP in cell-free Ca-alginate gel beads measured in those solutions

		DI	MS[1)]/MSYE	H.UCD[2)] + MS	H.UCD[2)]
Viscosity $\times 10^3$ (kg/m·s)		0.8007[3)]	2.87 ± 0.06	3.03±0.06	3.1 ± 0.1
$D_{aq} \times 10^{10}$ (m^2/s)	Chlorferon	7.49	2.09	1.98	1.94
	DETP	8.47	2.36	-	2.19
$D \times 10^{11}$ (m^2/s)	Chlorferon	7.77	3.44	3.08	2.22
	DETP	8.99	4.58	-	4.29

[1)] Hydrolyzed coumaphos solution was used for chlorferon supply in MS medium.
[2)] Hydrolyzed UCD solution was for chlorferon supply.
[3)] From reference (Geankoplis, 1993)

Effect of Substrate Concentration on Diffusion

Effects of the concentration of DETP and chlorferon on their diffusion into cell-free Ca-alginate gel beads were studied. Diffusion coefficients decreased as substrate concentrations increased. The experimental results are shown in Figure 6-3. The diffusion coefficient for DETP measured at a concentration of 500 mg/L was 73 % of that measured with 50 mg/L. In the case of chlorferon, the diffusion coefficient measured at a concentration of 600 mg/L was 72 % of that measured with 80 mg/L chlorferon.

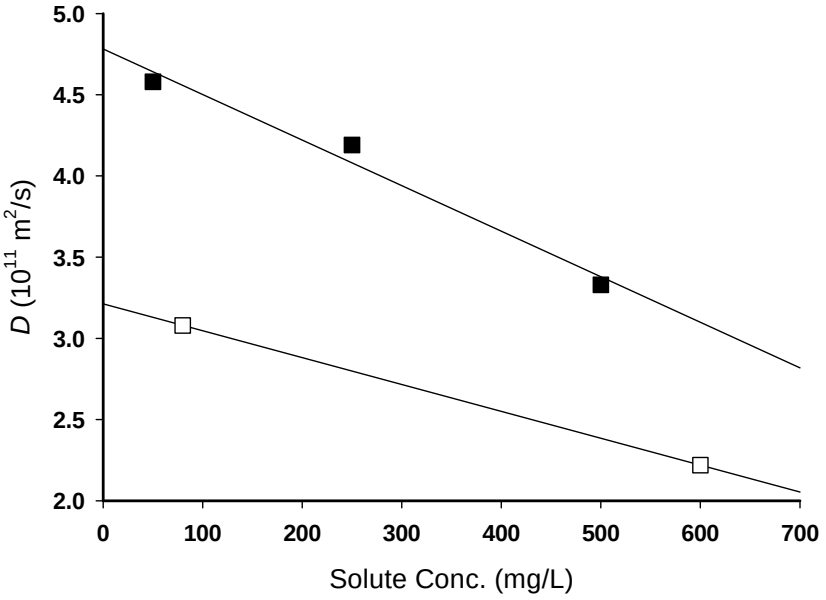


Figure 6-3. Influence of substrate concentration on the diffusion of DETP and chlorferon in cell-free Ca-alginate gel beads. Reaction conditions: temperature 30 °C; agitation speed 200 rpm (■ DETP in MSYE medium; □ chlorferon in hydrolyzed UCD + MS medium)

Effect of Agitation Speed on Diffusion

The effect of agitation speed on substrate diffusion was investigated using DETP as the diffusing substrate in cell-free Ca-alginate gel beads. As shown in Figure 6-4, the diffusion coefficient increased with increasing agitation speed. However, Ca-alginate gel beads were not stable at agitation speeds over 300 rpm. At 300 rpm, beads were found to be partially broken after one hour. Although agitation speed over 100 rpm was enough to overcome external mass transfer resistance (see Figure 6-1), maintaining the maximum speed within stability limits appears to enhance internal diffusion.

Effect of Cell Concentration on Diffusion

Diffusion coefficients for DETP and chlorferon were measured in cell-containing Ca-alginate gel beads. Diffusion coefficients decreased with increasing cell concentration. This was expected because of the diffusion hindrance effect caused by immobilized cells.

Several theoretical models to predict diffusion in cell-containing gels as a function of cell volume fraction (ϕ_c) are available. Among those models, the exclusion, random-pore and Maxwell models were compared with experimental results obtained in this study. The mathematical equations for each model are as follows:

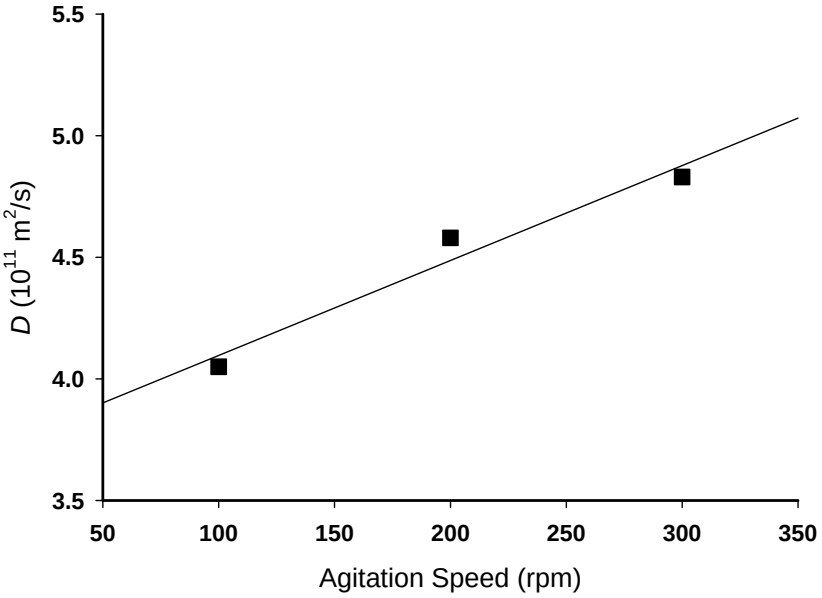


Figure 6-4. Influence of agitation speed on diffusion of DETP into cell-free Ca-alginate gel beads. Reaction conditions: bulk solution of MSYE medium; initial DETP concentration 50 mg/L; temperature 30 °C

Exclusion model: $$\frac{D_{eff}}{D_{eff\,0}} = 1 - \phi_c \qquad (6\text{-}19)$$

Random-pore model: $$\frac{D_{eff}}{D_{eff\,0}} = (1 - \phi_c)^2 \qquad (6\text{-}20)$$

Maxwell model: $$\frac{D_{eff}}{D_{eff\,0}} = \frac{1 - \phi_c}{1 + \frac{\phi_c}{2}} \qquad (6\text{-}21)$$

where D_{eff0} is the effective diffusivity determined with cell-free beads which can be calculated from D and ε using equation (2-2). All three models assume that the immobilized cells are impermeable, thus, substrate does not diffuse into the cells. Models assuming cells to be permeable consider the possibility of diffusional flux through the cells. In this case, the effective diffusion coefficient within the cells (D_c) is required (Westrin and Axelsson, 1991). Due to the complexity of predicting the D_c value, those models were not considered in this study.

The exclusion model incorporates only the exclusion effect of immobilized cells. The random-pore model originated from Kozeny's capillary model describing the porous solid as consisting of tortuous uniform capillaries. The Maxwell model describes cells as suspended impermeable sphere in the gel. These models were originally developed as a function of cell volume fraction (ϕ_c), but cell weight fraction (ϕ_{cw}) can be used when the volume fraction is unknown (Westrin and Axelsson, 1991). Since the organisms used for degradation of DETP and chlorferon were a mixed culture, the exact cell density could

not be determined to calculate cell volume fractions. Thus, cell weight fractions were used in this study.

For the experiments of DETP diffusion into cell-containing Ca-alginate beads, immobilized cell concentrations in the beads were varied in the range of 0 – 100 g/L. The bulk solution was MSYE medium and the initial DETP concentration was 50 mg/L. Normalized effective diffusivities for experimental data and results from theoretical models are shown in Figure 6-5 as a function of cell weight fraction (ϕ_{cw}). All three models overestimated the effective diffusivity. A relation between D_{eff} and ϕ_{cw} could be found from linear regression of the experimental data as follows:

$$\frac{D_{eff}}{D_{eff\,0}} = 1 - 2.5164\phi_{cw} \qquad (6\text{-}22)$$

Studies for chlorferon diffusion in cell-containing Ca-alginate beads were carried out in two different bulk solutions, MS medium with chlorferon supplied as hydrolyzed UCD solution and as hydrolyzed pure coumaphos solution. Cell concentrations in Ca-alginate gel beads were 40 g/L and 80 g/L. The initial chlorferon concentration was 50 mg/L. Different sources of chlorferon did not produce much variation in the diffusion coefficient. As with DETP, the models overestimated the effective diffusivity as shown in Figure 6-6. A linear relation between D_{eff} and ϕ_{cw} could be derived as follows:

$$\frac{D_{eff}}{D_{eff\,0}} = 1 - 2.314\phi_{cw} \qquad (6\text{-}23)$$

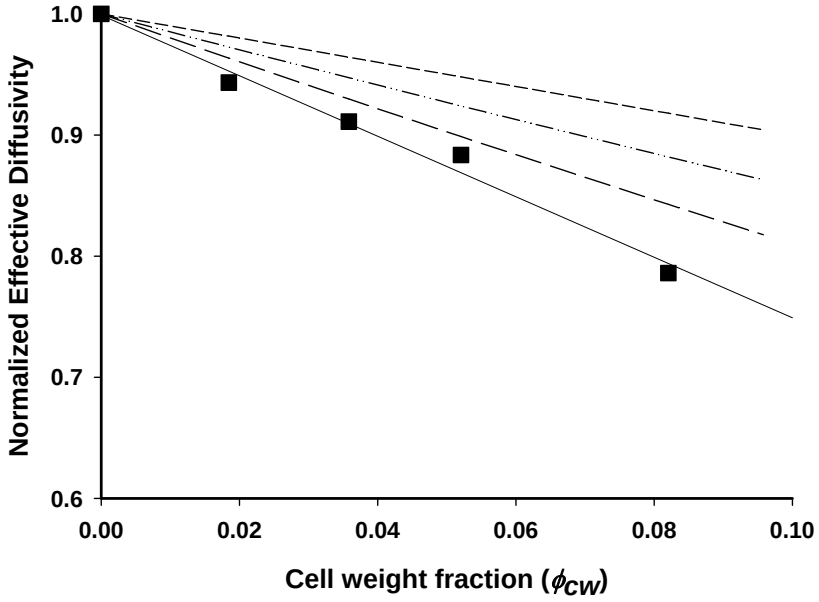


Figure 6-5. Normalized effective diffusivity of DETP as a function of cell weight fraction (ϕ_{cw}) and comparison of experimental correlation and model predictions. Reaction conditions: bulk solution of MSYE medium; initial DETP concentration 50 mg/L; temperature 30 °C; agitation speed 200 rpm (• experimental data; — linear regression of experimental data; --- Exclusion model; −··− Maxwell model; −−− Random–pore model)

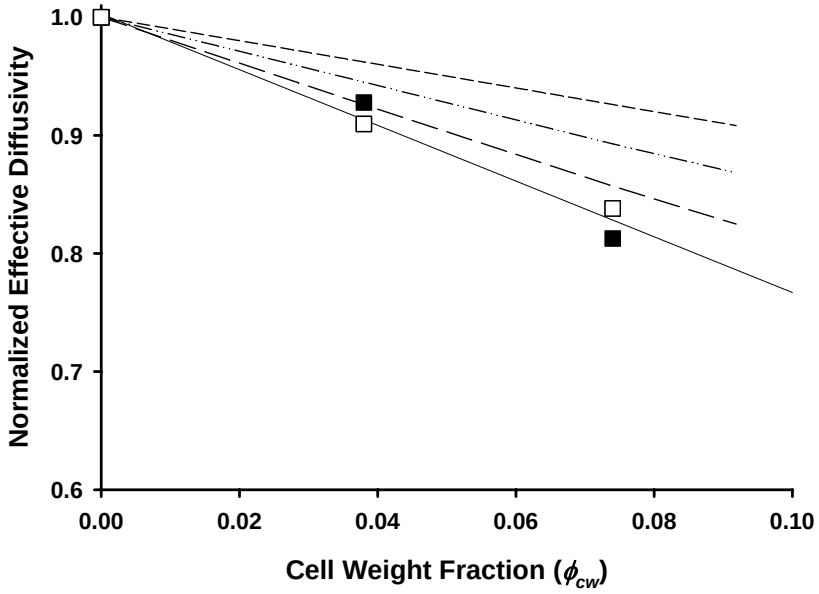


Figure 6-6. Normalized effective diffusivity of chlorferon into Ca-alginate gel beads as a function of cell weight fraction (ϕ_{cw}) and model predictions. Reaction conditions: initial chlorferon concentration 50 mg/L; temperature 30 °C; agitation speed 200 rpm (■ chlorferon supplied as hydrolyzed pure coumaphos solution in MS medium; □ chlorferon supplied as hydrolyzed UCD solution in MS medium; — linear regression of experimental data; --- Exclusion model; −··− Maxwell model; −−− Random–pore model)

Internal Mass Transfer Considerations

The effect of internal mass transfer resistance can be assessed by the Thiele modulus (ϕ) and effectiveness factor (η). The Thiele modulus is a single dimensionless parameter combining factors affecting concentration profiles in the porous particles, including the particle size, the effective diffusivity of substrate through the particle, and intrinsic kinetic parameters for the immobilized cells (Blanch and Clark, 1996). The Thiele modulus defined for Michaelis-Menten kinetics is as follows:

$$\phi = \frac{R}{3}\left(\frac{V_m}{K_m D_{eff}}\right)^{1/2} \qquad (6\text{-}24)$$

where R is the bead radius, V_m is the maximum reaction rate, K_m is the substrate half-saturation constant and D_{eff} is the effective diffusivity.

The squared Thiele modulus (ϕ^2) is the ratio of the intrinsic reaction rate in the absence of mass transfer limitation to the diffusion rate through the bead particle. Values of ϕ^2 close to 0 mean reaction limited conditions, and when ϕ^2 exceeds 1, mass transfer limitations become important. Assuming that the intrinsic characteristics of the cells remained unchanged after immobilization, the kinetic parameters determined from free cells could be used to calculate ϕ^2. Calculated values of ϕ^2 for DETP and chlorferon were 0.27 and 0.13, respectively. Since both values were close to 0, internal diffusion in the Ca-alginate gel beads did not limit the reaction rate. However, it was observed that

the activity of cells was enhanced by immobilization in Chapter V. Thus, intrinsic kinetic parameters of immobilized cells might be different from those of free cells. Since intrinsic kinetic parameters of immobilized cells can not be directly measured, the observable modulus,Φ, was defined using following equation (Blanch and Clark, 1996):

$$\Phi = \frac{v_{obs}}{D_{eff} S}\left(\frac{R}{3}\right)^2 \qquad (6\text{-}25)$$

where v_{obs} is the observed reaction rate in the immobilized cell system and S is the initial substrate concentration in bulk solution. Values of Φ calculated for chlorferon and DETP using the volumetric reaction rates obtained in Chapter V were 0.23 and 0.18, respectively. These low values of Φ indicate that the degradation reaction rates obtained with immobilized cells in Ca-alginate beads were not diffusion limited. A general criterion of mass transfer effects on the overall kinetics is that the internal effectiveness factor approaches unity when Φ is less than 0.3 (Blanch and Clark, 1996).

Significance of Diffusion Studies

Diffusion coefficients for chlorferon and DETP measured at various conditions are shown in Table 6-4. Agitation speed, the concentration of diffusing substrate, the viscosity of bulk solution, and the concentration of immobilized cells all influenced the diffusion coefficient. Diffusion coefficients for both chlorferon and DETP decreased with increasing immobilized cell concentration in accord with results for other immobili-

Table 6-4. Diffusion coefficients determined for chlorferon and DETP at various conditions

Substrate	Bulk Solution	Agitation (rpm)	Substrate Conc. (mg/L)	Cell Conc. (g/L)	$D \times 10^{11}$ (m^2/s)
DETP	DI	200	50	-	8.99 ± 0.06
	UCD		50	-	4.29 ± 0.06
	MSYE		50	-	4.58 ± 0.12
		100	50	-	4.05 ± 0.08
		300	50	-	4.83 ± 0.07
		200	250	-	4.19 ± 0.11
			500	-	3.33 ± 0.23
			50	20	4.40 ± 0.05
				40	4.33 ± 0.06
				60	4.28 ± 0.14
				100	3.96 ± 0.28
Chlorferon	H.UCD	200	600	-	2.22 ± 0.01
	H.UCD + MS		80	-	3.08 ± 0.23
	H.Cou. + MS		50	-	3.44 ± 0.11
	H.Cou. + DI			-	7.77 ± 0.11
	H.UCD + MS			40	2.95 ± 0.12
				80	2.7 ± 0.11
	H.Cou. + MS			40	3.23 ± 0.16
				80	3.11 ± 0.11

zed cell systems reported in the literature (Chen et al., 2003; Jovetic et al., 2001; De Backer, et al., 1992).

Increasing concentrations of DETP and chlorferon decreased their diffusion coefficients in Ca-alginate gels. Nguyen and Luong (1986) reported that diffusivity decreased in response to an increase in the glucose concentration in *k*-carrageenan gels. However, Arnaud and Lacroix (1991) reported no difference in diffusivity in *k*-carrageenan gels as lactose concentration increased, and no effect of concentration on diffusivity was found by Hannoun and Stephanopoulos (1986) with Ca-alginate/glucose and Ca-alginate/ethanol systems. Yankov (2004) also reported no influence of glucose and maltose concentration on their diffusivity in polyacrylamide gels.

There do not appear to be any general models for predicting diffusion characteristics for various combinations of diffusing substrates and matrices. In addition, diffusion coefficients reported in the literature are highly variable making comparisons even for the same substrate difficult. This is because diffusion in gels is affected by many factors which depend on experimental conditions and techniques used. Thus, studies of diffusion characteristics for each combination of diffusing substrate and matrix are necessary to determine diffusion coefficients for the analysis and design of reactors using immobilized cells (Brito et al., 1990a).

CHAPTER VII
CONCLUSIONS AND RECOMMENDATIONS

Increasing use of pesticides has significantly raised the potential for environmental contamination. Since organophosphate pesticides are the most widely used pesticides in the world, bioremediation of organophosphate compounds were the interest in this research. Coumaphos was selected as the model compound and the focus of this research was on degradation of chlorferon and DETP, the products of coumaphos hydrolysis. Indigenous organisms capable of degrading chlorferon or DETP present in UCD solution were isolated and grown to high cell density for degradation studies following a two step growth procedure. Optimum degradation conditions were determined using free cells. A calcium-alginate immobilized cell system was developed to investigate the effect of immobilization on degradation. Characteristics of diffusion of chlorferon and DETP into Ca-alginate gel beads were studied to assist in designing and operating bioreactor systems. Major conclusions from this work are as follows:

1) Consortia of indigenous microorganisms capable of degrading chlorferon and DETP were isolated separately. They were mostly Gram-negative rod type bacteria. Cell growth during the enrichment of chlorferon-degrading organisms was due to methanol added to solubilize the chlorferon. DETP-degrading organisms showed a flocculating nature of growth during enrichment and used DETP as a carbon source. Chlorferon inhibited both chlorferon-degrading and DETP-degrading organisms during enrichment

at 100 mg/L and 50 mg/L, respectively. Due to this inhibition effect, it was not possible to enrich a consortium of organisms for simultaneous degradation of chlorferon and DETP.

2) A two-step growth procedure was developed for degradation studies to provide large quantities of biomass acclimated to the target compound (chlorferon or DETP) and reaction medium since cells lost their degradation activity during growth in a rich medium. Without acclimation, a lag period of approximately one week was required before degradation was initiated. For degradation of chlorferon and DETP, MS medium and MSYE medium, respectively, were selected as the acclimation and reaction medium.

3) Optimum reaction conditions were found for chlorferon and DETP degradation using freely suspended cells. For chlorferon degradation, the optimum biomass concentration was 80 g/L. Reaction pH between 6.5 and 8.5 did not have a significant influence on the degradation reaction. For DETP degradation, the optimum biomass concentration was 60 g/L. The optimum range of reaction pH was 7.5 to 8. Since DETP degradation produced acidic product(s), pH decreased significantly during the degradation reaction. The degradation rate at pH 6.5 was only 30 % of that obtained at pH 7.5.

4) Reaction kinetics for chlorferon and DETP were determined using enzyme kinetic models because cell growth was not observed during the degradation reactions. Chlorferon degradation followed substrate inhibition kinetics with parameter values estimated to be $V_m = 0.06 \pm 0.01$ mg/g-biomass· h, $K_m = 21 \pm 7$ mg/L, and $K_{Si} = 118 \pm 45$ mg/L. The DETP degradation reaction followed simple Michaelis-Menten kinetics with

parameter values estimated to be V_m = 1.52 ± 0.1 mg/g-biomass·h and K_m = 610 ± 106 mg/L.

5) An immobilized cell system was developed using the enriched cell cultures immobilized in Ca-alginate gel beads. Optimum bead loadings in the reactor were 20 % for chlorferon degradation and 30 % for DETP degradation.

6) Degradation rates were enhanced by immobilization. The chlorferon degradation rate for immobilized cells in UCD solution was approximately five times higher than for free cells. DETP degradation was conducted in MSYE medium and the degradation rate increase was 15 %. Similar results were found for coumaphos degradation using recombinant *E.coli* immobilized in Ca-alginate gel beads. The coumaphos degradation rate with immobilized *E.coli* was enhanced approximately 2.5 times in UCD solution but the rate increase in pure coumaphos solution was only approximately 15 %. Based on these results, it was concluded that the enhanced degradation of immobilized cells in UCD solution was due to protection of the cells from inhibitory substances present in UCD solution. Physiological changes of cells by Ca-alginate immobilization may have contributed to the slightly increased reaction rate in pure solution.

7) Diffusion coefficients for chlorferon and DETP into Ca-alginate gel beads were determined experimentally at the same conditions used for degradation studies. The diffusion coefficient for chlorferon in a cell-containing Ca-alginate gel bead (cell concentration of 80 g/L) was 3.08×10^{-11} m^2/s and for DETP (cell concentration of 60 g/L) was 4.28×10^{-11} m^2/s.

8) Diffusivities of chlorferon and DETP were influenced by several factors, such as viscosity of the bulk solution, agitation speed, and the concentrations of diffusing substrate and immobilized cells. Diffusion coefficients increased with increasing agitation speed and decreasing substrate concentration. Increased cell concentrations in gel beads caused lower diffusivity. Theoretical models to predict diffusivities as a function of cell weight fraction overestimated the effective diffusivities for both chlorferon and DETP. Linear relations were derived from experimental data as $\frac{D_{eff}}{D_{eff\,0}} = 1 - 2.5164\phi_{cw}$ for the diffusion of DETP and $\frac{D_{eff}}{D_{eff\,0}} = 1 - 2.314\phi_{cw}$ for the diffusion of chlorferon.

9) Calcium-alginate gel beads with radii of 1.65 – 1.7 mm used in this study were not subject to diffusional limitations. External mass transfer resistances were negligible based on Biot number calculations. Observable modulus and effectiveness factors indicated that internal mass transfer resistance was negligible and the degradation rate inside Ca-alginate gel beads was reaction-limited.

Studies of coumaphos degradation were extended in this research by developing systems for degradation of the hydrolysis products, chlorferon and DETP. However, further research is required to determine a complete degradation pathway, including fundamental biochemical and microbial information, for mineralization of coumaphos.

Although these studies demonstrated that the Ca-alginate immobilized cell systems enhanced the efficiency of bioremediation processes, a method to strengthen the

Ca-alginate gel beads is required since the beads weakened rapidly in UCD solution. Due to the nature of natural polysaccharides, Ca-alginate beads are weak in environmentally realistic conditions and their longevity is a major limiting factor for many applications. Other immobilization methods will be needed for large-scale applications.

NOMENCLATURE

Bi = Biot number (dimensionless)

D_{eff0} = Effective diffusivity of substrate in cell-free beads (m^2/s)

D_{eff} = Effective diffusivity of substrate in the gel bead (m^2/s)

D_{aq} = Diffusivity of substrate in the bulk liquid (m^2/s)

k_L = Liquid-solid mass transfer coefficient (m/s)

K_m = Substrate half-saturation constant (mg/L)

K_p = Partition coefficient (dimensionless)

K_{si} = Substrate inhibition constant (mg/L)

L_p = Initial cell concentration inside the particle (g-cell/g-bead)

m = Mass (g)

M_p = Bead loading in the reactor (g-beads)

N_{Re} = Reynolds number (dimensionless)

N_{Sc} = Schmidt number (dimensionless)

N_{Sh} = Sherwood number (dimensionless)

r = Radius (cm)

R = Gel bead radius ($0 \le r_p \le R$)

r_i = Radius of impeller in the reactor (cm)

S = Substrate concentration (mg/L)

V = Volume (L)

V_m = Maximum reaction rate (mg/g-biomass·h)

V_{mo} = Molar volume of substrate

X = Cell concentration (g-cell/L)

Greek Letters

Φ = Observable modulus (dimensionless)

ε = Porosity (dimensionless)

ϕ = Thiele modulus (dimensionless)

ϕ_c = Cell volume fraction

ϕ_{cw} = Cell weight fraction

η = Effectiveness factor (dimensionless)

μ = Viscosity

v = Reaction rate in the absence of mass transfer resistance

v_{obs} = Observed reaction rate in immobilized cell system

v_s = Slip velocity

ρ = Density

τ = Tortuosity (dimensionless)

Subscripts

0 = Initial

b = Bulk liquid

c = Cell

e = Cell-free

eq = Equilibrium

exp = Experimental

f = Free

i = Immobilized

p = Gel bead particle

pred = Predicted

r = Reactor

v = Void volume in gel bead particle

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