

**BIOREMEDIATION OF THE ORGANOPHOSPHATE  
METHYL PARATHION USING GENETICALLY ENGINEERED  
AND NATIVE ORGANISMS**

A Thesis

by

ADRIANA ZULAY DIAZ CASAS

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

August 2004

Major Subject: Biological and Agricultural Engineering

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August 2004

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## ABSTRACT

Bioremediation of the Organophosphate Methyl Parathion  
Using Genetically Engineered and Native Organisms. (August 2004)  
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Toxic waste disposal problems have become enormous due to the proliferation of xenobiotic compounds for use in agricultural, industrial and numerous other applications. Organophosphate (OP) pesticides are commonly used in agriculture and their toxicity is associated with inhibition of cholinesterase in the exposed organism. Some OPs have been shown to produce OP-induced delayed neuropathy (OPIDN). The overall goal of the work described in this thesis was to develop bacterial consortia to remediate hazardous substances at significantly higher rates than found with natural systems. Specifically, degradation of methyl parathion (MP) by hydrolysis with a genetically engineered *Escherichia coli* was investigated along with degradation of one of the resulting products, p-nitrophenol (PNP), by *Sphingobium chlorophenicum* ATCC 53874. Simultaneous degradation of both MP and PNP was investigated using a consortium of a genetically engineered *Escherichia coli* and a native *S. chlorophenicum*. Concentrations of MP and PNP were measured by high performance liquid chromatography (HPLC). Non-growing freely suspended recombinant OPH<sup>+</sup> *E. coli* cells efficiently degraded MP without addition of nutrients for growth. Maximum reactor productivity was found with a biomass concentration of 25 g/L. Substrate inhibition did not occur up to 3 g MP/L. The simple Michaelis-Menten kinetic model for enzymatic reactions provided a good fit of the degradation data with  $V_m=11.45 \mu\text{mol}/\text{min}\cdot\text{g}\text{-biomass}$  and  $K_m=2.73 \text{ g/L}$ . *B. cepacia* failed to degrade PNP under the experimental conditions evaluated, so further studies were not conducted. Growing cultures of *S. chlorophenicum* degraded PNP at concentrations up to 0.1 g/L

without a lag phase in mineral salts glutamate medium. Parameters such as initial pH, growth medium and growth stage for addition of PNP were important degradation factors. The bacterium exhibited substantial growth in the degradation process. Hydroquinone (HQ) or nitrocatechol (NC) were not identified as products of PNP degradation. The recombinant OPH<sup>+</sup> *E. coli* and *S. chlorophenicum* consortium failed to degrade PNP when starting with higher concentrations of MP. The presence of organic solvent in the bacterial consortium degradation medium negatively affected the degradation of PNP. The genetically engineered organism efficiently degraded high concentrations of MP, but the resulting high concentration of intermediate product (PNP) inhibited growth of the native type organism. Biodegradation by consortia of genetically engineered non-growing and native-type organisms generally will be limited by the growing native-type organism.

To my parents Carlos and Lucila;  
to my sisters Yanira, Milena, Tatiana;  
and to my brother Aldemar;  
their love has been the strength in my life.

(A mis padres Carlos y Lucila;  
a mis hermanas Yanira, Milena, Tatiana;  
y a mi hermano Aldemar;  
su amor ha sido la fuerza en mi vida).

To Angelos, who has brought happiness to my life

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## LIST OF ABBREVIATIONS

AChE - Acetylcholinesterase

ATCC – American Type Culture Collection

BT - Benzenetriol

DMTP-Dimethylthiophosphate

EPA- Environmental Protection Agency

GC- Guanine Cytosine

HPLC – High Performance Liquid Chromatography

HQ - Hydroquinone

LB - Luria-Bertani Broth

MP – Methyl Parathion

NACs – Nitroaromatic Compounds

NC – Nitrocatechol

Opd – Organophosphate Degrading

OP - Organophosphate

OPH – Organophosphate Hydrolase

OPIDN- Organophosphate Induced Delayed Neuropathy

PCP- Pentachlorophenol

PNP – p-Nitrophenol

## CHAPTER I

### INTRODUCTION

During the last century advances in synthetic chemistry have given chemists the ability to make numerous novel compounds, some of which are xenobiotic (Xu et al., 1999). The release of xenobiotic compounds into the environment and the problem of toxic waste disposal have become enormous due to the proliferation of these xenobiotic compounds for use as pesticides, solvents, explosives, refrigerants and dyes in industrial, urban and agricultural applications (Doung et al., 1997). Many xenobiotic compounds, particularly those used as insecticides, are toxic (Xu et al., 1999). Even though many insecticides degrade rapidly in the soil, they can be potentially hazardous as a consequence of accidental spills, runoff from applications in agricultural areas, and discharge from pesticide containers and waste disposal systems (Rani and Lalithakumari, 1994). Hertel (1993), Shimazu et al. (2001) and Zhongli et al. (2001) mention that organophosphate (OP) compounds represent the largest group of chemical insecticides used in plant protection throughout the world. In the United States, OPs represent about half of the total insecticides used, with annual applications over 75 million pounds (Bravo et al. 2002). Because of the rapid growth in industrial and agricultural chemical usages, vast quantities of soil and groundwater have been contaminated with hazardous compounds (Chauhan et al., 2000). Recent analyses of groundwater have indicated that the general population is not adequately protected from pesticide contamination (Walker and Keasling, 2002) and that pesticides are found in groundwater sooner than generally can be predicted with traditional management models (Pivetz et al., 1996).

The United States, as a signatory to the 1993 Chemical Weapons Convention, has over 30,000 metric tons of chemical warfare agents slated to be destroyed by incineration methods.

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This thesis follows the style of *Transactions of the ASAE*.

Incineration and chemical treatment are the currently approved methods for disposal of these materials. However, the destruction of chemical warfare agents by incineration has significant political barriers, and incineration facilities currently used have had discharges of chlorinated organics, heavy metals and unreacted chemical warfare agents in smoke-stack emissions (Walker and Keasling, 2002). Given the potential environmental disadvantages of physical and chemical detoxification methods, biodegradation would appear to be a more acceptable alternative. This issue is of such social importance that predictions by the Organization for Economic Cooperation and Development indicated that the worldwide market demand for bioremediation technologies would reach \$75 billion (Doung et al., 1997).

With the discovery in the 1960s that many soil microorganisms are capable of metabolizing xenobiotic compounds, the use of biological processes to degrade hazardous materials became a viable and acceptable possibility. The use of microbial metabolic pathways poses a potential for elimination of environmental pollutants since it provides a safe and economic alternative to disposal in landfills and to physico-chemical strategies (Pieper and Reineke, 2000). However, biodegradation rates usually are slow because of the novelty of these compounds for the microbes. Since xenobiotics do not occur in nature, microorganisms have not evolved appropriate metabolic pathways to metabolize them, causing growth to be slow (Xu et al., 1999 and Pieper and Reineke, 2000). Some xenobiotics are transformed incompletely or inefficiently, and no degradative routes have evolved for others. Where degradation pathways exist, complex mixtures of contaminants often prevent degradation (Pieper and Reineke, 2000). In addition, complete biodegradation often requires a consortium of organisms since a single species of organism generally is not capable of metabolizing all the products resulting from the initial biodegradation reactions. Besides these factors, organisms capable of degradation must be present at the site where they are needed, and they must perform under available environmental conditions (Pieper and Reineke, 2000).

Thus, the vast accumulation of xenobiotic compounds in the environment corroborates the fact that the natural metabolic diversity of autochthonous microbes is not sufficient to protect the biosphere from anthropogenic pollution. Therefore, continued effort is needed to develop biodegradation processes to break down environmental pollutants to nontoxic by-products (Pieper and Reineke, 2000).

The overall goal of this work was to develop a bacterial consortium for remediation of hazardous substances at significantly higher rates than currently is obtained with native organisms. Various genetic approaches have been developed and used to optimize the enzymes, metabolic pathways and organisms relevant for biodegradation (Pieper and Reineke, 2000). Among these, Kim (1998) and Kim et al. (2002) investigated the degradation of coumaphos using a recombinant strain of *Escherichia coli* containing the organophosphate degrading (*opd*) gene for organophosphate hydrolase (OPH). Significantly higher degradation rates were obtained compared to those rates obtained with the microbial consortium naturally present in coumaphos dip waste. Also, Cho (2001) successfully and rapidly eliminated the genotoxic potential and toxicity of methyl parathion (MP) by OPH. Our approach was to use a multi-step process in which the selected microorganisms were grown rapidly under favorable conditions to achieve a high cell density then used for biodegradation of MP, a model OP compound. The strategy is based on a rational combination of organisms to provide different catabolic pathways. Ultimately, complete metabolic routes for xenobiotics may be achieved and the formation of toxic metabolites can be avoided.

This study focused on degradation of MP and one of its degradation products, p-nitrophenol (PNP). MP is an OP insecticide that creates potential human risk. PNP and dimethylthiophosphate (DMTP) have been shown to be the hydrolysis products of MP using the OPH enzyme (Shimazu et al., 2001). PNP, like MP is toxic to human health (Walker and Keasling, 2002).

## 1. Organophosphate Compounds

The agricultural industry has been using chemical pesticides since the early 1900's. German chemists developed a new class of pesticides, OP compounds, during the Second World War, some of which also proved to be chemical warfare agents. Since then, more than 1,000 OP compounds have been synthesized. Some have been recognized to be useful OP pesticides and others have been stockpiled as potent chemical warfare agents. Stockpiles of OP chemical warfare agents in the United States are estimated to be about  $2.5 \times 10^4$  tons, while the Soviet Union inventories are about  $4 \times 10^4$  tons. The immediate disposal of these agents has received worldwide approval; however, projected dates for the complete detoxification of these hazardous chemicals are questionable (Caldwell, 1991).

OP pesticides are a group of highly toxic agricultural chemicals extensively used in plant protection. OP pesticides such as parathion, MP, and methamidophos, are widely used around the world despite their high toxicity (Zhongli et al., 2001). Over 40,000 tons of OP pesticides are applied in the United States annually (Shimazu et al., 2001). It has been estimated that each year up to 800,000 individuals are affected by pesticide exposure. OP pesticides are of particular interest since they represent the major proportion of agricultural pesticides utilized today. Poisoning might occur from both occupational or incidental exposure where absorption occurs by direct contact with air, food and water (Caldwell, 1991).

Currently, incineration is the most efficient method for disposal of chemical compounds. However, environmental groups have opposed incineration as a disposal method because of the release of byproducts which may pose significant environmental hazards. Transportation of these toxic compounds from storage to disposal sites is both time consuming and dangerous, considering the threat of potential accidents. Further as expected, the population has openly opposed the installation of more incinerators within their communities near storage sites (Caldwell, 1991). There are several other methods of disposal available, but most of these have some undesirable limitations. Landfills are an adequate method, but



leaching into the soil and groundwater may cause further contamination (Caldwell, 1991). Other disposal methods involve removal, alteration, or contaminant isolation. Usually, for treating contaminated soil, these techniques consist of excavation followed by incineration or containment. Moreover, these remediation technologies are expensive, and most of the time they do not destroy the contaminating compound but rather transfer it from one environment to another (Hurst et al., 1997).

### 1.1 Methyl Parathion

MP (*O*-dimethyl*O*-(4-nitro-phenyl) phosphorothioate) has a molecular formula of  $C_8H_{10}NO_5PS$  with a molecular mass of 263.23. Pure MP is a white crystalline solid or powder; technical (80%) grade is a light to dark tan liquid. Pure MP solubility in water is 55-60 mg/L at 25 °C; it is readily soluble in most organic solvents, soluble in ethanol, chloroform and aliphatic solvents, and slightly soluble in light petroleum. The odor of MP is like rotten eggs or garlic. Technical MP is available as a solution containing 80% active ingredient, 16.7% xylene, and 3.3% inert ingredients (Hertel, 1993). MP is one of the highly active thiophosphorus ester insecticides developed in the 1940s by the German pesticide company Bayer. MP is produced worldwide by many companies. The production of MP in 1966 was 31,700 tonnes, from which 14,800 tonnes were produced in the United States (Hertel, 1993). It is registered in at least 38 countries and widely used throughout the world (Pesticide News, 1995).

MP is used to control chewing and sucking insects such as aphids, boll weevils, and mites in a wide variety of crops, including cereals, fruits, vines, vegetables, ornamentals, cotton and field crops (Pesticide News, 1995; Garcia et al., 2003). It is a broad-spectrum non-systemic pesticide that kills pests by stomach poisoning, is an acaricide, and has some fumigant action (Garcia et al., 2003; Pesticide News, 1995; Hertel, 1993). MP was originally registered in the United States in 1954. However, its use has been restricted in this country since 1978. MP is used throughout the southern and Midwestern parts of the United States. As a restricted-use pesticide, the application of MP requires appropriately trained certified pesticide applicators.

Recently, the use of MP has voluntarily been cancelled in many of the most significant food crops by the United States Environmental Protection Agency (EPA). In the United States, annual agricultural application peaked at 12,503 metric tons in 1971 and was 3,471 metric tons in 1982 (Garcia et al., 2003).

Contamination of fields, crops, water and air occurs through off-target spraying as a consequence of agricultural and forestry activities. For this reason exposure may occur through air, water and food-borne residues of MP (Hertel, 1993). MP is not very persistent in the environment; it is not bioconcentrated and is not transferred through food-chains. It is efficiently degraded by a number of microorganisms and other forms of wild life. This insecticide is likely to cause damage to ecosystems only in instances of heavy over-exposure resulting from accidental spills (Hertel, 1993).

Degradation of MP appears to be faster in the presence of sediment and in fresh water rather than salt water. The rate of degradation depends on the presence and acclimation of microbial populations in the body of water (Exttoxnet, 1996). MP has a half-life in water environments of 175 days, and 10 days to two months in soils. Temperature and exposure to sunlight increases the rate of degradation. When large concentrations of MP reach the soil, for instance in an accidental spill, degradation will occur only after many years (Pesticide News, 1995). MP does not volatilize significantly at ambient conditions (Exttoxnet, 1996). Oxidative degradation of MP to the less stable methyl paraoxon may occur by ultraviolet radiation or sunlight. MP also undergoes chemical hydrolysis in aquatic environments; however, this degradation reaction is not a significant contribution to the disappearance of MP. In towns in the center of agricultural areas in the United States, MP concentrations in natural waters ranged up to 0.46 µg/L, with the highest levels found during the summer (Hertel, 1993).

## **2. p-Nitrophenol**

Nitroaromatic compounds (NACs) are widely distributed in the environment because of their numerous applications. They are extensively used as herbicides, fungicides, insecticides, explosives and precursors for dyes and plasticizers. They are also released into the environment as hydrolytic products of some OP insecticides or industrial wastes (Bhushan et al., 2000). Most nitrophenols find their way into the environment during manufacturing and processing (Bhatti et al., 2002). Of these NACs, PNP is a manufactured chemical that does not occur naturally in the environment. PNP is used as a fungicide for leather and has applications in engineering polymers (Bhatti et al., 2002).

PNP readily breaks down in surface waters but takes a long time to degrade in deep soil and in groundwater. Therefore, PNP accumulates in the soil as a product from the hydrolysis of various OP insecticides, such as parathion and MP, and nitrophenolic herbicides. As a result of this accumulation, PNP may enter rivers and groundwater resources where it can cause adverse effects to ecosystems due to its acute toxicity (Bhatti et al., 2002, Chauhan et al., 2000, and Bhushan et al., 2000). Because of its toxicity, its removal from water resources has been widely studied (Bhatti et al., 2002). Moreover, nitrophenols are considered priority pollutants by the EPA. PNP has been widely studied as a model for biodegradation of nitrophenols because it can be found in both terrestrial and aquatic environments (Leung et al., 1997 and Leung et al., 1999).

## **3. Health Effects of Organophosphate Compounds**

The primary biochemical effect associated with high level exposure to OP insecticides is inhibition of acetylcholinesterase (AChE), resulting in acetylcholine accumulation (Hertel, 1993; Shimazu et al., 2001 and Garcia et al., 2003). The normal function of AChE is to terminate neurotransmission due to acetylcholine; it is liberated at cholinergic nerve endings in response to nervous stimulation. Loss of AChE activity may lead to a variety of effects resulting from excessive nervous stimulation,

culminating in respiratory failure and death. Hypotension, bradycardia, bronchoconstriction, and bronchial fluid accumulation, symptoms that result from the inability of respiratory muscles to work, are observed following lethal amounts of MP. Cyanosis and central respiratory depression can be observed as well. In less severe cases of intoxication, bradycardia, muscle rigidity, muscle hypotonia, bronchial spasm, and constriction might occur (Hertel, 1993). Clinical symptoms of poisoning with MP include pallor, sweating, dizziness, vomiting, diarrhea, abdominal cramps, headache, blurred vision, convulsions, dilation of the pupils, tears, salivation, cardiac arrest, and, in extreme cases death (Pesticide News, 1995 and Garcia et al., 2003).

MP is a highly toxic OP ester insecticide (Hertel, 1993). It is easily absorbed via all routes of exposure (oral, dermal, inhalation) and is rapidly distributed to the tissues of the body. Conversion of MP to methyl paraoxon occurs within minutes of administration. Methyl paraoxon is 10 times more toxic than MP (Dzyadevych et al. 2002). Metabolism and detoxification occur through the liver. The mechanisms of detoxification of MP or methyl paraoxon are mainly through oxidation, hydrolysis, and demethylation or dearylation with reduced glutathione (Hertel, 1993). Effects such as impaired memory and concentration, disorientation, severe depression, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking, drowsiness and insomnia have been reported in workers repeatedly exposed to MP. Studies suggest that there is a decrease in blood cholinesterase activity without clinical manifestations following repeated, long-term exposures (Hertel, 1993).

Some OP compounds also may cause a delayed neurotoxicity called OP induced delayed neuropathy (OPIDN) (Cho, 2001). MP has also been reported to have DNA-alkylating properties. Results of mutagenicity tests have been both positive and negative. The results of most of the *in vitro* mutagenicity studies with both bacterial and mammalian cells were positive; whereas, the *in vivo* studies produced equivocal results (Hertel, 1993). The genotoxicity of MP was later confirmed by Cho (2001). Although the toxicity and the

degradation of MP have been documented in many previous studies, the health effects of DMTP, one of its hydrolysis products is not well known (Cho. 2001). PNP, the other hydrolysis product is toxic to plants, animals and humans. Studies on animals suggest that PNP may cause a blood disorder. Acute exposure to PNP may lead to methemoglobin formation, liver and kidney damage, anemia, skin and eye irritation, and systemic poisoning. PNP is a suspected carcinogen (Walker and Keasling, 2002).

#### **4. Biodegradation/Bioremediation**

Bioremediation overcomes the limitations of traditional methods of hazardous chemical disposal by bringing about the actual destruction of many organic contaminants at reduced cost. In consequence, over the last 20 years, bioremediation has grown from a virtually unknown technology to a technology that is considered for the remediation of a wide range of contaminating compounds (Hurst et al., 1997). Characteristics of microorganisms such as their small size, ubiquitous distribution, high specificity, surface area, potentially rapid growth rate and unrivaled enzymatic and nutritional versatility cast them as recycling agents. Moreover, the diversity of inorganic and organic materials present on Earth match diversity of habitats whose physical and chemical characteristics span wide ranges of pH, temperature, salinity, oxygen tension, redox potential, water potential, etc. This distribution of resources between environments gave origin to a selective evolutionary diversification of microorganisms, resulting in an evolved microbial world capable of exploiting all the naturally occurring metabolic resources on Earth (Hurst et al., 1997).

Biodegradation is a metabolic process that involves the complete breakdown of an organic compound. When this compound is broken down into its inorganic components, the process is referred to as mineralization. Xenobiotic compounds are those having a molecular structure to which microorganisms have not been exposed; therefore, they may be recalcitrant or resistant to bioremediation or not completely degraded. Biodegradability represents the susceptibility of substances to be altered by microbial processes. The alteration may occur by intra- or extracellular enzymatic attack that is essential for growth of the microorganisms.

The attacked substances are used as a source of carbon, energy, nitrogen, or other nutrients or as final electron acceptor. Cometabolic reactions occur when enzymatic attack is not necessarily beneficial to the microorganism, i.e., a physiologically useful primary substrate induces production of enzymes that fortuitously alter the molecular structure of another compound (Hurst et al., 1997).

Biodegradation may occur under either aerobic or anaerobic conditions. Under aerobic conditions, oxygen is used as a final electron acceptor; and under anaerobic conditions, microorganisms use compounds such as nitrate ( $\text{NO}_3$ ), sulfate ( $\text{SO}_4^{2-}$ ) or iron ( $\text{Fe}^{3+}$ ) as the final electron acceptor instead of oxygen (Hurst et al., 1997). The rate of biodegradation depends on environmental factors, numbers and types of microorganisms present, and the chemical structure of the target compound (Hurst et al., 1997). The process of biodegradation generally involves the breakdown of organic compounds to produce more cell biomass and less complex compounds which are further converted to water and either carbon dioxide or methane.

Biodegradation can be intrinsic if the appropriate environmental conditions, nutrients and microorganisms are present. This is the preferred method because of its lower cost, but enhanced (engineered) bioremediation may be required if the process is not naturally sustained and the rates of degradation are low (Hurst et al., 1997).

#### **4.1 Methyl Parathion Degradation by Different Bacteria**

In nature, microorganisms have evolved degradative pathways as a result of continuous or repeated exposure to xenobiotic chemicals such as OPs. However, because of the enhancement of microbial degradation of many chemicals, the efficacy of several pesticides including OPs has been reduced (Rani and Lalithakumari, 1994). In particular, loss of insecticidal activities has been reported in soils that have received continuous applications, resulting in the enhanced degradation of these compounds by soil microorganisms (Chaudhry

et al., 1988). Ramanathan and Lalithakumari (1996) showed that some microorganisms can use MP as a carbon source. Studies on a natural microbial community showed that concentrations of MP up to 5 mg/L increased biomass and reproductive activity, but higher soil concentrations were found to reduce microbial reductive potential. A MP positive effect was observed in bacteria and actinomycetes, while fungi and yeasts were less able to utilize the compound (Hertel, 1993).

Adhya et al. (1981) reported the rapid hydrolysis of MP and fenitrothion by a *Flavobacterium* sp. Sharmila et al. (1989) stated that MP is susceptible to degradation by hydrolysis to PNP and DMTP in soil and water environments, by nitro group reduction to methyl aminoparathion, or both. Hydrolysis is the leading pathway in non-flooded soil, while MP is degraded essentially by nitro group reduction in predominantly anaerobic ecosystems such as flooded soil. In a few instances, hydrolysis is the major or only pathway of MP degradation in soils even under flooded conditions (Sharmila et al. 1989). Chaudhry et al. (1988) isolated two mixed bacterial cultures by soil enrichment that were capable of utilizing MP as a sole source of carbon. The results from their study indicated that mixed cultures are more stable in retaining their ability to completely degrade MP than are isolated bacteria. Misra et al. (1992) has isolated a *Flavobacterium* sp. (ATCC 27551) from diazinon-treated rice fields that could use MP as a sole source of carbon. A *Bacillus* sp. was isolated from MP-treated flooded soil but required yeast extract for the degradation of MP in a mineral salts medium. Rani and Lalithakumari (1994) and Ramanathan and Lalithakumari (1999) investigated the degradation of MP by *Pseudomonas putida* and *Pseudomonas* sp. A3, respectively, finding that MP is utilized as both carbon and phosphorus sources by both bacteria. *Pseudomonas putida* also utilized one of the intermediates of MP degradation, PNP, as a sole carbon source. Ramanathan and Lalithakumari (1999) reported that *Pseudomonas* sp. A3 completely removed an initial concentration of 0.25 g/L MP from the medium in 40 h; however, when the initial concentration was 1 g/L, only 45% of the MP was degraded in 56 h, which gave a degradation rate of 0.51  $\mu\text{mol/L}\cdot\text{min}$ .

An alternative approach to degradation of OPs is to use enzymes produced by these microorganisms. Shimazu et al. (2001) isolated an OPH from soil microorganisms that has been shown to hydrolyze a wide range of OP pesticides. Enzymatic hydrolysis of MP leads to the formation of DMTP and PNP as byproducts (Fig. I-1), thus reducing its toxicity by 120-fold (Shimazu et al., 2001). The gene sequence of OP pesticide hydrolase genes from *P. diminuta* GM and *Flavobacterium* sp. Strain ATCC 27551 have been studied. The two genes were found to share 86.3% identity on the nucleic acid level within the coding region (Zhongli et al., 2001).

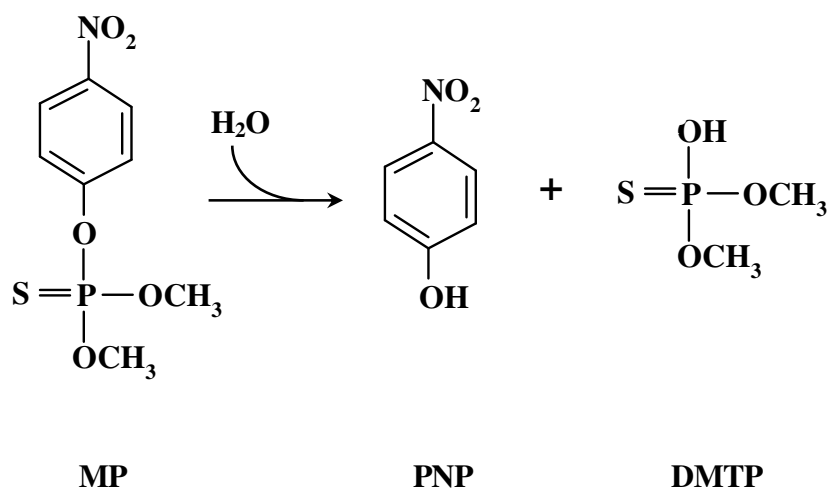


Figure I-1. MP hydrolysis pathway. MP, methyl parathion; PNP, p-nitrophenol; DMTP, dimethylthiophosphate (Shimazu et al., 2001).



## 4.2 Organophosphate Hydrolase

The OP trimesters and phosphofluoridates which are used as pesticides or chemical warfare agents have existed for only about fifty years. Since the early 1960s, it has been observed that soil microbial communities are capable of metabolizing pesticides by enzymatic reactions (Caldwell, 1991). OPH isolated from soil microorganisms has been shown to have a wide range of hydrolytic capability on OP pesticides. MP and parathion toxicity can be reduced by nearly 120-fold by enzymatic hydrolysis, which leads to the formation of dialkylthiophosphates and PNP as byproducts (Shimazu et al., 2001). In general, the hydrolytic cleavage of the OP bond is the initial step in OP metabolism. This reaction is of considerable importance because it renders the molecule biologically inactive (Rani and Lalithakumari, 1994).

OPH activity was first detected in the soil bacterial strain, *Pseudomonas diminuta*, by observation of parathion hydrolysis. The hydrolytic enzyme activity was later found to be associated with a protein encoded by a plasmid-born gene (Caldwell, 1991). *Flavobacterium* sp. ATCC 27551 also has been shown to possess this hydrolytic enzyme activity. The *opd* gene, which expresses OPH activity, from both microbial strains was reported to be homologous by hybridization studies (Cho et al., 2000). The functional significance of the plasmid-born gene expressing OPH has not yet been reported. Nonetheless, many OP pesticides are degraded enzymatically in the soil and the resultant metabolites have been characterized. Bacteria, yeast, fungi and mammals have shown hydrolysis of OP pesticides. However, only OPH from *Pseudomonas diminuta* has been fully characterized (Caldwell, 1991).

OPH has a number of advantages over many di-isopropyl-fluorophosphatases, OP hydrolases, or paraoxonases. OPH possesses much broader substrate specificity and will tolerate significant variation in the structure of its substrates. This enzyme is capable of hydrolyzing OP compounds, including those that have P-O bonds as in diethylparaoxon (“paraoxon”), P-S bonds as in malathion, P-N bonds as in acephate, and P-CN bonds as in tabun (Fig. I-2). OPH is also capable of hydrolyzing sarin, soman and mipafox through the P-F bond (Dave et al., 1993). OPH has been shown to hydrolyze a broad spectrum of OP trimesters, thioesters and fluorophosphonates in both whole cell extracts and as the purified enzyme. The hydrolytic capability of this enzyme on OPs is extremely high (Dave et al., 1993).

Because of the high catalytic rate of OPH and its capability to hydrolyze a wide variety of OP pesticides and chemical warfare agents, this enzyme has generated significant interest in its potential for bioremediation applications. The use of living systems where natural biological processes are responsible for degradation has been exploited in the detoxification of chemical wastes (Caldwell, 1991). This process requires maintenance of the organism; however, the direct utilization of specific enzymes can provide another feasible alternative. The *opd* gene encoding OPH in *Pseudomonas diminuta* has been extensively studied (Hong, 1997). The gene has been expressed in *E. coli* to determine its feasibility as an active component in stable systems capable of degrading OP compounds (Hong et al., 1998).

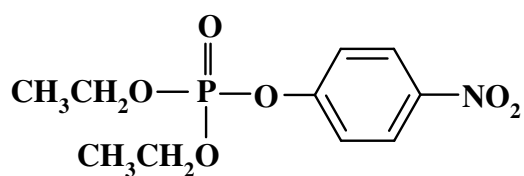
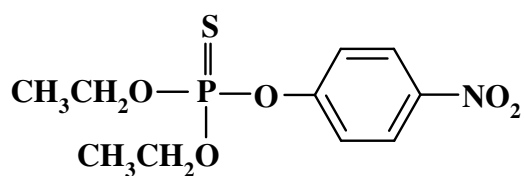
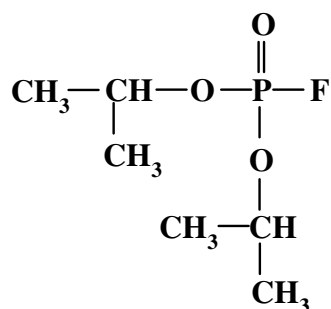
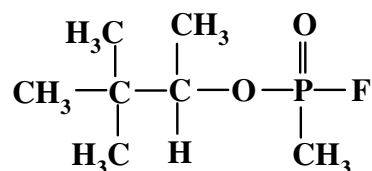
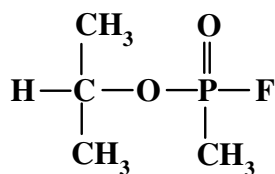
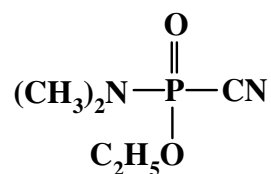
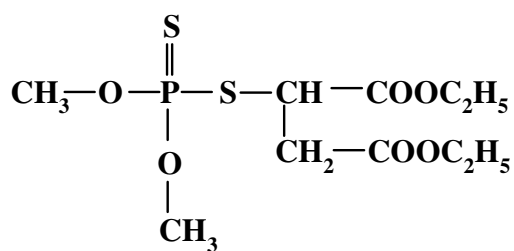
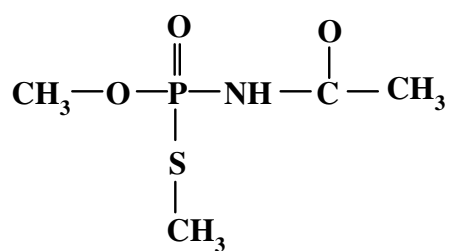
**Paraoxon****Parathion****Diisopropylphosphorofluoridate****Soman****Sarin****Tabun****Malathion****Acephate**

Figure I-2. Structure of representative phosphotriester and phosphonate substrates subject to enzymatic hydrolysis by organophosphate hydrolases (Dave et al., 1993 and Hong, 1997).

### 4.3 p-Nitrophenol Degradation by Different Bacteria

Biodegradation of nitrophenols has been well documented as well as their respective metabolic pathways; however, little information is available on kinetics of PNP degradation and effects of metabolic intermediates on its degradation. The toxicity and the poor biodegradability exhibited by these compounds are the main disadvantages in the application of bioremediation for their treatment (Bhushan et al., 2000). Bhushan et al. (2000) studied the rate of PNP degradation by different PNP-degrading bacteria in relation to the accumulation of metabolic intermediates and their effects on PNP degradation. Bhushan et al. (2000) also, investigated PNP degradation kinetics by *Ralstonia* sp. SJ98, *Arthrobacter protophormiae* RKJ100, and *Burkholderia cepacia* RKJ200. They found that all three bacteria utilized PNP as a sole carbon and nitrogen source and that *Ralstonia* sp. SJ98 showed the highest values for both substrate saturation constant and maximum degradation rate. Chauhan et al. (2000) discovered that *Arthrobacter protophormiae* strain RKJ100 was capable of utilizing PNP as well as 4-nitrocatechol (NC) as carbon and nitrogen sources.

Although the nitro group of PNP enhances the resistance of the aromatic ring to biodegradation, bacterial strains capable of degrading PNP have been isolated (Chauhan et al., 2000 and Leung et al., 1997). Usually, aromatic compounds are activated for further reactions by the introduction of two hydroxyl-groups, either in *ortho*- or *para*-position to one another, which in the case of hydrophobic aromatics is usually achieved by multi-component dioxygenases (Pieper and Reineke, 2000). Nitroaromatic compounds can be degraded by aerobic bacteria through a variety of oxidative or reductive pathways. Nitroaromatic compound degradation pathways involve either the removal or the reduction of the nitro group and conversion of the resulting molecule into a substrate for oxidative ring fission (Hurst et al., 1997).

Thus, two major pathways have been proposed for the aerobic bacterial degradation of PNP. Aerobic degradation of PNP can be initiated by formation of either hydroquinone (HQ) or

NC (Chauhan et al., 2000). The HQ degradation pathway, found in *Moraxella* sp. and *Pseudomonas putida* involves the formation of HQ by an initial monooxygenase-catalyzed reaction where the nitro group of PNP is replaced by an -OH group with the release of nitrite. HQ is then converted to beta-ketoadipic acid via gamma-hydroxymuconic semialdehyde and maleylacetic acid (Spain and Gibson, 1991). The NC degradation pathway, found in *Arthrobacter* sp., *Flavobacterium* sp. and *Rhodococcus* sp. involves formation of NC by a 2-monooxygenase followed by removal of the nitro group as nitrite via a NC-4-monooxygenase (Leung et al., 1997). However, Chauhan et al. (2000) who studied degradation of PNP and NC by *Arthrobacter protophormiae* RKJ100 found that PNP degradation proceeded with the formation of p-benzoquinone (BQ) and HQ and was further degraded via the beta-ketoadipate pathway.

Among the bacterial strains able to degrade PNP, Barik et al. (1978) isolated a particular strain of *Pseudomonas* sp., *Burkholderia cepacia* ATCC 29354, from parathion-amended flooded soil. The organism degraded PNP via a pathway involving ring hydroxylation with NC as the stable intermediate. No growth of the bacterium was observed despite degradation of PNP. Doung et al. (1997) investigated the stoichiometry and kinetics of the hydroxylation of PNP to NC by the same bacterial strain in a batch system. It was noted that PNP removal did not correspond to an accumulation of NC, implicating another relatively short-lived biochemical pathway for the breakdown of PNP. Spain et al. (1984) studied the acclimation of microbial communities exposed to PNP in laboratory test systems and in a freshwater pond finding that degradation of PNP required acclimation in both cases. Zaidi and Mehta (1995) studied the degradation of PNP and the effect of a supplementary carbon source on PNP degradation by *Corynebacterium* ZA, *Pseudomonas* MS and *Pseudomonas* GR in lake and industrial wastewater. Their results suggested that a supplementary carbon source increases or decreases the rate of degradation depending on the bacteria.

Several studies of immobilized cells for PNP degradation have been carried out. Errampalli et al. (1999) observed the survival of free and immobilized cells of *Moraxella* sp. G21 in

sterile and non-sterile soil contaminated with PNP. Cells survived for a month, and immobilized *Moraxella* sp. G21 cells and free cells produced similar PNP mineralization values. Bhatti et al. (2002) studied the use of nonwovens as a cell retainer for PNP degradation in a continuous flow system. It was found that cultivation of the PNP-degrading cells prior to attachment was necessary to achieve degradation and that the use of nonwoven supports allowed consistent high-rate PNP degradation. In another study, plasmid-harboring operons encoding enzymes for PNP transformation to p-ketoadipate were transformed into *P. putida* allowing the organism to use 0.5 mM PNP as a carbon and energy source (Walker and Keasling, 2002).

#### 4.3.1 Aromatic Compound Degrading Bacteria

Crawford (1987) discovered certain bacteria of the genus *Flavobacterium* able to degrade PCP, a *Sphingobium chlorophenicum* deposited as *Flavobacterium* sp. (ATCC 53874) and a redeposit of *Sphingobium chlorophenicum* ATCC 39723. Several Gram-negative PCP-degrading bacteria previously classified as *Flavobacterium* sp. have been reclassified as *Sphingomonas* sp. (Leung et al., 1999). Cassidy et al. (1999) state that members of the genus *Sphingomonas* can degrade environmentally important xenobiotics, including chloro- and nitro-phenols and that some *Sphingomonas* sp. have multiple mechanisms for dehalogenation, transformation of nitroaromatics and subsequent aromatic ring cleavage of these compounds. PCP-4-monooxygenase from *S. chlorophenicum* ATCC 39723 exhibits broad substrate specificity. This enzyme may also hydroxylate the para position of halo-, nitro-, amino- and cyano-substituted phenols, forming hydroquinones and releasing halide, nitrite, hydroxylamine and cyanide, respectively (Xun et al., 1992 and Cassidy et al., 1999). PCP-4-monooxygenase was reported to catalyze the transformation of PCP to 2,3,5,6-tetrachlorohydroquinone (Leung et al., 1997 and Lange et al., 1996). However, later Dai et al. (2003) showed that the first step in the PCP degradation pathway in *S. chlorophenicum* is the conversion to tetrachlorobenzoquinone.

Leung et al. (1997) investigated the transformation of PNP by *S. chlorophenolicum* UG30 finding that NC is an intermediate product. This suggested that PCP-4-monooxygenase does not catalyze the first step of PNP transformation by this strain (Leung et al., 1997). To verify this, Leung et al. (1999) cloned the gene encoding PCP-4-monooxygenase from *S. chlorophenolicum* UG30 to study its potential role in PNP degradation, confirming that PCP-4-monooxygenase is not the primary enzyme in the initial step of PNP metabolism by *S. chlorophenolicum* UG30 but indicating that the enzyme may be involved in the second step of PNP degradation. Cassidy et al. (1999) examined the ability of several PCP-mineralizing strains (*S. chlorophenolicum* UG30, and *S. Chlorophenolicum* ATCC 39723 and RA2) to metabolize PNP. All of the strains degraded PNP with nitrite release. This suggests that UG30 degrades PNP by the NC pathway.

Dechlorination of PCP by bacteria has been extensively studied (Leung et al., 1999 and Wang et al., 2002). Xu et al. (1999) studied the effect of metals and found that only  $\text{Fe}^{2+}$  supported enzyme activity. Topp et al. (1988) and Chang and Su (2003) studied the effect of carbon source on PCP degradation indicating that supplementary carbon decreased acclimation time and facilitated PCP metabolism by glucose or glutamate-grown *Flavobacterium* cells and that, as expected, yields were higher when cells received more carbon source. Leung et al. (1997) studied the transformation of PNP by PCP-degrading *S. chlorophenolicum* UG30 and *S. chlorophenolicum* strains RA2 and ATCC 39723 in either mineral salts-glutamate or mineral salts-glucose medium. It was observed that mineralization of PNP only occurred in mineral salts-glucose medium. Particular studies with UG30 showed that this strain did not transform or mineralize PNP in a growth medium lacking glucose or glutamate and that pre-exposure of UG30 cells to PNP eliminated an initial lag phase in PNP transformation (Leung et al., 1997). Later, Alber et al. (2000) evaluated degradation of mixtures of PCP and PNP by *Sphingomonas* sp. Rapid disappearance of both substrates at concentrations of 0.1 g/kg soil and higher were observed, and occasionally complete degradation of PNP occurred.

Crawford and Ederer (1999) described the genus *Sphingomonas* as Gram-negative, non-spore-forming, aerobic, yellow-pigmented, straight rods. It was also mentioned that the yellow pigmentation in *Sphingomonas* is due to the carotenoid nostoxanthin. Also, members of this genus are characterized by the presence of sphingoglycolipids. *Sphingomonas* species also contain octadecanoic acid, 2-hydroxymyristic acid, cis-9-hexadecenoic acid, and hexadecanoic acid as major fatty acids, the ubiquinone Q10 as the major respiratory quinone, and DNA containing 62-67% GC (Table I-1). Organisms in the genus *Sphingomonas* secrete gellan-related polysaccharides. Finally, Crawford and Ederer (1999) mention that this genus is best grown on dilute media. The four *Sphingomonas* species shown in Table I-1 were characterized by a very similar protein profile and a slow growth rate (Crawford and Ederer, 1999).

Table I-1. Phenotypes of the PCP-degrading *Sphingomonas* species.

Strain	Origin (USA)	PCP (mg/L)	Shape	Gram stain	Pigment	Motility	Fimbriae	Sphingolipids	Octadecenoate	%GC
ATCC 33790	NY	300	Rod	Neg	+	-	Nd	+	62	66±1
ATCC 39723	MN	100-200	Rod	Neg	+	-	+	+	57	66±1
SR3	FL	175	Rod	Neg	-	+	+	+	59	64.2
RA2	CO	300	rod	neg	+	+	nd	+	62	64±1

+ or – indicates the presence or absence of a characteristic. The presence of sphingolipids and octadecenoate is indicative of the genus *Sphingomonas* (Crawford and Ederer, 1999).

Although many of the previous studies on degradation of MP and PNP provide excellent information about the bacteria involved and degradation pathways, none have directly studied the hydrolysis kinetics of MP by recombinant *Escherichia coli* or have fully assessed the degradation of PNP by *Burkholderia cepacia* and *Sphingobium chlorophenicum*. This research has studied the effect of biomass and substrate concentrations on the degradation of



MP by recombinant *Escherichia coli* and has established conditions for degradation of PNP by *Burkholderia cepacia* and *Sphingobium chlorophenicum*.

The overall purpose of this study was to develop a bacteria consortium for the remediation of hazardous chemicals using MP and its hydrolysis product PNP as model compounds. Hydrolysis of MP can be accomplished using a dense, non-growing population of a recombinant OPH<sup>+</sup> *E. coli* strain that functions without the addition of nutrients required for growth. Degradation of PNP can be accomplished using *Burkholderia cepacia* deposited as *Pseudomonas* sp. (ATCC 29354) and *Sphingobium chlorophenicum* deposited as *Flavobacterium* sp. (ATCC 53874).

The specific objectives of this study were to

1. evaluate hydrolysis of the organophosphate methyl parathion by recombinant *Escherichia coli*
2. assess the degradation of p-nitrophenol by *Burkholderia cepacia* and *Sphingobium chlorophenicum* and
3. assess the degradation of both methyl parathion and p-nitrophenol by a bacterial consortium.

## CHAPTER II

### BIODEGRADATION OF THE ORGANOPHOSPHATE METHYL PARATHION BY RECOMBINANT *ESCHERICHIA COLI*

#### 1. Overview

The widespread use of organophosphate (OP) compounds, such as methyl parathion (MP), in agricultural, urban, and industrial applications poses an environmental challenge for remediation and detoxification of OP neurotoxins. It has been suggested that biodegradation becomes an attractive option for destruction of OP since it utilizes a natural process and offers the potential for less costly treatment. However, biodegradation rates usually are slow because the compound being destroyed is toxic or recalcitrant. This study investigated hydrolysis of MP using a recombinant strain of *Escherichia coli*, which contains the organophosphate degrading (*opd*) gene for organophosphate hydrolase (OPH). The effect of biomass concentration up to 100 g/L was evaluated for degradation of MP at a concentration of 1g/L. Results showed that the concentration of biomass for maximum reactor productivity was 25 g/L. With this biomass concentration, the degradation rate was  $69 \pm 3 \mu\text{mol/L}\cdot\text{min}$ . The effect of substrate concentration on hydrolysis kinetics also was evaluated. Over a range of substrate concentrations up to 3 g/L, substrate inhibition did not occur. MP degradation was confirmed by reduction of the high performance liquid chromatography (HPLC) peak for MP and the appearance of the p-nitrophenol (PNP) peak, which increased as MP was degraded. However, the appearance of PNP was less than the theoretical PNP formation based on stoichiometry. The fate of PNP in the hydrolysis medium was investigated under the slightly alkaline reaction conditions and the possibility that some PNP was further metabolized by OPH<sup>+</sup> cells was also evaluated. The results indicated

that the lower than theoretical PNP concentration from hydrolysis was due to absorption and/or degradation of PNP by OPH<sup>+</sup> *E. coli*.

## 2. Introduction

As the demand for agricultural products increases, so inevitably does the need for pesticides. Currently, OP compounds are one of the most widely used classes of pesticide in industrialized countries (Shimazu et al., 2001). Despite the rapid degradation of these insecticides in the soil, they can be potentially hazardous as a result of accidental spills, runoff from areas of application, and discharge from pesticide containers and waste (Rani and Lalithakumari, 1994). MP is the most hazardous OP insecticide allowed in the United States food supply (Shimazu et al., 2001) and is chemically similar to nerve agents used in chemical warfare (Cho, 2001). MP toxicity is primarily associated with the inhibition of cholinesterase activity and resulting effects on the nervous system (Cho, 2001). Although the toxicity of OPs is associated with anticholinesterase activity; some compounds also produce a delayed toxicity termed OP-induced delayed neuropathy (OPIDN). MP can also induce a genotoxic effect in bacterial and mammalian cells. OPIDN is characterized by a delayed onset of pathological symptoms, such as ataxia and muscle paralysis, 7 to 14 days following the exposure (Cho, 2001). Cho (2001) evaluated the neurotoxic effects of MP and its degradation products using an OPH enzyme from genetically engineered *E. coli* to degrade MP and eliminate its neurotoxicity. He found that metabolites produced were not mutagenic, confirming the genotoxicity of MP and supporting the potential utility of OPH in the bioremediation of MP.

Because of these health concerns, MP has been classified a restricted pesticide and its use in most food crops in the United States has been cancelled (Garcia et al., 2003). MP is a restricted OP pesticide. The World Health Organization has classified technical MP as a class Ia 'extremely hazardous' pesticide in normal use, based on an oral 50% lethal dose in

rats of 14 mg/kg. It is highly toxic by inhalation and ingestion and moderately toxic by dermal adsorption as it is readily adsorbed through the skin (Hertel, 1993). MP has adverse effects on many different beneficial insects (Hertel, 1993).

It is highly toxic for aquatic invertebrates with most 50 % lethal concentrations ranging from less than 1 µg/L to 40µg/L. Most fish species in both fresh and sea water have 50% lethal concentrations between 6 and 25 mg/L, with a few species substantially more sensitive to MP. MP has been detected at a concentration of 59 µg/kg in the ovaries of spotted sea trout (*Cynoscion nebulosus*) collected in Texas. MP was detected in 34 out of 55 suspectedly poisoned apiaries examined in Connecticut. Concentrations of MP found in dead bees and in brood comb ranged from 0.04 to 5.8 mg/kg (Hertel, 1993). MP is also highly to very highly toxic to birds (Extoxnet, 1996). In 1992, a massive bird kill occurred in Costa Rica after MP was applied by plane in a cotton field (Pesticide News, 1995).

In the United States recently, there have been a number of important prosecutions involving MP. Over 1,500 homes and businesses in Mississippi and Ohio were sprayed with MP by unlicensed operators. The authorities relocated over 1,100 people in temporary accommodations and clean up costs could reach US \$50 million. On April 30 1997, the United States EPA canceled the registrations of emulsifiable concentrate formulations (Pesticide News, 1995).

Currently, the primary methods approved for disposal of hazardous wastes are incineration and chemical treatment, but these methods are costly and often create new environmental problems. Kim et al. (1999), Hertel (1993) and Spain and Gibson (1991) suggested that biodegradation to benign products is an attractive option for destruction of OP since it utilizes natural processes and offers the potential for less costly treatment. However, biodegradation rates usually are low because the compound being destroyed is toxic or recalcitrant, which causes growth to be slow as well. Numerous decomposition products may be produced so that complete biodegradation often requires a consortium of organisms to metabolize the

resulting products. Considerable research has been directed toward the development of alternative processes for biodegradation of OPs. Among these, Kim et al. (2002) investigated the degradation of coumaphos using a recombinant strain of *Escherichia coli* containing the *opd* gene for OPH. Significantly higher degradation rates were obtained compared to those obtained with the microbial consortium naturally present in coumaphos dip waste.

The overall goal of our work is to develop technology for biodegradation of hazardous substances at significantly higher rates than currently are obtained with native organisms. In this study, the objective was to evaluate and demonstrate the use of *Escherichia coli* containing the *opd* gene for OPH for the remediation of MP. MP was selected because its biodegradation has been well characterized and because of its persistence in the environment. The focus of this study was to determine the effect of biomass concentration on the activity of freely suspended, non-growing OPH<sup>+</sup> *E. coli* cells for MP degradation and to determine the kinetic model. This study describes the enhanced efficiency of degrading MP using a dense, non-growing population of a recombinant OPH<sup>+</sup> *E. coli* strain that functioned without the addition of nutrients required for growth.

### **3. Materials and Methods**

#### **3.1 Bacterial Strain**

*Escherichia coli* DH5 $\alpha$  was prepared as described by Dave et al. (1993). Standard recombinant DNA techniques were employed in the construction of plasmid and phage vectors expressing OPH for *E. coli*. 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. The resulting *opd* gene was transformed into *E. coli* strain DH5 $\alpha$  [*supE44*,  $\delta$ *lacU169*( $\Phi$ 80*lacZ* $\delta$ *M15*), *hsdR17*, *recA1*, *endA1*, *gryA96*, *thi-1*, *relA1*], which was made competent for transformation by the CaCl<sub>2</sub> method.

### 3.2 Biomass Preparation

An isolated colony of transformed *E. coli* was cultured in a 5 mL tube of Luria-Bertani (LB) broth (tryptone, 10 g/L; yeast extract, 5g/L; NaCl, 5 g/L) supplemented with 1 mg/L ampicillin then transferred at a concentration of 5% to TB medium (pancreatic digest of casein, 12 g/L; yeast extract, 24 g/L; K<sub>2</sub>HPO<sub>4</sub>, 9.4 g/L; KH<sub>2</sub>PO<sub>4</sub>, 2.2 g/L), supplemented with 1 mM CoCl<sub>2</sub>, 4 ml/L glycerol and 1 mg/L ampicillin. The culture was grown at 30 °C in a rotary shaker (200 rpm) for 14 h (late exponential phase). Ampicillin, glycerol, CoCl<sub>2</sub> and LB medium were obtained from Fisher Chemicals (Fair Lawn, NJ) and TB medium was obtained from Difco (Sparks, MD). The resulting biomass was harvested by centrifugation at 4,000 rpm (4 °C) for 15 min. The pellet obtained from centrifugation was washed with potassium phosphate buffer (50 mM, pH 7.2) and centrifuged at 4,000 rpm (4 °C) for another 15 min. The resulting biomass was used for experiments.

### 3.3 Methyl Parathion and Buffer Medium

MP crystal (99.5% purity) was obtained from Chem Service (West Chester, PA) and was used without further purification. Since MP solubility in water is very low (0.055 g/L) (Hertel, 1993), a suspension was prepared in acetonitrile (10g MP/L acetonitrile) and the solution was mixed by agitation for 30 min before use. Buffer medium was prepared with tap water and 100 mM CHES. The final pH was adjusted with KOH to 8.0, the optimum for OPH<sup>+</sup> cell activity at 30 °C (Kim et al., 2002).

### 3.4 Reaction System

Shake flask experiments were conducted to determine the effect of biomass concentration on the activity of freely suspended non-growing OPH<sup>+</sup> *E. coli* cells for MP degradation and to determine the kinetic model. For experiments to determine the effect of biomass concentration, MP dissolved in acetonitrile was added to give a final concentration of 1 g/L. For experiments to determine the effect of substrate concentration, the optimum biomass

concentration was used with varying amounts of MP and a constant concentration of acetonitrile of 10%. Experiments for degradation of MP were conducted under batch reactor conditions in 250-mL Erlenmeyer flasks with a working volume of 50 mL. Reactions were carried out at 30 °C with rotary shaking at 300 rpm. Experiments were conducted in triplicate. The reaction medium consisted of buffer solution (pH 8.0) containing varying amounts of MP and biomass. Samples (0.2 mL) were taken from the bulk phase at intervals during each experiment for determination of MP and PNP concentrations. MP degradation was confirmed by reduction of the HPLC peak for MP and appearance of the PNP peak.

### 3.5 Analytical Methods

Growth phases of recombinant *E. coli* cells in TB medium were determined by following the increase in optical density at 600 nm using a spectrophotometer (Spectronic 20D<sup>+</sup>, Milton Roy, Rochester, NY). A 1.0 mL sample was placed in a polystyrene 1.5 mL cuvette and absorbance measured.

Hydrolysis of MP in the bulk solution (buffer medium and biomass) was determined by following the reduction in its concentration along with production of PNP using HPLC as described by Funch (1981). A 0.2 mL sample was diluted with 0.8 mL of acetonitrile in a 1.5 mL polypropylene Eppendorf micro test tube. The tube was centrifuged for 15 min using a micro centrifuge. Analysis of the supernatant was done using HPLC (photodiode array detector 996 and separation module 2690, Waters, Milford, MA) equipped with an auto sampler and C<sub>8</sub> column (25 cm x 4.6 mm, Burdick & Jackson, Muskegon, MI) with acetonitrile and water (60:40) as the mobile phase at a flow rate of 1 mL/min. Absorbance was monitored at 280 nm. Under these conditions, MP was eluted after a residence time of 5.2 min and PNP after 3.5 min. A standard for DMTP was not available; therefore, it was not possible to positively identify and calibrate a peak for DMTP.

### 3.6 Numerical Calculations

The initial rate for each experiment was calculated from the slope of a plot of substrate concentration versus time. Kinetic parameters for substrate utilization were estimated from initial rate data using non-linear parameter estimation software (Origin version 6, Microcal Software Inc, Northampton, MA).

## 4. Results and Discussion

### 4.1 Initial Studies

OP pesticides are not very soluble in aqueous systems and thus require an organic solvent to achieve higher concentrations (Caldwell, 1991). MP solubility in water is very low (0.055 g/L); therefore, a stock solution with a concentration of 10 g/L was prepared using methanol as the solvent. The MP stock solution was stored in the dark at room temperature. Standard solutions at varying concentrations were prepared from MP stock solution to calibrate the HPLC. After storing the stock solution for a month and a half, a new MP stock solution was prepared. HPLC standards were prepared using the old MP stock solution. After analyzing samples from hydrolysis experiments, it was observed that concentrations of MP from HPLC analysis were much higher than initially present in the reaction medium. This indicated that MP concentrations in the HPLC standards were lower than calculated, i.e., MP appeared to be unstable in methanol. To overcome this problem, MP stock solutions were prepared with acetonitrile, and no inconsistencies in the data were observed.

For enzyme assays, the biomass or enzyme normally is added to the reaction medium prior to the addition of substrate to allow the biomass or enzyme to acclimate to the reaction medium. However, to obtain uniform dispersion of the substrate in the reaction medium, the MP stock solution was added to the buffer before biomass was added. Preliminary experiments showed that when MP stock solution was added to the buffer



medium, the solution turned slightly yellow, indicating the presence of PNP. It was also observed that the initial MP concentration measured by HPLC was less than the amount added during medium preparation. To determine the actual MP concentration degraded by the biomass, samples of MP hydrolysis reaction medium were taken immediately before and after addition of OPH<sup>+</sup> cells. MP concentrations measured immediately after addition of biomass were used to calculate degradation rates

The recombinant OPH<sup>+</sup> *E. coli* cells were maintained on LB ampicillin agar plates. After a month, colonies were transferred to fresh LB ampicillin agar plates. MP degradation experiments performed using transferred recombinant OPH<sup>+</sup> *E. coli* colonies showed lower degradation rates than those obtained with untransferred OPH<sup>+</sup> *E. coli* colonies. Therefore, further MP hydrolysis experiments were carried out using fresh recombinant OPH<sup>+</sup> *E. coli* without any transfers.

Kim (1998) evaluated the hydrolysis of coumaphos by recombinant OPH<sup>+</sup> *E. coli*. The recombinant *E. coli* cells were grown initially in LB medium, harvested in the late exponential phase, and transferred to TB medium for biomass production using a 5% inoculum. Inoculum concentrations of 1, 2.5, 5 and 10% were evaluated for transfer to TB medium. Concentrations of 5 and 10% gave the highest yield of biomass, with no difference between 5 and 10%. Thus an inoculum concentration of 5% was selected. Yields of biomass were determined based on OD values. However, TB medium components had high absorbance at the wavelength used for OD; therefore, samples were diluted 5 times in order to minimize interference from TB medium components.

## **4.2 Effect of Biomass Concentration**

Shake flask experiments were conducted to determine the effect of biomass concentration on the activity of freely suspended non-growing OPH<sup>+</sup> *E. coli* cells for MP degradation. Kim et al. (2002) investigated the hydrolysis of coumaphos by

recombinant OPH<sup>+</sup> *E. coli*. He evaluated concentrations of biomass up to 200 g/L. Preliminary experiments showed that the degradation rate of MP was faster than it was for coumaphos. Therefore, for determination of the effect of biomass concentration on MP hydrolysis, concentrations up to 100 g/L were evaluated. Results showed that the overall reaction rate increased with increasing biomass concentration up to 25 g/L and then remained constant at higher concentrations. The specific rate decreased with increasing biomass concentration. From these results, the optimum biomass concentration was found to be 25 g/L (Fig. II-1).

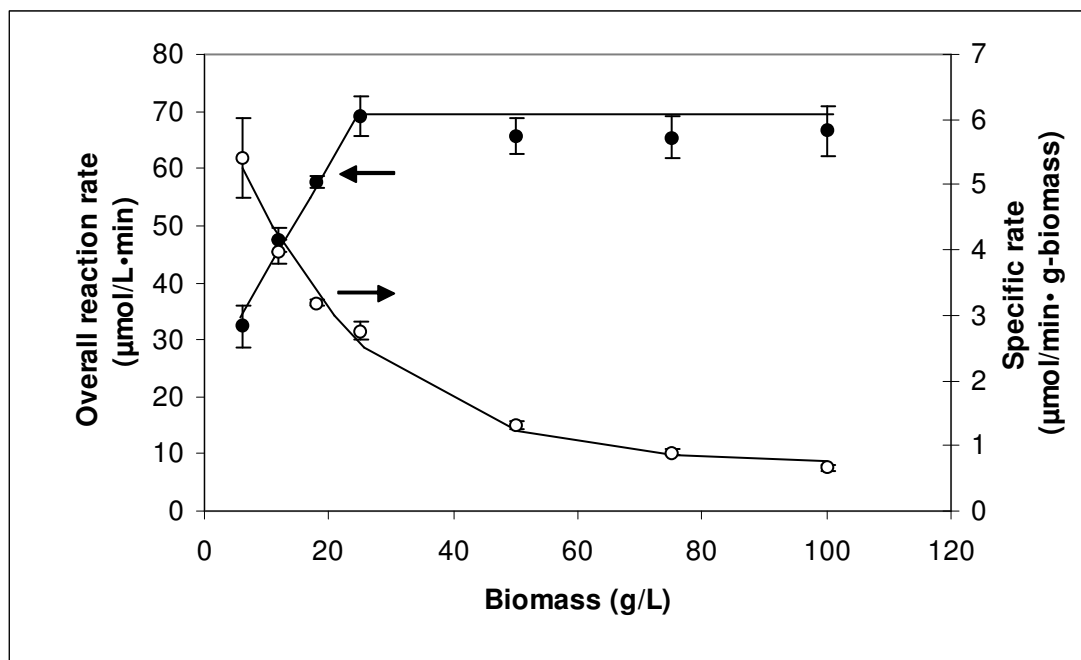


Figure II-1. Effect of concentration of freely suspended recombinant OPH<sup>+</sup> *E. coli* on degradation of MP. (○) specific rate; (●) overall reaction rate. Reaction conditions: substrate, MP (1 g/L); initial pH, 8.0 using 100 mM CHES and KOH pellets; temperature, 30 °C; agitation rate, 300 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

The maximum degradation rate for MP in buffer was found to be  $69 \pm 3 \mu\text{mol/L}\cdot\text{min}$  using freely suspended OPH<sup>+</sup> *E. coli* cells (Fig. II-1). This is about a 135-fold increase over the rate of  $0.51 \mu\text{mol/L}\cdot\text{min}$  found by Ramanathan and Lalithakumari (1999) for degradation with native cells. The high rate of degradation of MP by recombinant OPH<sup>+</sup> *E. coli* is not unexpected. OPH has been shown to hydrolyze a broad spectrum of organophosphorus trimesters, thioesters and fluorophosphonates in whole cell extracts and as the purified enzyme (Dave et al., 1993).

### 4.3 Degradation Kinetic Model

To determine the MP degradation kinetic model followed by OPH<sup>+</sup> cells, initial experiments were performed using varying substrate concentrations obtained by adding varying amounts of stock solution to buffer. Thus, as MP concentration increased, the concentration of acetonitrile also increased. MP concentrations were varied from 0.25 to 2.5 g/L, with acetonitrile concentrations varying from 2.5% to 25%. Under these conditions, the reaction appeared to follow substrate inhibition kinetics. However, it was possible that this inhibition was due to acetonitrile rather than to substrate. Therefore, it was necessary to investigate the effect of acetonitrile on the activity of OPH<sup>+</sup> cells.

Effects of acetonitrile concentration on MP degradation rate were evaluated using 3 different concentrations of acetonitrile (Fig. II-2). The maximum degradation rate was found to be at 10% of acetonitrile, with degradation rates for 2.5% and 25% both lower. These results suggest that acetonitrile may be required to activate the reaction system. The cellular localization of OP hydrolyzing enzymes has not been carefully examined in most biological systems, but membrane and lipid associations have been recently reported for some enzymes (Dave et al., 1993). Probably acetonitrile increases the permeability of the cells to MP, but higher concentrations of acetonitrile are inhibitory to the enzyme.

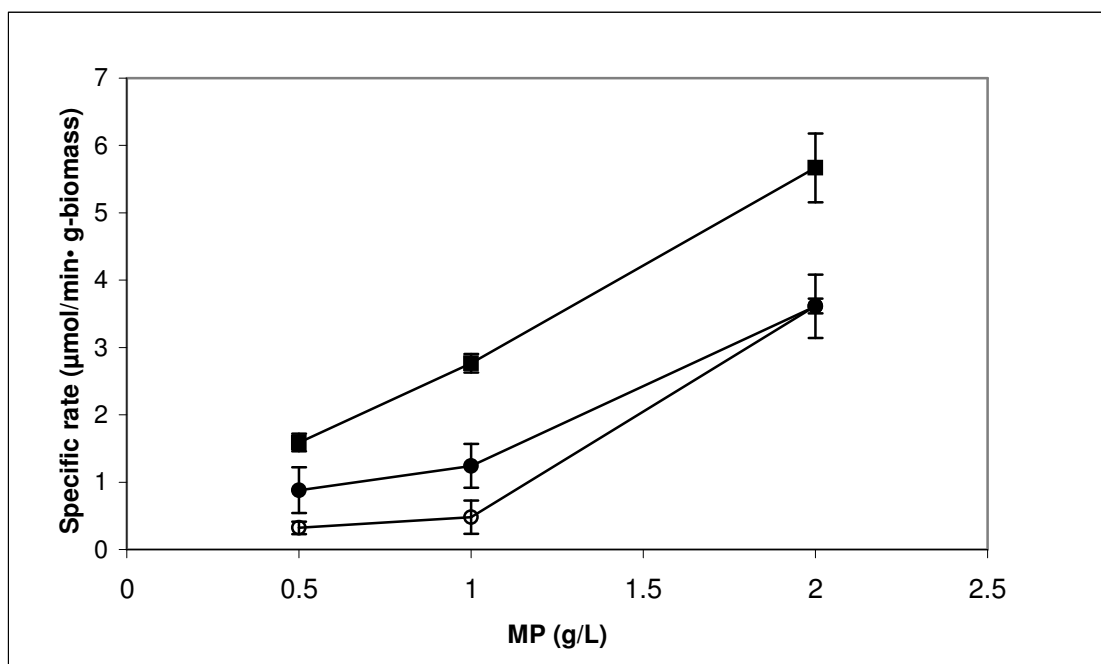


Figure II-2. Effect of acetonitrile concentration on MP degradation rate. (●) 2.5% acetonitrile (■) 10% acetonitrile (○) 25% acetonitrile. Reaction conditions: substrate, MP; initial pH, 8.0 using 100 mM CHES and KOH pellets; temperature, 30 °C; agitation rate, 300 rpm; shake flasks; 25 g/L biomass concentration. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

The effect of substrate concentration on hydrolysis kinetics was evaluated using a constant concentration of acetonitrile of 10% (Fig. II-3). For sample preparation of higher MP concentrations, it was necessary to use a stock solution concentration of 100 g/L. However, adding the corresponding volume of buffer followed by the corresponding volume of acetonitrile and finally adding the corresponding volume of MP stock solution did not always give good solubilization of MP in the reaction medium. Experiments performed with this procedure showed very inconsistent results. To enhance MP solubilization in the buffer medium, the required volume of stock solution was diluted with the corresponding volume of acetonitrile and then added to the buffer medium. Results of the effect of substrate concentration (Fig II-3) indicate that substrate inhibition did not occur up to a substrate

concentration of 3 g/L. Therefore, a simple Michaelis-Menten kinetic model was used to describe the degradation reaction:

$$v = \frac{V_m S}{K_m + S} \quad (\text{II-1})$$

where  $S$  is the substrate (MP) concentration (g/L),  $V_m$  is the maximum reaction rate ( $\mu\text{mol}/\text{min}\cdot\text{g}\text{-biomass}$ ) and  $K_m$  is the substrate saturation constant (g/L). The simple Michaelis-Menten kinetic model for enzymatic reactions was selected since the non-growing cells act more like an immobilized enzyme system (Kim et al., 2002). The maximum velocity,  $V_m$ , was estimated to be  $11.45 \mu\text{mol}/\text{min}\cdot\text{g}\text{-biomass} \pm 1.45$  and  $K_m$  was estimated to be  $2.73 \text{ g/L} \pm 0.59$ .

To investigate PNP as a product inhibitor, the activity of freely suspended OPH<sup>+</sup> cells in buffer medium saturated with PNP was evaluated. Results showed that there was no change in the rate of MP hydrolysis indicating that PNP does not inhibit the reaction. The effect of the other hydrolysis product, DMTP, could not be evaluated since a standard reagent was not available.

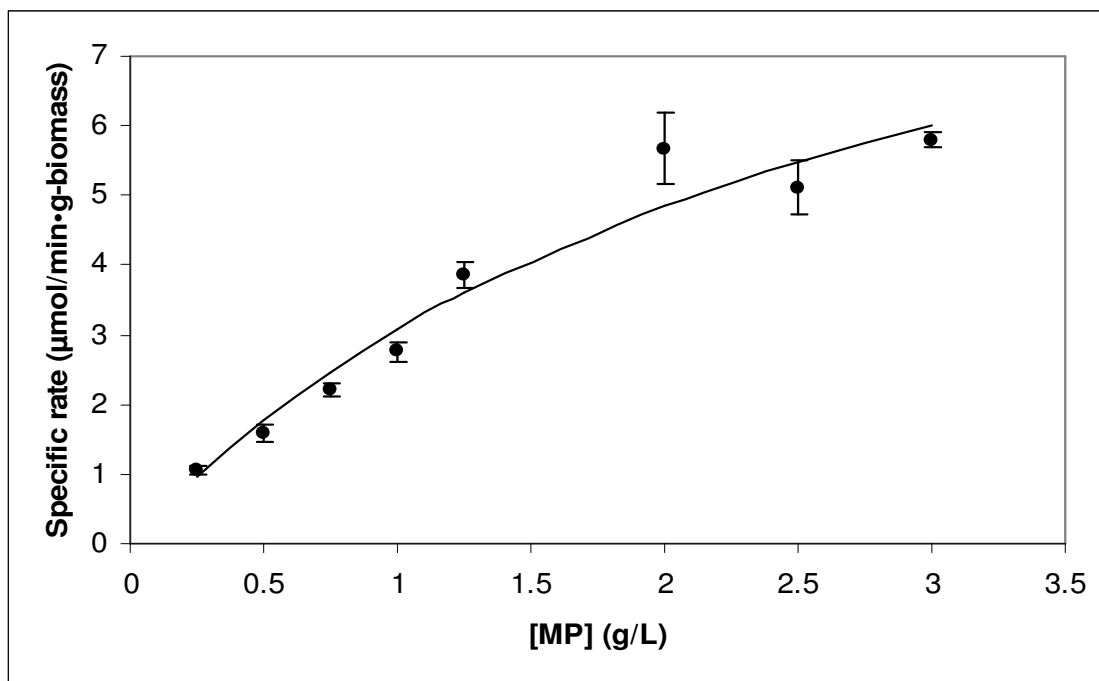


Figure II-3. Effect of substrate concentration on MP degradation rate. (●) Experimental data (—) fitted data. Reaction conditions: substrate, MP; initial pH, 8.0 using 100 mM CHES and KOH pellets; temperature, 30 °C; agitation rate, 300 rpm; shake flasks; 25 g/L biomass concentration. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

#### 4.4 Fate of MP in Buffer Medium

During the experiments it was observed, that the initial MP concentration measured by HPLC was less than the amount added during medium preparation. Thus, it was suspected that MP was being broken down by the slightly alkaline buffer medium. To investigate this possibility, the medium was incubated at hydrolysis conditions but without OHP<sup>+</sup> cells for a period of 8 hours. The results showed that MP concentration decreased from 0.85 g/L initially to 0.2 g/L at about 5 hours. After 5 h, no change in MP concentration was observed. Also, during the first 30 min, no significant change in MP concentration was observed. HPLC analysis showed a small peak eluting at a residence time identical to that of PNP. However, the PNP concentration remained constant for the period of time evaluated and was

not proportional to the amount of MP lost. Further investigations need to be made to determine the fate of MP in the buffer medium.

#### 4.5 PNP Production

As shown in Figure II-4, as MP concentration decreased, PNP concentration increased. As expected, once MP has been degraded completely, PNP is no longer produced. These results are consistent with the results of Cho (2001); the OPH enzyme activity induced hyperbolic production of PNP and exponential decay of MP.

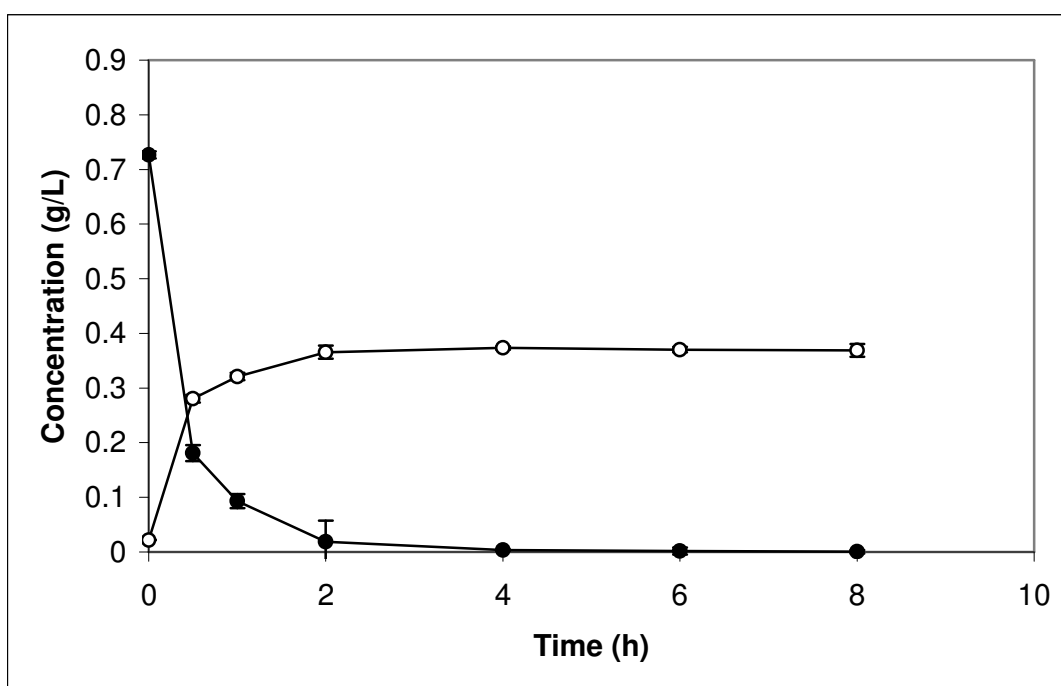


Figure II-4. Rates of MP disappearance and PNP appearance for freely suspended recombinant OPH<sup>+</sup> cells. (●) MP; (○) PNP. Reaction conditions: substrate, MP 1 g/L; initial pH, 8.0 using 100 mM CHES and KOH pellets; temperature, 30 °C; agitation rate, 300 rpm; shake flasks; biomass concentration, 25 g/L. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

However, the appearance of PNP was less than the theoretical PNP production based on disappearance of MP (Fig. II-5). Cho (2001) evaluated the capacity of an OPH enzyme from genetically engineered *E. coli* to degrade MP. He also found that the total amounts of MP and its metabolite, PNP, detected in GC analyses did not account for the total amount of MP that was used for the biodegradation assay. Cho (2001) suggested that the PNP and MP which could not be accounted for may have been adsorbed/absorbed into the *E. coli* biomass, remained in the LB medium while it was dissolved, mineralized, and/or transformed to metabolites that could not be detected with GC/NPD analysis.

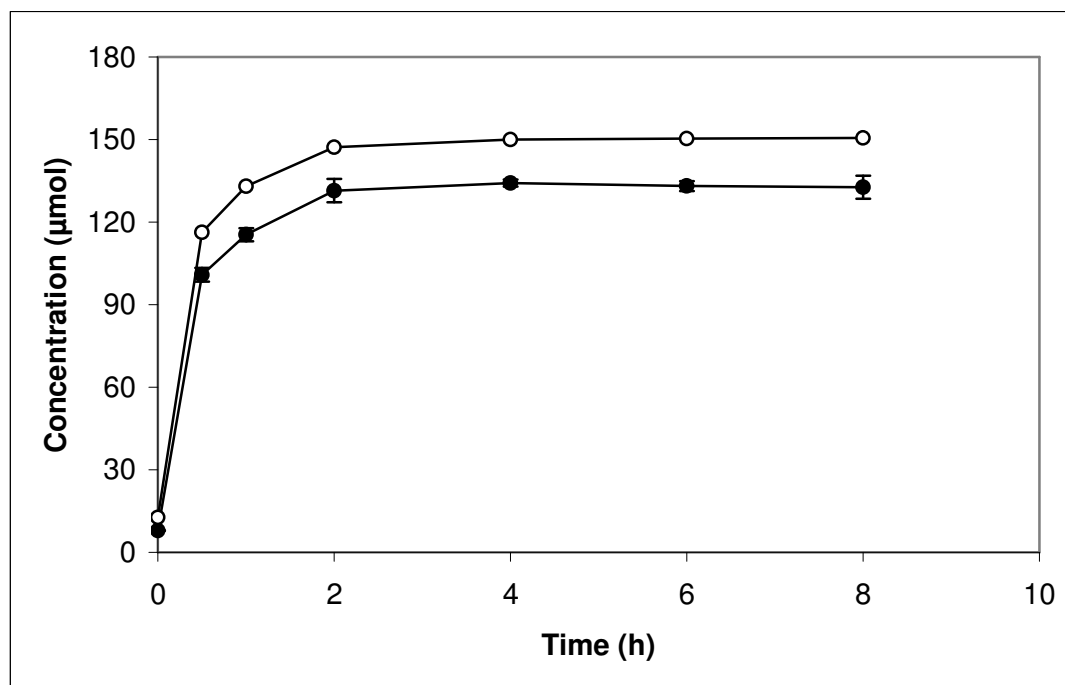


Figure II-5. MP degradation and PNP production for freely suspended recombinant OPH<sup>+</sup> cells on degradation of MP. (●) PNP experimental; (○) PNP theoretical. Reaction conditions: Substrate, MP 1g/L; initial pH, 8.0 using 100 mM CHES and KOH pellets; temperature, 30 °C; agitation rate, 300 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.



The fate of PNP in the hydrolysis medium was investigated. To determine whether PNP was being degraded under the slightly alkaline reaction conditions, PNP was added to the buffer medium and incubated for 8 hours. The PNP concentration remained constant over the time period evaluated, indicating the lower than theoretical PNP concentration from hydrolysis was not due to base-catalyzed reactions. Three smaller unidentified peaks that increased along with PNP appeared in the chromatograms from hydrolysis experiments (Fig. II-6). Since the lower than theoretical PNP concentration from hydrolysis was not due to base-catalyzed reactions, then the possibility that some PNP was further metabolized by OPH<sup>+</sup> cells was also evaluated. To determine this, PNP was added to the buffer medium with OPH<sup>+</sup> cells and incubated for 8 hours. Analysis of the samples by HPLC showed that the initial PNP concentration measured by HPLC was 90% of the amount added during medium preparation, but the concentration was reduced by 25% over the time period evaluated. This indicates the lower than theoretical PNP concentration from hydrolysis was due to absorption and/or degradation of PNP by OPH<sup>+</sup> *E. coli*.

HPLC sample analysis from MP hydrolysis by OPH<sup>+</sup> *E. coli* showed a constant peak eluted at a residence time of 2 min, initially suspected to be DMTP. HPLC analysis from PNP degradation in the buffer medium also showed a peak eluted after 2 min. HPLC analysis of the hydrolysis medium did not show this peak, suggesting that this peak may be a metabolic product from PNP degradation.

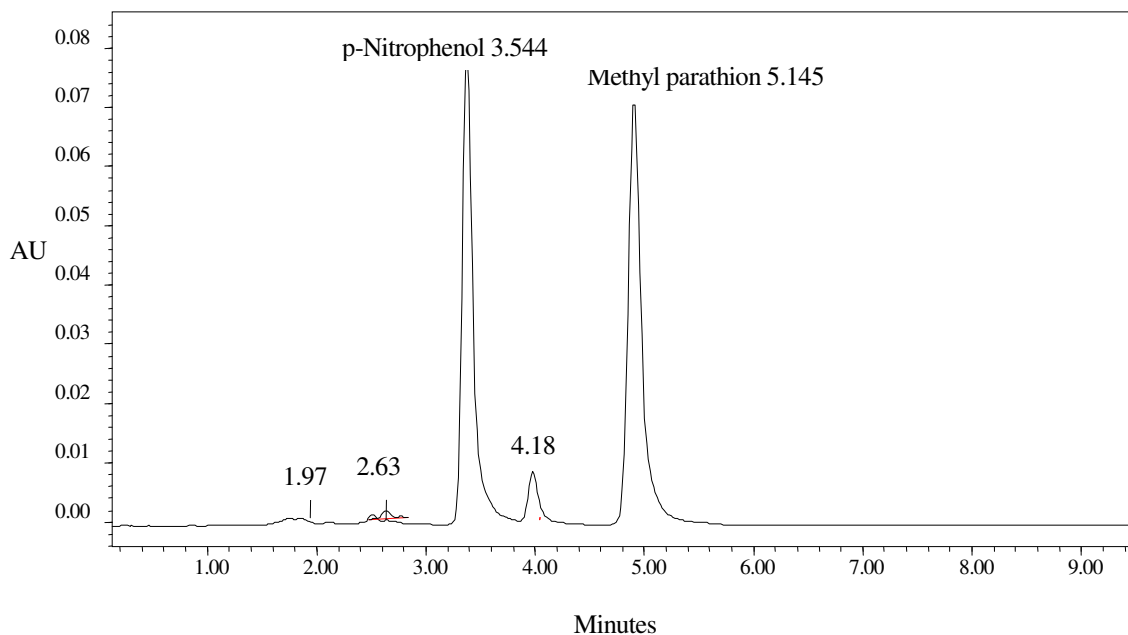


Figure II-6. Typical chromatogram of hydrolysis product. Reaction conditions: substrate 0.5 g/L of MP; initial pH, 8.0 using 100 mM CHES and KOH pellets; temperature, 30 °C; agitation rate, 300 rpm; biomass concentration, 25 g/L; shake flasks.

#### 4.6 Stability of OPH<sup>+</sup> Cells

Since the long term application of this technology is to have a continuous bioreactor process for bioremediation, repeated use of biomass was evaluated. After a hydrolysis reaction was completed, biomass was recovered from the medium by centrifugation and then washed with potassium phosphate buffer as described earlier. Results from a subsequent hydrolysis reaction showed a decrease in the initial rate of degradation by 20% compared to the activity of freshly grown biomass.

### 5. Conclusions

Cho (2001) demonstrated the potential for using non-growing OPH<sup>+</sup> *E. coli* cells for biodegradation of hazardous substances such as MP. By eliminating methyl groups from the parent chemical with the OPH enzyme, MP metabolites lose the capacity for inducing a

genotoxic response (Cho, 2001). The results of this study corroborate the potential for using non-growing OPH<sup>+</sup> *E. coli* cells for biodegradation of MP at significantly higher rates than currently are obtained with native organisms. The maximum biodegradation rate was found to be  $69 \pm 3 \mu\text{mol/L}\cdot\text{min}$ , which was 135 times higher than reported for a native OPH<sup>+</sup> organism ( $0.51 \mu\text{mol/L}\cdot\text{min}$  reported by Ramanathan and Lalithakumari, 1999).

Acetonitrile was found to enhance the hydrolysis rate up to concentrations of about 10%, but a concentration of 25% was found to be detrimental to the reaction. For an acetonitrile concentration of 10%, no inhibition by substrate or PNP was observed. Therefore, the simple Michaelis-Menten kinetic model was used to describe the rate of reaction at constant acetonitrile concentration. To evaluate higher concentration of OPs, the use of methanol could be considered since the activity of OPH does not decrease with increasing concentrations of methanol up to 40% (Caldwell, 1991). The use of methanol as an organic solvent for MP should be further investigated.

## CHAPTER III

### BIODEGRADATION OF P-NITROPHENOL

#### 1. Overview

Nitroaromatic compounds are extensively distributed in the environment. They are released as insecticides and as a result of hydrolysis of pesticides or industrial wastes (Bhushan et al., 2000 and Leung et al., 1997) in particular, p-nitrophenol (PNP) has been shown to be one of the hydrolysis products of the organophosphate (OP) methyl parathion (MP) (Shimazu et al., 2001). PNP is a toxic compound and suspected carcinogen. It is considered a priority pollutant by the United States Environmental Protection Agency (EPA) (Walker and Keasling, 2002). It has been shown that some members of the genus *Sphingomonas* can degrade nitroaromatic compounds (Cassidy et al. 1999). The present study evaluated the degradation of PNP by *Burkholderia cepacia* ATCC 29354 and *Sphingobium chlorophenolicum* ATCC 53874, a redeposit of *Sphingobium chlorophenolicum* ATCC 39723.

In preliminary trials, PNP degradation by *B. cepacia* was not observed in ATCC medium 666 or mineral salts broth; therefore detailed studies focused on the degradation of PNP by *S. chlorophenolicum*. The utilization of PNP by *S. chlorophenolicum* in minimal media such as mineral salts, mineral salts plus nitrogen source, mineral salts plus carbon source, ATCC medium 1687 (which includes glutamate as a carbon source) and CHES buffer was evaluated. PNP degradation was observed solely in ATCC medium 1687 suggesting that the process of degradation of PNP by *S. chlorophenolicum* required glutamate. It was also observed that the degradation of PNP did not exhibit a lag time. Degradation of PNP in complex growth media was evaluated, but media such as TB, LB, tryptone and yeast extract inhibited PNP degradation.

In this study, effects of type of carbon source, *S. chlorophenicum* growth stage for addition of PNP, PNP concentration and initial pH on PNP degradation were evaluated. Based on preliminary results, ATCC medium 1687 was utilized for most studies. Results showed that faster PNP degradation rates were obtained when PNP was added to the culture early in the growth phase indicating the process of degradation required cell growth. Degradation was slightly faster with a glutamate concentration of 10 g/L than for a concentration of 5 g/L. PNP degradation was not complete within 140 hours when the initial concentration was 0.3 or 0.5 g/L, but it was complete at concentrations of 0.1 g/L or less. PNP degradation rates were higher for an initial pH of 8.0 than for pH of 7.4. *S. chlorophenicum* failed to continuously degrade PNP without adjustment of nutrients and initial pH of the degradation medium. Neither hydroquinone (HQ) nor nitrocatechol (NC) were detected as degradation products, but an unidentified product peak having a retention time of 2.4 min and absorbance at 360 nm was detected.

## **2. Introduction**

Today's industrial and agricultural activities have resulted in contamination of soil and ground water resources with hazardous chemicals (Chauhan et al., 2000). Among these contaminants, nitrophenols are found extensively in the environment as a result of their use as herbicides, fungicides, explosives and insecticides. Nitrophenols entering rivers and ground water can be harmful to biological activity due to their acute toxicity (Leung et al., 1997 and Bhushan et al., 2000). PNP, a compound that does not occur naturally in the environment, is used in agriculture, dyes, pigments, engineering polymers and pharmaceuticals. PNP is toxic to plant, animal and human health. Prolonged or acute exposure to PNP may lead to methemoglobin formation, liver and kidney damage, anemia, skin and eye irritation, and systemic poisoning (Bhatti et al., 2002). PNP is a suspected carcinogen and is considered a priority pollutant by the United States Environmental Protection Agency (Walker and Keasling, 2002).

PNP easily breaks down in surface aquatic environments but it takes longer periods of time in deep soil and groundwater (Bhatti et al., 2002). PNP has been widely used as a model for biodegradation since it can be found in both aquatic and terrestrial environments (Leung et al., 1997). Nitroaromatic compounds can be degraded by bacteria through either oxidative or reductive pathways. These pathways involve either the removal or the reduction of the nitro group followed by oxidative ring fission. Degradation products are further converted to dicarboxylic acids which can enter other metabolic pathways. Reductive pathways usually involve the transformation to hydroxylamino or aminoaromatic intermediates. Oxidative pathways can be initiated by the action of either monooxygenase or dioxygenase enzymes. Dioxygenases catalyze the initial attack on 2,4-dinitrotoluene, 1,3-dinitrobenzene, nitrobenzene, 3-nitrobenzoate and 2-nitrotoluene, releasing nitrite and forming dihydroxy aromatic compounds. Monooxygenases catalyze the replacement of the nitro group by a hydroxyl group resulting in the liberation of the nitro group as nitrite (Hurst et al., 1997). There are two known PNP degradation pathways. One is by monooxygenation to hydroquinone (HQ) and the other one by dioxygenation to nitrocatechol (NC). The nitro group is released prior to ring cleavage (Hurst et al., 1997).

Barik et al. (1978) and Doung et al. (1997) studied the degradation of PNP by *Pseudomonas* sp. ATCC 29354 finding that PNP was converted to NC. Doung et al. (1997) also found that the bacterial population did not change over time. Members of the genus *Sphingomonas* are also capable of degrading naturally occurring compounds and environmentally important xenobiotics such as chloro- and nitro- phenols. They have mechanisms for dehalogenation and transformation of the compound resulting in aromatic ring cleavage (Cassidy et al., 1999). The enzyme PCP-4-monooxygenase has broad substrate specificity and hydroxylates the para position of halo-, nitro-, amino- and cyano-substituted phenols, forming hydroquinones and releasing halide, nitrite, hydroxylamine and cyanide, respectively (Xun et al., 1992, Leung et al., 1999 and Wang et al., 2002).

Among the *Sphingomonas* strains, *Sphingomonas* sp. UG30, *S. chlorophenicum* RA2 and ATCC 39723 have been shown to mineralize PNP (Leung et al., 1999). Cassidy et al. (1999) found that these bacterial strains metabolize PNP with the release of nitrite and mineralization of carbon to CO<sub>2</sub> and that they can also transform NC. They confirmed also that PCP-4-monooxygenase of *Sphingomonas* sp. UG 30 para-hydroxylates phenols substituted in the ortho position with an electron withdrawing group as with *S. chlorophenicum* ATCC 39723.

*Sphingomonas* sp. UG30 has been shown to transform PNP to NC. Leung et al. (1999) cloned the *pcpB* gene from *Sphingomonas* sp. UG30 that encodes PCP-4-monooxygenase to study its role in PNP degradation. They observed that the involvement of PCP-4-monooxygenase in PNP degradation occurred in the second step, degradation of NC. PCP-4-monooxygenase catalyzed the hydroxylation of NC to 1,2,4-benzenetriol. They also observed that part of the nitro substituent of NC was cleaved with release of nitrite. PCP-4-monooxygenase hydroxylated PNP to a very slight extent. Therefore, PCP-4-monooxygenase is not involved in the initial step of PNP metabolism by *Sphingomonas* sp. UG30. The UG30 *pcpB* sequence is similar to the PCP-4-monooxygenase genes from *S. chlorophenicum* ATCC 39723 and ATCC 33790; therefore, enzyme functionalities should be similar (Leung et al., 1999).

The overarching goal of this work is to develop technology for biodegradation of hazardous substances using MP as a model compound. PNP has been shown to be one of the hydrolysis products of MP using the OPH enzyme (Shimazu et al., 2001). The design of biocatalysts that perform in reliable conditions requires information about the bacteria and pathways involved (Pieper and Reineke, 2000). Thus, to optimize bioremediation conditions, studies to understand the interaction between PNP and degrading bacteria were needed. Moreover, the ability of PCP-degrading strains to mineralize PNP represents a novel and unexplored method for treatment of PNP-containing wastes (Leung et al., 1997). Therefore,

this study has focused on the degradation of PNP by *B. cepacia* ATCC 29354 and *S. chlorophenolicum* ATCC 53874.

### 3. Materials and Methods

#### 3.1 Bacterial Strains

Lyophilized cells of both *Sphingobium chlorophenolicum* deposited as *Flavobacterium* sp. ATCC 53874 (a redeposit of ATCC 39723 which no longer degrades PCP) and *Burkholderia cepacia* deposited as *Pseudomonas* sp. ATCC 29354 were purchased from the American Type Culture Collection (Manassas, VA).

#### 3.2 Media

The *B. cepacia* ATCC 29354 culture was hydrated and maintained in ATCC medium 666 ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 0.5 g/L;  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 2.0 g/L; peptone, 5.0 g/L; sucrose 15 g/L;  $\text{FeSO}_4$  0.5 g/L). Final pH was adjusted to 7.2 with NaOH.

The *S. chlorophenolicum* ATCC 53874 culture was hydrated and maintained in ATCC medium 1687 ( $\text{K}_2\text{HPO}_4$ , 0.65 g/L;  $\text{KH}_2\text{PO}_4$ , 0.19 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/L;  $\text{NaNO}_3$ , 0.5 g/L; sodium glutamate, 5.0 g/L) supplemented with 0.02 mM  $\text{FeSO}_4$ . Final pH was adjusted to 7.4 with NaOH.

For production of biomass of *S. chlorophenolicum* both Luria-Bertani broth (LB) (tryptone, 10 g/L; yeast extract, 5g/L; NaCl, 5 g/L) and TB (pancreatic digest of casein, 12 g/L; yeast extract, 24 g/L;  $\text{K}_2\text{HPO}_4$ , 9.4 g/L;  $\text{KH}_2\text{PO}_4$ , 2.2 g/L) media supplemented with  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/L and  $\text{FeSO}_4$  0.02 mM and 0.2 mM were evaluated. Modifications of ATCC medium 1687 were evaluated to determine if the biomass yield could be increased. The concentration of glutamate was increased to 47.6 g/L;  $\text{FeSO}_4$  was evaluated at 0.02 mM and 0.2 mM, and media with and without glycerol at 4g/L were evaluated. Growth of *S. chlorophenolicum* was



also investigated in ATCC medium 1687 doubling the concentration of glutamate and  $\text{NaNO}_3$  and supplemented with 20 g/L of tryptone or yeast extract and 0.02 mM  $\text{FeSO}_4$ . ATCC medium 1687 was also modified by replacing glutamate with cellobiose, sucrose, fructose, glycerol, or glucose.

$\text{K}_2\text{HPO}_4$ ,  $\text{Na}_2\text{HPO}_4$  and NaOH were obtained from EM Science (Gibbstown, NJ);  $\text{KH}_2\text{PO}_4$  and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  were obtained from Sigma (St. Louis, MO);  $\text{NaNO}_3$  was obtained from J.T. Baker (Phillipsburg, NJ);  $\text{Ca}(\text{NO}_3)_2$  was obtained from Mallinckrodt Baker (Paris, KY); sodium glutamate in the form of L-(+) glutamic acid, glycerol, LB medium, PNP crystal (99% purity), sucrose, tryptone, yeast extract, peptone and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  were obtained from Fisher Chemicals (Fair Lawn, NJ) and TB medium was obtained from Difco (Sparks, MD).

### 3.3 Biomass Preparation

For *S. chlorophenolicum* ATCC 53874 experiments, an isolated colony was cultured in a 5 mL tube of ATCC medium 1687 and transferred at a concentration of 5% to mineral salts solutions with different types of carbon source. The pH was adjusted to 7.4 with NaOH. Media were supplemented with 0.02 mM  $\text{FeSO}_4$ . The culture was grown at 30 °C in a rotary shaker (200 rpm) to the middle exponential phase. Filter sterilized PNP was then added to the culture to give a final concentration of 0.1 g/L. Disappearance of PNP and appearance of resulting metabolites were confirmed by HPLC. To determine the optimum growth stage for degradation of PNP, PNP dissolved in water was added at the early, middle and late exponential growth phases of the culture to give a final concentration of 0.1 g/L. To select an optimum pH value for the degradation of PNP, the type of carbon source selected was supplemented with 0.02 mM  $\text{FeSO}_4$  and final pH adjusted to 7.4 or 8.0 with NaOH.

For *B. cepacia* ATCC 29354 experiments, an isolated colony was cultured in a 5 mL tube of ATCC medium 666 and transferred at a concentration of 5% to the same medium. Growth conditions were carried out as stated above for *S. chlorophenolicum*. The pellet obtained from centrifugation was washed with nitrogen-free mineral salts broth with the pH adjusted to 7.2

(MSB:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001 g/L;  $\text{K}_2\text{HPO}_4$ , 0.1 g/L; pH 7.2) and centrifuged at 4,000 rpm (4 °C) for another 15 min. The resulting biomass was used for experiments. To determine the optimum growth stage for degradation of PNP, PNP dissolved in water was added to 2 and 4 day old cultures at a final concentration of 0.02 g/L.

### 3.4 p-Nitrophenol Degradation Medium

Since PNP solubility in water is very high (16 g/L), a solution having a concentration of 10 g/L in water was prepared. The solution was sterilized by filtration and stored in the refrigerator. Filter sterilized PNP was added to the culture to give the desired concentration. To determine the utilization of PNP by *S. chlorophenolicum*, media consisting of mineral salts plus carbon source, mineral salts plus nitrogen source, and mineral salts plus both carbon and nitrogen sources were evaluated. Mineral salts and nitrogen source were as given for ATCC medium 1687, cellobiose was used as a carbon source, and glutamate was used to provide both carbon and nitrogen. To determine the effect of concentration of both carbon source and substrate, the optimum growth stage was used with varying amounts of carbon source and PNP. To evaluate repeated use of the biomass, additional PNP was added after confirming disappearance of the previous addition of PNP. For *B. cepacia* ATCC 29354 experiments, the degradation medium consisted of nitrogen-free MSB with final pH adjusted to 7.2 using KOH (Dung et al., 1997).

### 3.5 Reaction System

Shake flask experiments were conducted to determine the activity of both *S. chlorophenolicum* and *B. cepacia* freely suspended cells on PNP degradation. For *S. chlorophenolicum*, the reaction medium was the one selected for biomass preparation (initial pH 8.0 and glutamate as carbon source) supplemented with 0.02 mM  $\text{FeSO}_4$ . For *B. cepacia* the reaction medium consisted of nitrogen-free MSB solution (pH 7.2). Experiments for biodegradation of PNP were conducted under batch reactor conditions in 250-mL Erlenmeyer flasks with a working volume of 100 mL. Reactions were carried out at 30 °C with rotary

shaking at 200 rpm. Experiments were conducted in triplicate. Samples (1.0 mL) were taken from the bulk phase at intervals during each experiment for determination of PNP concentration.

### 3.6 Analytical Methods

Growth phases of *S. chlorophenicum* and *B. cepacia* freely suspended cells were determined by following the change in optical density at 600 nm using a spectrophotometer (Spectronic 20D<sup>+</sup>, Milton Roy, Rochester, NY). A 1.0 mL sample was placed in a polystyrene 1.5 mL cuvette and absorbance measured. Samples grown in LB and TB media were diluted 5 fold to reduce interference from medium components.

Degradation of PNP in the bulk solution (medium and biomass) was determined by following the reduction in its concentration as well as appearance of metabolic products using HPLC analysis. A 1.0 mL sample was placed in a polypropylene Eppendorf micro test tube and centrifuged for 15 min. The supernatant was filtered using a disposable syringe filter (13 mm, PTFE, 0.2  $\mu$ M). Analysis of the supernatant was done using the HPLC system described above with a C<sub>18</sub> column (25 cm x 4.6 mm, Burdick & Jackson, Muskegon, MI) with acetonitrile and water (60:40) as the mobile phase at a flow rate of 1 mL/min. Absorbance was monitored at 300 nm. Under these conditions, HQ was eluted after a residence time of 2.6 min, NC after 3.1 min, and PNP after 3.5 min. The PNP degradation pathway by the microorganisms was determined by the production of either NC or HQ. HQ was obtained from Sigma (St. Louis, MO) and NC was obtained from Chem Service (West Chester, PA).

## 4. Results and Discussion

### 4.1 *Burkholderia cepacia* ATCC 29354

#### 4.1.1 Medium Development

During preparation of medium ATCC 666, a precipitate was formed during autoclave sterilization. Formation of precipitate in the medium was undesirable, since it interfered with optical density measurements for biomass determination. In addition, precipitate would be harvested along with the biomass.

To overcome the precipitation problem, ATCC technical service recommended allowing the precipitate to settle overnight after autoclaving and using only the supernatant. For growth, however, this gave a very low *B. cepacia* biomass yield. The low yield of biomass was likely due to the lack of nutrients.

It was observed that the formation of precipitate was caused either by the addition of peptone or FeSO<sub>4</sub> prior to sterilization. To determine whether FeSO<sub>4</sub> was causing precipitation, FeSO<sub>4</sub> stock solution was prepared in water and filtered sterilized. ATCC medium 666 was supplemented with 0.02 M FeSO<sub>4</sub> after autoclaving, but precipitate still formed. To determine if peptone caused precipitate formation, it was replaced with tryptone. Under these conditions, the medium was clear and no precipitation was observed.

Growth of *B. cepacia* was studied using the modified medium: ATCC medium 666 with tryptone instead of peptone and supplemented with 0.02 M FeSO<sub>4</sub> after autoclaving. Initially, it appeared that growth was complete after 14 hours, and the biomass yield was no better than that obtained with the clarified medium. However, growth was allowed to continue, and it was found that *B. cepacia* continued to grow slowly for up to 100 hours (Fig. III-1).

Growth stages were not clearly differentiated; the bacterium seemed to have a short exponential phase then continued to grow slowly. Barik et al. (1978) studied the degradation of PNP by a 5 day old culture of *B. cepacia* ATCC 29354. Doung et al. (1997) also investigated the degradation of PNP by this bacterium. He grew *B. cepacia* for two days prior to the addition of PNP. Even though these studies mentioned the time of incubation of *B. cepacia* for degradation of PNP, they did not specify the growth stage of the bacterium.

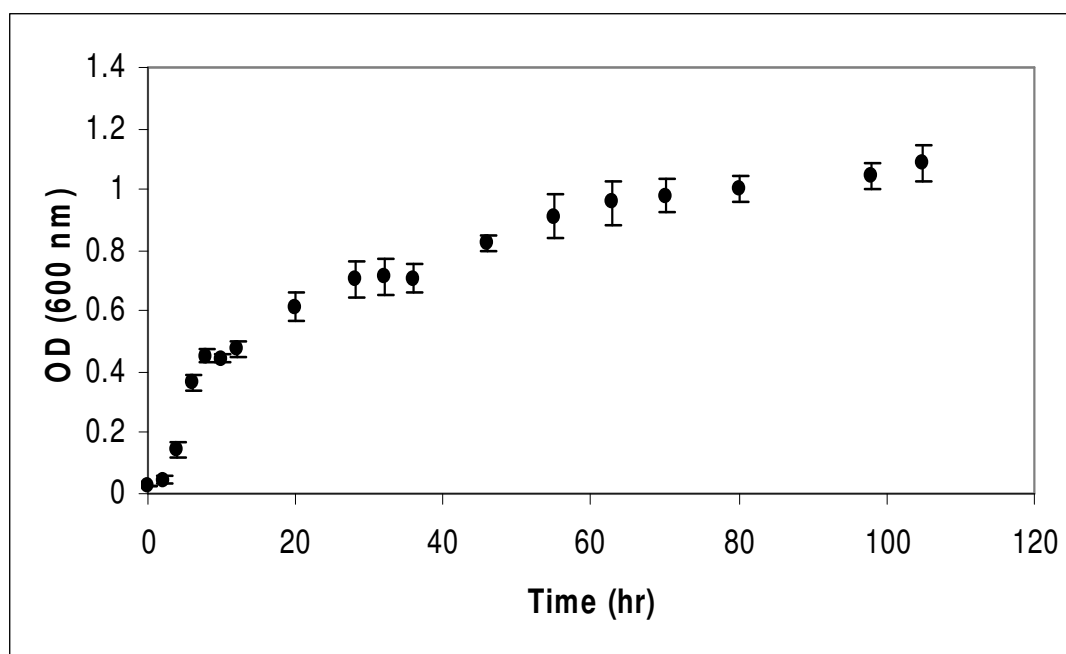


Figure III-1. Growth of *Burkholderia cepacia* ATCC 29354 in ATCC medium 666 with substitution of peptone by tryptone and supplemented with  $\text{FeSO}_4$  0.02 M. Reaction conditions: working volume, 100 mL; inoculum, 5% of *B. cepacia* grown in ATCC medium 666; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

#### 4.1.2 PNP Degradation

Prior studies had shown that PNP could be degraded by *B. cepacia* in mineral salts with and without prior exposure to PNP (Barik et al. 1978 and Doung et al., 1997). Experiments were conducted to determine the best stage of growth for degradation of PNP by *B. cepacia* and whether adaptation to PNP was necessary. Because growth did not follow the typical pattern, PNP was added after 1, 2, or 4 days of growth. Both the original ATCC medium 666 supernatant and the modified medium were used.

Results showed no degradation of PNP for up to one week for any of the six conditions evaluated. Barik et al. (1978) used a PNP concentration of 0.015 g/L and incubated for 60 days. This suggests that degradation of PNP by *B. cepacia* is very slow. Doung et al. (1997) found that the degradation of PNP by *B. cepacia* followed a Michaelis-Menten model with a  $V_m$  of 7.9  $\mu\text{M/h}$  and  $K_m$  146  $\mu\text{M}$ . Barik et al. (1978) reported that less than 50% of PNP had disappeared in more than 40 days, which indicates a much lower degradation rate than found by Doung et al. (1997). In this study, initially PNP was added to a final concentration of 0.1 g/L. Since Barik et al. (1978) and Doung et al. (1997) used much lower concentrations, tests also were conducted using a final PNP concentration of 0.02 g/L. Again, no PNP degradation was observed.

Even though no degradation was observed in the above experiment, the biomass was harvested and washed as previously stated to evaluate the effect of higher cell density on PNP degradation. The resulting biomass was added to MSB with a PNP concentration of 0.025 g/L to give a final cell density of 30 g/L. This experiment was also carried out without adaptation of *B. cepacia* to PNP. For all cases, no PNP degradation was observed up to 210 hours ( $\approx$  9 days) (Fig. III-2).

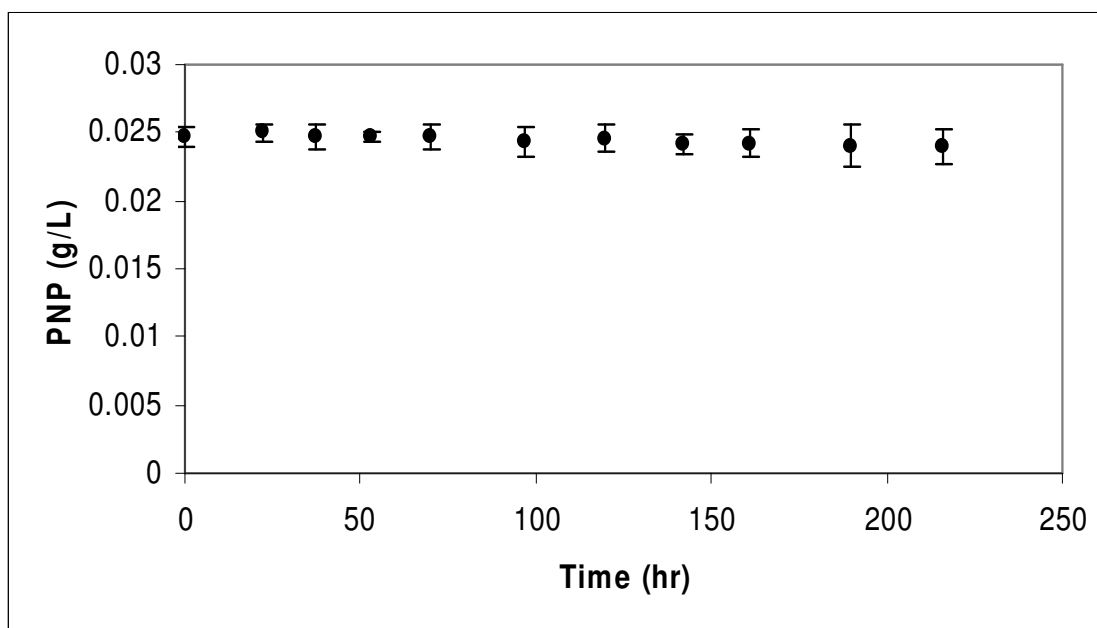


Figure III-2. PNP degradation for freely suspended *B. cepacia* cells. Reaction conditions: Substrate, PNP 0.025 g/L; initial pH, 7.2; ATCC medium 666 with substitution of peptone by tryptone and supplemented with  $\text{FeSO}_4$  0.02 M; temperature, 30 °C; agitation rate, 200 rpm; shake flasks; biomass concentration, 30 g/L. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

The results of this study indicated that either the incubation period was not long enough to show any degradation of PNP or the PNP concentration was inhibitory to *B. cepacia*. Based on these results, further work with *B. cepacia* was terminated.

## 4.2 *Sphingobium chlorophenolicum* ATCC 53874

### 4.2.1 Growth Conditions

For growth studies of *S. chlorophenolicum*, all experiments were conducted with ATCC medium 1687 unless noted otherwise. Isolated colonies were culture in 5 ML tubes and transferred at a concentration of 5% to fresh medium. Tubes inoculated with colonies from

the same plate did not show consistent growth. Because of the variation in growth curves, harvesting at a specific time after inoculation would not yield cells that were at the same stage of growth; therefore, isolated colonies were cultured in 5 ML tubes, transferred at a concentration of 5% to fresh medium when the OD reached about 1.0, then used as the inoculum for a set of experiments. Optical density was used to determine the growth stage, with early exponential phase corresponding to an absorbance reading of 0.5, middle exponential phase to an absorbance reading of 1 to 1.2 and late exponential phase to an absorbance reading of 1.8 (Fig. III-3).

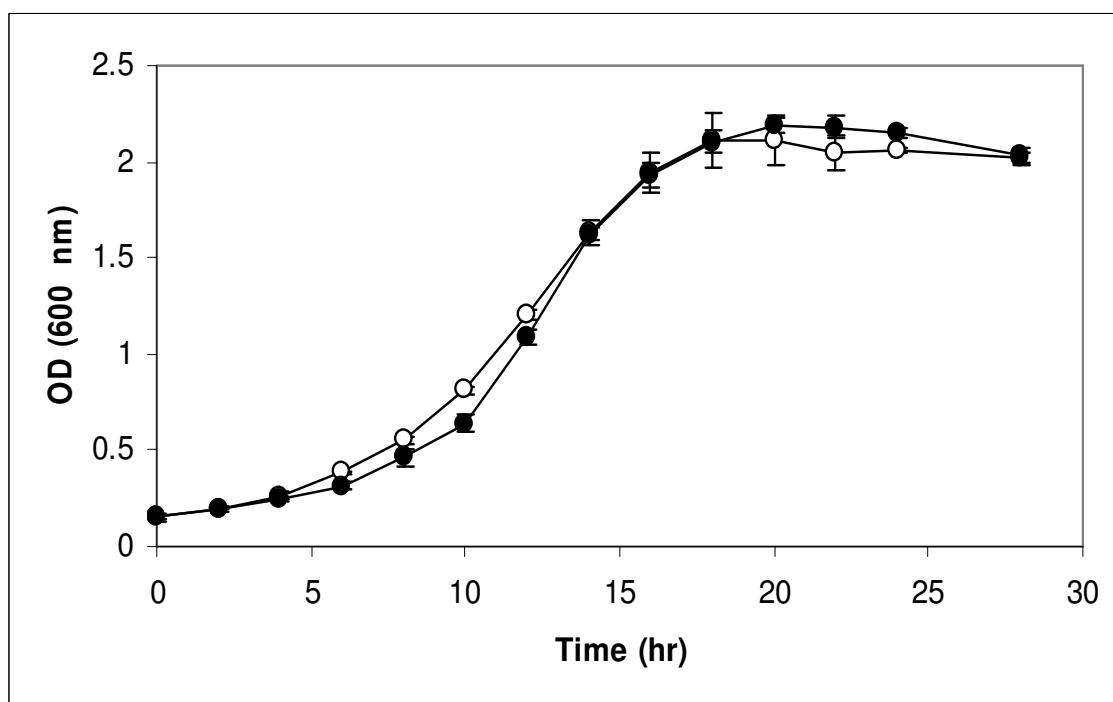


Figure III-3. Growth of *S. chlorophenicum* ATCC 53874 in ATCC medium 1687. (○) 5 g/L of glutamate; (●) 10 g/L of glutamate. Reaction conditions: working volume, 100 mL; inoculum, 5% of *S. chlorophenicum* grown in ATCC medium 1687; initial pH, 7.4; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.



#### 4.2.2 PNP Degradation Conditions

Experiments were designed to evaluate degradation of PNP by *S. chlorophenolicum*. The bacterium was grown to the early exponential phase, harvested and added to different media to determine if PNP was used as a carbon or nitrogen source. Media tested were mineral salts, mineral salts plus nitrogen source (NaNO<sub>3</sub>), mineral salts plus carbon source (cellobiose) and ATCC 1687. To establish conditions for degradation, the effect of growth stage for addition of PNP and the effect of initial pH were investigated as well.

As shown in Figure III-4, neither PNP degradation nor *S. chlorophenolicum* growth was observed when PNP was the sole carbon or nitrogen source or both. PNP degradation and *S. chlorophenolicum* growth were observed with ATCC medium 1687, which includes glutamate. PNP disappearance was completed in 60 hours (Fig. III-5). For this concentration of PNP, no lag phase was observed, suggesting that the PNP degradation pathway does not require induction when the degradation medium is ATCC 1687. *S. chlorophenolicum* did not utilize PNP as the sole carbon or nitrogen source, instead its degradation required the presence of carbon and nitrogen sources different than PNP.

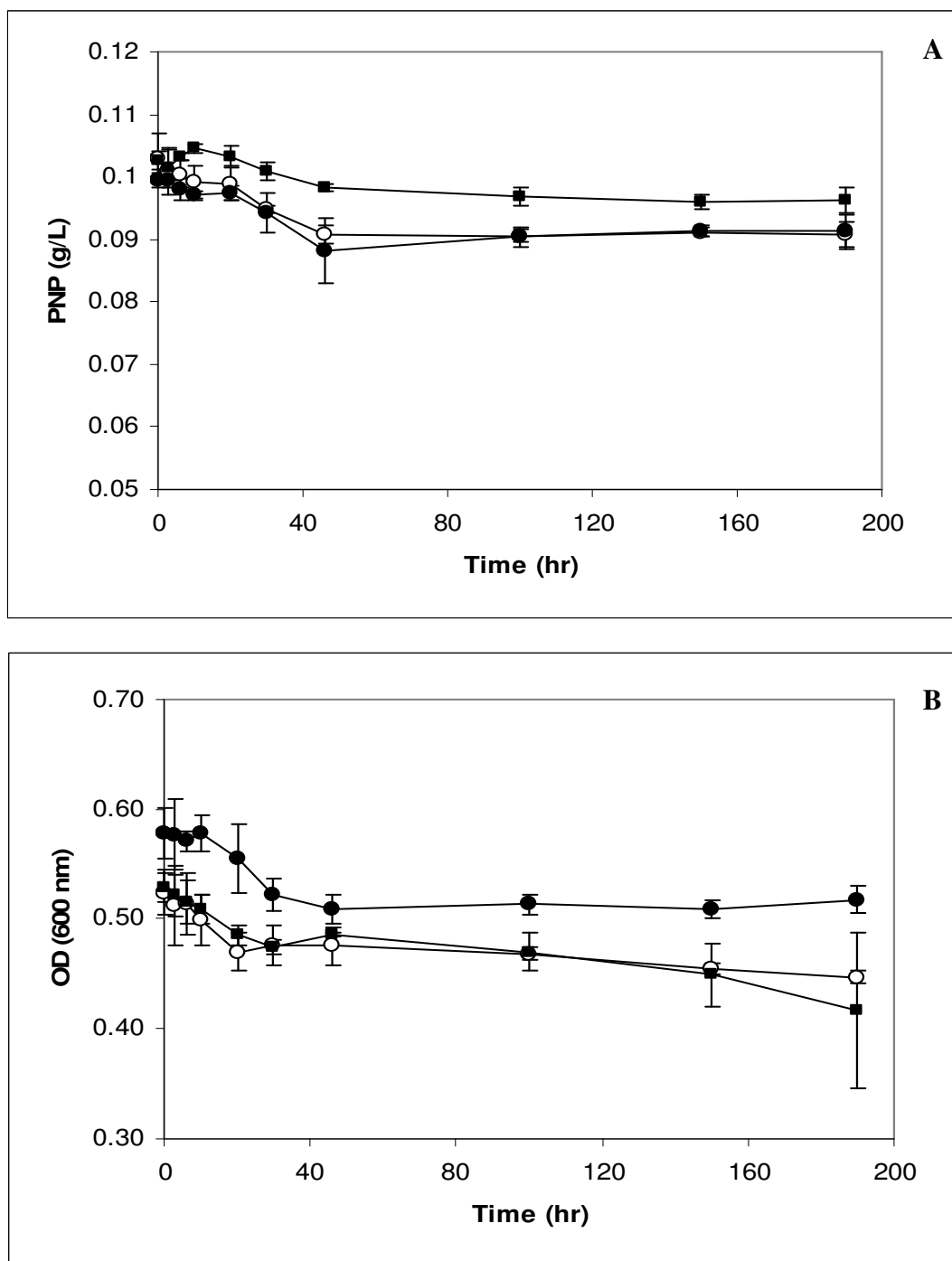


Figure III-4. PNP degradation for freely suspended *S. chlorophenicum* in different media. (A), PNP concentration; (B), growth; (○) mineral salts; (●) mineral salts plus carbon source; (□) mineral salts plus nitrogen source. Reaction conditions: working volume, 100 mL; harvesting time, early exponential phase; initial cell optical density, 0.5; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

To investigate if the process of PNP degradation occurred by enzyme induction, the bacterium was grown to the early exponential phase and PNP was added to give a final concentration of 0.01 g/L. After disappearance of PNP was confirmed, the biomass was harvested and added to mineral salts medium, containing PNP at a final concentration of 0.02 g/L. PNP degradation was not observed for up to 100 hours, indicating that the pathway for degradation of PNP by *S. chlorophenolicum* is not induced by PNP.

The ability of *S. chlorophenolicum* to degrade PNP at different stages of growth was evaluated by adding PNP during the early, middle and late exponential phases. As shown in Figure III-5, degradation rates were about the same when PNP was added during the early or middle exponential phase; however, when PNP was added during the middle exponential phase degradation was not complete. The lowest PNP degradation and growth rates were observed when PNP was added to the culture during the late exponential phase, and degradation occurred only after a long lag phase.

The maximum yield of biomass was lower when PNP was added to the medium compared to that obtained when PNP was not added. Growth of *S. chlorophenolicum* was observed when PNP was added to the glutamate degradation medium during the early and middle growth stages, but no growth occurred with addition during the late growth stage. These results indicated that PNP degradation occurs when *S. chlorophenolicum* is growing. As observed in Figure III-5 when the bacterium exhibits a higher growth rate, the degradation is also higher, suggesting that PNP degradation is proportional to growth.

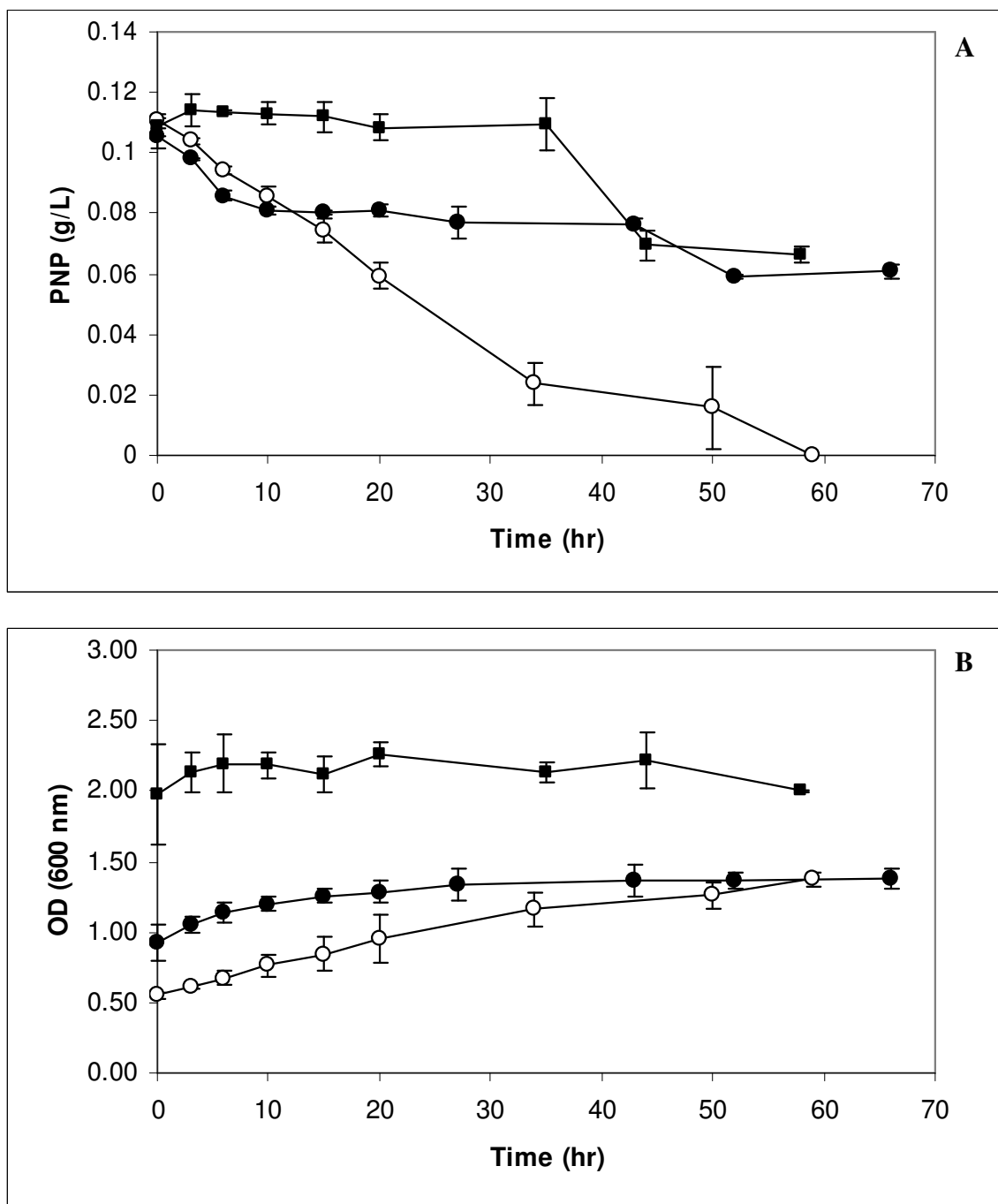


Figure III-5. Degradation of PNP when added at different *S. chlorophenicum* growth stages. (A), PNP concentration; (B), growth. (○) early exponential phase; (●) middle exponential phase; (■) late exponential phase. Reaction conditions: working volume, 100 mL; degradation medium, ATCC medium 1687; initial pH, 7.4; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

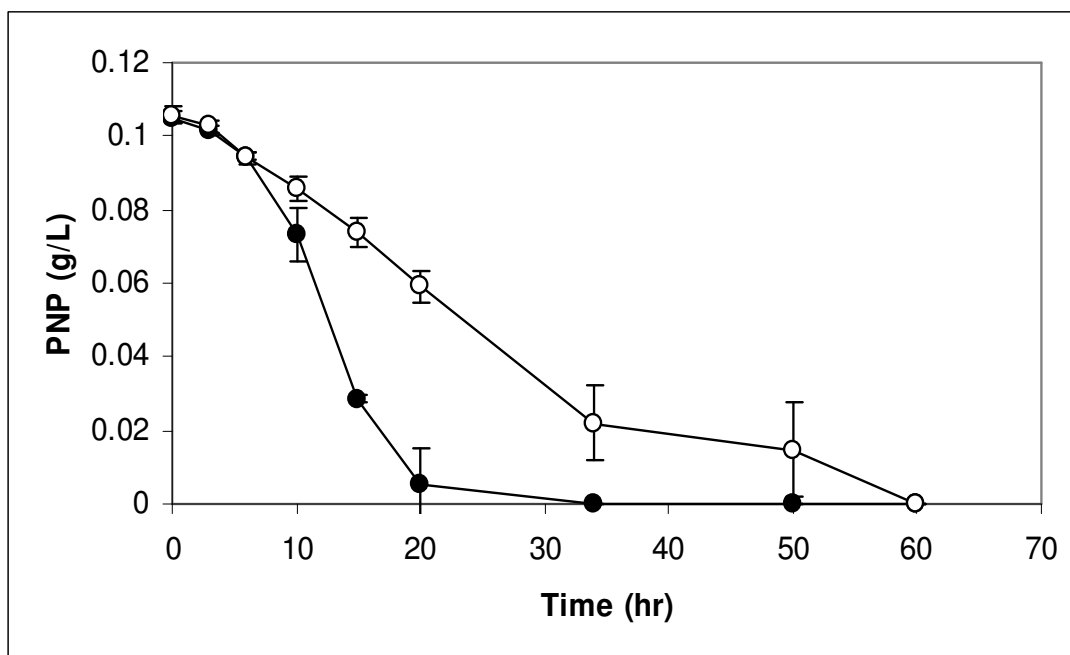


Figure III-6. Effect of pH value on PNP degradation for freely suspended *S. chlorophenolicum*. (○) initial pH 7.4; (●) initial pH 8.0. Reaction conditions: working volume, 100 mL; reaction medium, ATCC 1687; growth stage for addition of PNP, early exponential phase; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

The effect of initial pH on PNP degradation rates was evaluated. Results showed that complete degradation of PNP occurred faster with an initial pH of 8.0 compared to when it was 7.4 (Fig. III-6). However, initial rates were the same for both pH values.

In this study the degradation product of PNP by *S. chlorophenolicum* was neither NC nor HQ. Sample analysis by HPLC showed a peak eluted at 2.4 min. Under the same analysis conditions, HQ is eluted after 2.6 min and NC after 3.1 min. The samples, along with standards for NC and HQ were analyzed at wavelengths of 210, 240, 280, 300, 320, 360 and 400 nm. The peak was best resolved at 360 nm. HQ did not absorb at 360 nm whereas NC did. These results suggested that the degradation product was neither HQ because of the wavelength nor NC because of its elution time. To confirm that the degradation product peak did not correspond to either NC or HQ, a PNP degradation sample was spiked with both compounds. Comparison of spiked and unspiked chromatograms (Fig. III-7) shows that the NC standard peak did not overlap the degradation product peak. There was a decrease in the peak area representing the degradation product due to dilution by spiking.

The peak was not observed during the initial stages of degradation, but appeared when about half the PNP had disappeared, after which it continued to increase as PNP was degraded. The area of the peak was constant after disappearance of PNP was complete for some samples and began to slowly decrease for others. This suggests that the degradation product of PNP was being slowly metabolized by *S. chlorophenolicum*.

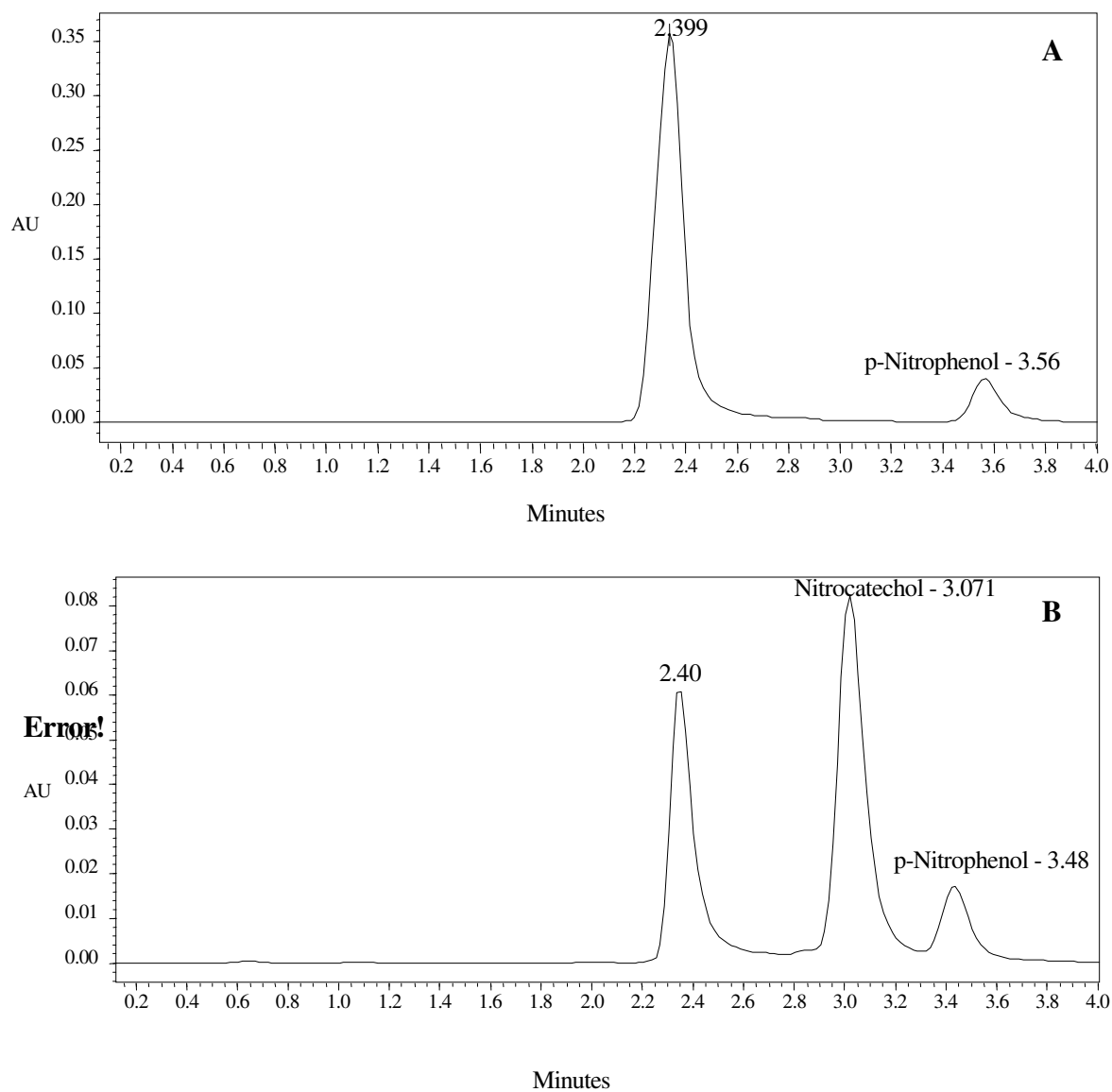


Figure III-7. Chromatogram of PNP degradation by *S. chlorophenolicum* in ATCC medium 1687. (A), Unspiked; (B), spiked with hydroquinone and nitrocatechol. Reaction conditions: working volume, 100 mL; initial pH, 7.4; temperature, 30 °C; agitation rate, 200 rpm; shake flasks.

When PNP was added to glutamate medium at a concentration of 0.1 g/L, no lag phase for its degradation was observed. This suggests that the degradation pathway by *S. chlorophenolicum* does not need to be induced. This is in contrast to results reported by Leung et al. (1997) who observed a lag phase prior to degradation of PNP by *S. chlorophenolicum* UG30. Crawford (1987) isolated *S. chlorophenolicum* ATCC 53874 from a PCP-contaminated site and induced the pathway for PCP degradation. It is interesting that this bacterium requires induction for PCP degradation but not for degradation of PNP. That PCP-degrading *Sphingomonas* strains are also capable of degrading and mineralizing nitrophenolic compounds may be due to the chlorine and nitro substituents, as both are electron-withdrawing and pose a similar bioenergetic challenge to enzymes that mediate their release from the compound (Cassidy et al., 1999).

*S. chlorophenolicum* did not utilize PNP as the sole carbon or nitrogen source at a concentration of 0.1 g/L. However, the same strain utilized PCP as its sole carbon and energy source (Crawford, 1987). Leung et al. (1997) and Cassidy et al. (1999) showed that *Sphingomonas* sp. UG30, *S. chlorophenolicum* ATCC 39723 and RA2 degraded PNP with release of nitrite. The three strains mineralized PNP to some extent at a concentration of 0.02 g/L (Leung et al. 1997). Mineralization was observed if glucose rather than glutamate was given as a carbon source (Cassidy et al. 1999). Leung et al. (1997) and Cassidy et al. (1999) suggested that *S. chlorophenolicum* UG30 was assimilating nitrogen from PNP in mineral salts glucose medium by cleaving the nitro group of PNP and releasing it as nitrite.

Both Cassidy et al. (1999) and Leung et al. (1999) found that the main role of PCP-4-monooxygenase in PNP degradation is in the oxidative removal of nitrite from NC to convert it to 1,2,4-benzenetriol (Fig. III-8). The enzyme does not convert PNP to NC. They concluded that *Sphingomonas* sp. UG30 metabolizes PNP via production of NC. Regarding the degradation product, the difference between this study and those of Leung et al. (1999) and Cassidy et al. (1999) may have been a result of the degradation medium used. Leung et al. (1999) and Cassidy et al. (1999) degraded PNP in mineral salts glucose medium (PNP was



given as nitrogen source), whereas the degradation medium used in this study was ATCC 1687, which provided glutamate and  $\text{NaNO}_3$  as nitrogen sources.

PNP degradation was observed only in glutamate medium. Leung et al. (1997) stated that the mineralization and transformation of PNP by UG30 required the presence of a supplementary carbon source. Topp et al. (1988) transferred PCP-induced, glutamate grown cells to mineral salts medium containing PCP with and without glutamate. They found that *Flavobacterium* sp. did not significantly remove PCP in the absence of supplementary carbon. Without glutamate they observed that regardless of prior induction of PCP degradation, the transfer resulted in cellular damage and a long lag phase preceding PCP removal. The addition of glutamate promoted full recovery of activity. They stated that a supplementary carbon source reduced acclimation periods and facilitated PCP metabolism by *Flavobacterium* sp. These statements are consistent with our findings in that *S. chlorophenicum* ATCC 53874 required the presence of carbon and nitrogen sources different from PNP to degrade it.

PNP degradation occurred only when growth occurred in glutamate medium. This result is again consistent with Topp et al. (1988), who studied the degradation of PCP by *Flavobacterium* sp. They found that the disappearance of PCP was accompanied by significant growth on almost all the supplementary carbon sources evaluated. The enhancement in PCP degradation by addition of a supplementary carbon source occurs through stimulation of the activity of an induced population (Topp et al. 1988).

*S. chlorophenicum* grows optimally within a pH range of about 6.9-8.5, preferably from about 7.0 to 7.4 (Crawford, 1987). However, the effect of pH showed that complete degradation of PNP occurred faster when initial pH was 8.0 compared to when it was 7.4.

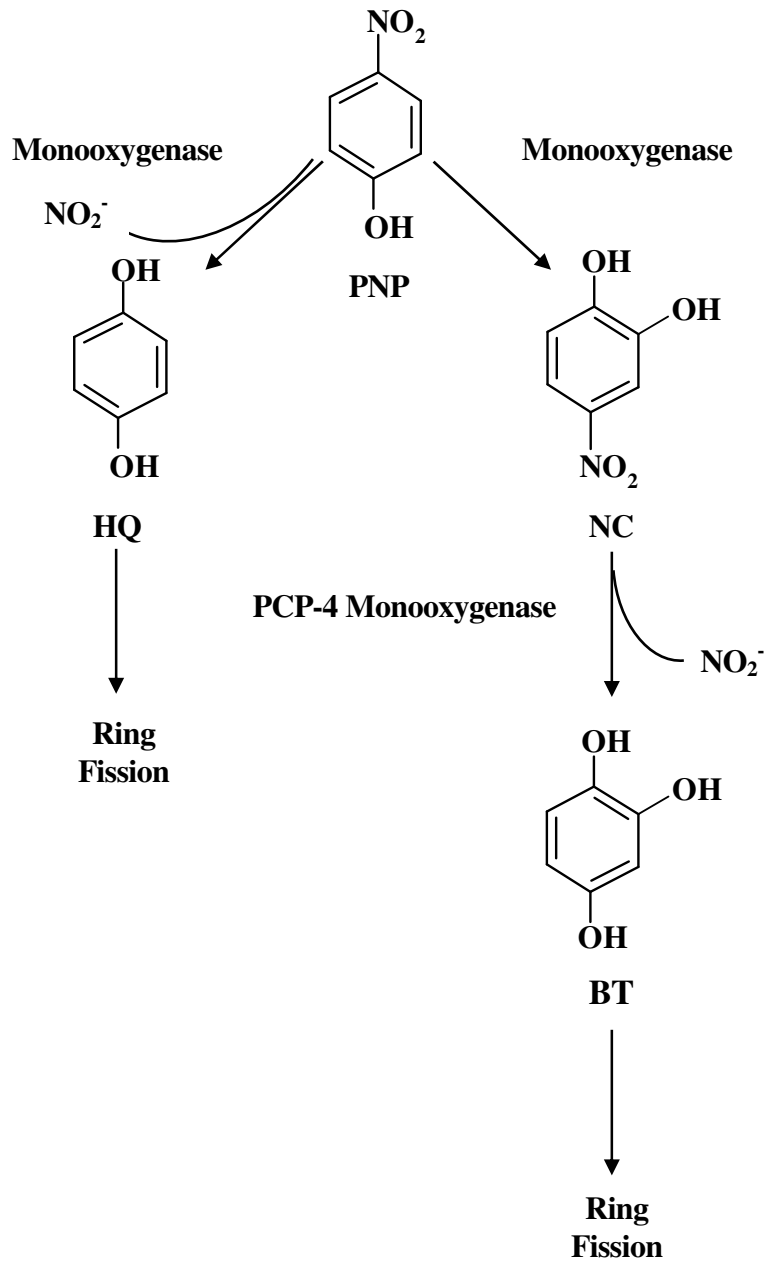


Figure III-8. Proposed involvement of PCP-4-monooxygenase in the degradation of PNP by *S. chlorophenolicum* UG30. PNP, *p*-nitrophenol; NC, nitocatechol; BT, 1,2,4-benzenetriol; HQ, hydroquinone (Leung et al., 1999).

### 4.2.3 Increased *S. chlorophenicum* Biomass Production

Experiments were conducted to find conditions for production of larger quantities of biomass with PNP degradation capability. Isolated colonies were cultured in 5 mL tubes of ATCC medium 1687 then transferred at a concentration of 5% to various growth media. Cells were grown to the middle exponential phase, and PNP was added to the cultures to a final concentration of 0.02 g/L. All media evaluated were supplemented with FeSO<sub>4</sub> and MgSO<sub>4</sub>·7H<sub>2</sub>O.

Growth of *S. chlorophenicum* in TB medium gave a maximum absorbance reading value of about 4.5 and a maximum reading of about 2 when grown in LB medium (Fig. III-9). These results indicated that the bacterium efficiently utilizes TB medium components for growth. However, no PNP degradation was observed up to 52 hours. Although no PNP degradation was observed, additional batches of cells were grown to the middle exponential phase, harvested as previously described and resuspended in ATCC 1687, TB or LB media to test for PNP degradation. No degradation was observed in any medium. From these results, it appears that growing *S. chlorophenicum* in TB or LB media inhibited its capability to degrade PNP.

Growth of *S. chlorophenicum* in modified ATCC medium 1687 plus either tryptone or yeast extract also increased biomass yield compared to ATCC medium 1687. The maximum absorbance reading was 4 to 4.5 (Fig. III-9). There was little difference in biomass yield when supplementing the medium with either tryptone or yeast extract. PNP was added when the culture reached an absorbance reading of 2.5, but no degradation was observed up to 120 hours.

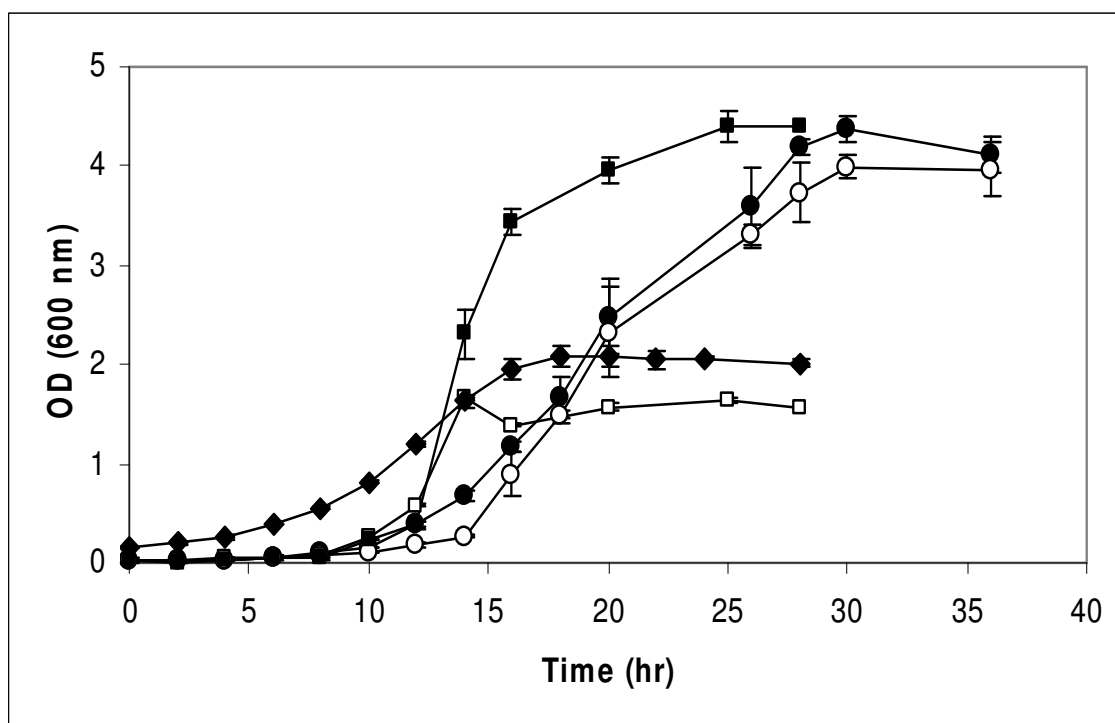


Figure III-9. Growth of *S. chlorophenolicum* ATCC 53874 in different media. (○) glutamate plus yeast extract; (●) glutamate plus tryptone; (■) TB; (□) LB; (◆) ATCC 1687;. Reaction conditions: working volume, 100 mL; inoculum, 5% of *S. chlorophenolicum* grown in ATCC medium 1687; initial pH, 7.4; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

Other modifications of ATCC medium 1687 evaluated to determine if the biomass yield could be increased were increasing the concentration of glutamate from 5 g/L to 47.6 g/L (corresponding to the total concentration in TB medium), increasing the concentration of  $\text{FeSO}_4$  and adding glycerol. Results from this set of experiments indicated that growth was linear following a lag phase rather than exponential (Fig. III-10). For all these cases no exponential growth phase was identified, instead it was observed a linear growth after a lag phase. The culture reached a maximum absorbance reading of about 1.6 between 32 and 37 hours. There were no differences in the yield of biomass for increased  $\text{FeSO}_4$  concentration or supplementation with glycerol. Even though growth phases were not clearly identified, the medium was evaluated for degradation of PNP. No degradation of PNP occurred up to a

week. ATCC medium 1687 was evaluated doubling the concentration of glutamate,  $\text{MgSO}_4$ ,  $\text{NaNO}_3$  and  $\text{FeSO}_4$ . Results showed that the growth of the bacterium in these media was linear and at 88 hours continued to grow for which the absorbance reading was 3.4. ATCC medium 1687 was also evaluated increasing all its components by 10 fold. For this case, no growth was observed. The culture reached an absorbance reading of 0.35 in the first 9 hours and then remained constant up to a week. This result indicates that the excessive amounts of nutrients in the medium inhibited growth.

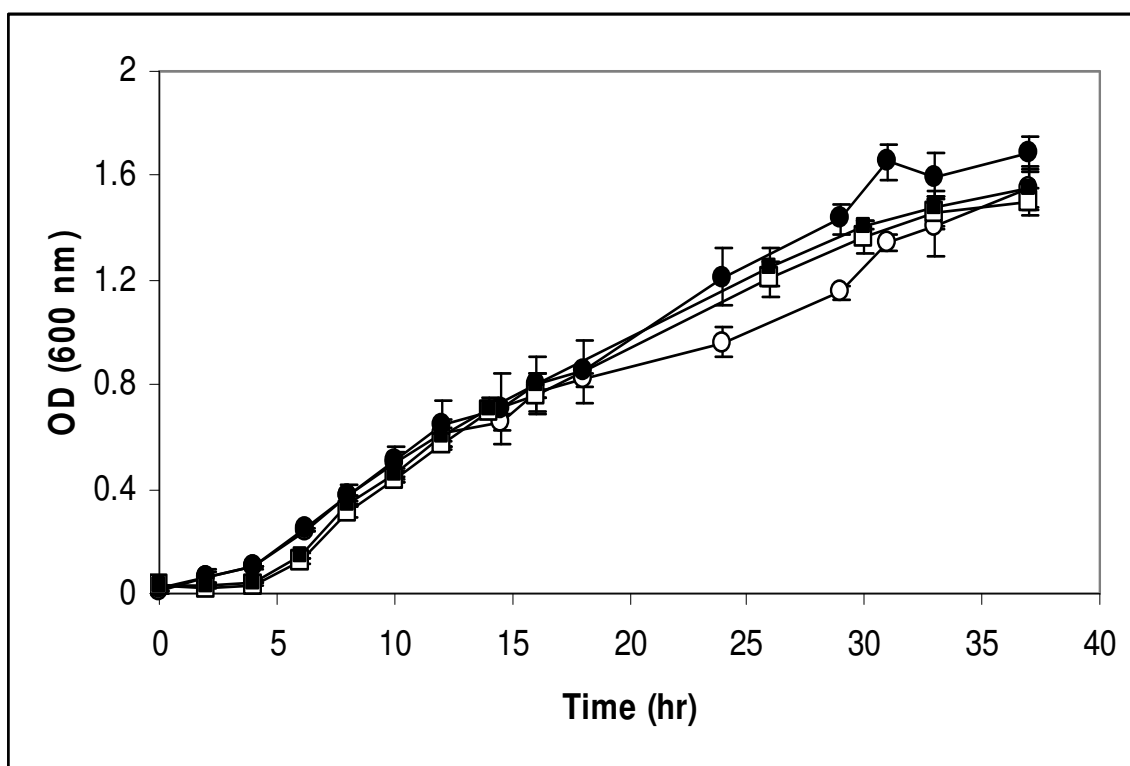


Figure III-10. Growth of *S. chlorophenicum* ATCC 53874 in glutamate, 47.6 g/L. (○) no glycerol; (●) glycerol, 4 g/L; (■)  $\text{FeSO}_4$ , 0.2 mM; (□)  $\text{FeSO}_4$ , 0.02 mM. Reaction conditions: working volume, 100 mL; inoculum, 5% of *S. chlorophenicum* grown in ATCC medium 1687; initial pH, 7.4; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

ATCC medium 1687 was modified by replacing glutamate with cellobiose, sucrose, fructose, glycerol, or glucose to evaluate their suitability as carbon sources for growth of *S. chlorophenicum*. Results for growth of the bacterium in each medium are shown in Figure III-11. The highest yield of biomass was obtained when cellobiose or glucose were added as a carbon source with a maximum absorbance reading of 2, the same as the biomass yield obtained in ATCC medium 1687. For these two carbon sources, the early exponential phase corresponded to an absorbance reading of 0.5, middle exponential phase to an absorbance reading of 1 to 1.2 and late exponential phase to an absorbance reading of 1.8. When fructose or glycerol was given as a carbon source the maximum absorbance reading was about 1.5. The early exponential phase corresponded to an absorbance reading of 0.5, middle exponential phase to an absorbance reading of about 0.8 and late exponential phase to an absorbance reading of 1.2. No growth of *S. chlorophenicum* was observed when sucrose was given as a carbon source. Crawford (1987) also found that *S. chlorophenicum* did not utilize either sucrose or fructose. However, the organism did utilize fructose in this study.

For all carbon sources evaluated, a longer lag phase was obtained compared to that when the carbon source was only glutamate. It is possible that the cells needed to acclimate to the new medium conditions since *S. chlorophenicum* was grown in ATCC medium 1687 and then transferred to the test medium. The longest lag phase was observed for fructose, then glycerol and cellobiose, and the shortest lag was with glucose.

To evaluate the capability for PNP degradation by *S. chlorophenicum* grown with different carbon sources, cultures were grown to the middle exponential phase then PNP was added at a concentration of 0.1 g/L. However, growth times were inconsistent compared to the data obtained for growth in Figure III-11. No degradation was observed in any of the media studied up to 54 hours. *S. chlorophenicum* cells from all these media were plated on ATCC medium 1687 agar. Colonies from modified media appeared very light maroon color, contrary to when cells were grown in the original medium where colonies appeared yellow.

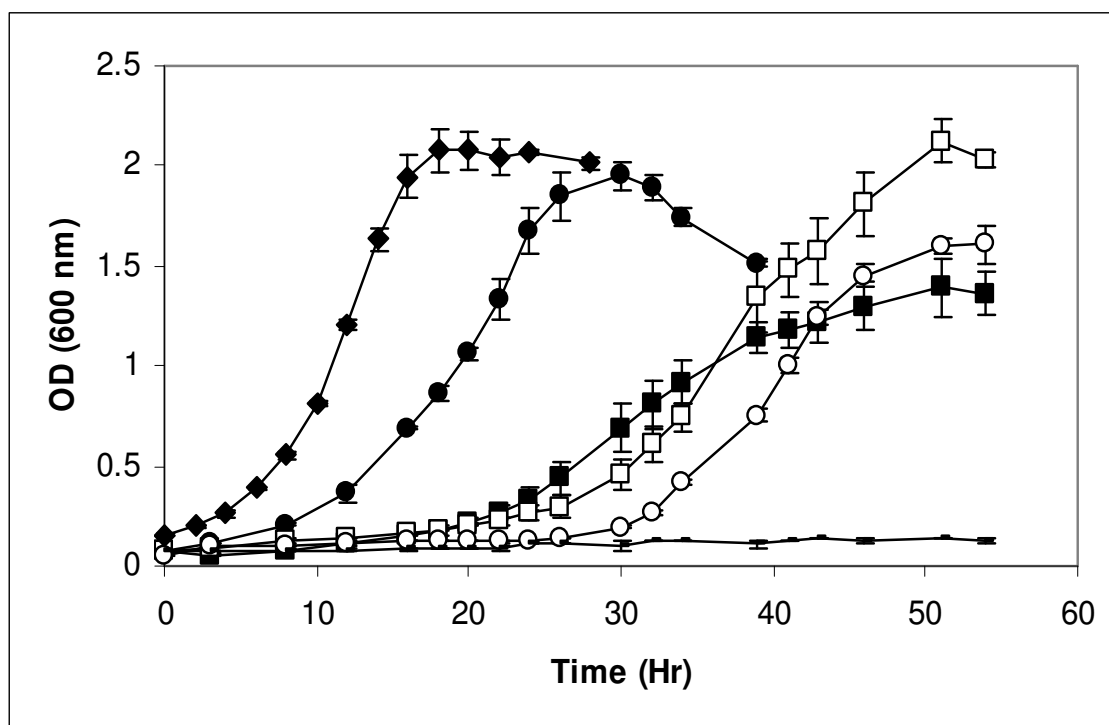


Figure III-11. Growth of *S. chlorophenolicum* in mineral salts medium with different carbon sources. (◆) glutamate, 5 g/L; (○) fructose, 5 g/L; (●) glucose, 5 g/L; (■) glycerol, 5g/L; (□) cellobiose, 5 g/L; (-)sucrose, 5 g/L. Reaction conditions: working volume, 100 mL; inoculum, 5% of *S. chlorophenolicum* grown in ATCC medium 1687; initial pH, 7.4; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

The possibility of cross contamination was considered, and experiments were repeated under strict aseptic conditions using the same inoculum source. Data for both growth and biomass color continued to be inconsistent for the modified media. The original medium (glutamate as carbon source) was used as a control, and it provided consistent data in terms of growth time, biomass color, and ability to degrade PNP. Therefore, it did not appear that contamination of the culture had occurred.

Degradation of PNP was observed only with growth of *S. chlorophenolicum* on the original medium with glutamate. For this reason, ATCC 1687 was chosen for further studies.

#### 4.2.4 Effect of Both Glutamate and PNP Concentration

Brown et al. (1986) stated that a *Flavobacterium* strain utilizes PCP as a sole source of carbon and energy. The absolute rate of PCP utilization is biomass-dependent and relatively low levels of PCP inhibit biomass production. To be able to degrade higher PCP concentrations, they grew the bacterium in carbon-limited continuous cultures with two carbon sources, PCP and cellobiose, which limited its grow simultaneously. Their rationale was that biomass grown on cellobiose would simultaneously utilize PCP. Therefore, experiments were conducted using two concentrations of glutamate and four concentrations of PNP to determine if that glutamate-produced biomass could simultaneously degrade PNP.

Results are shown in Figure III-12. PNP was degraded at an initial concentration as high as 0.5 g/L. However, complete degradation and no lag times were observed only for concentrations up to 0.1 g/L, above which the rate and extent of PNP degradation decreased. For all PNP concentrations evaluated, when the initial glutamate concentration was 10g/L, the degradation was slightly faster than with 5 g/L. When the initial PNP concentration was 0.3 or 0.5 g/L, degradation was very slow over the time period evaluated. At those concentrations, degradation was about 0.1 g/L in 195 hours. Growth of *S. chlorophenolicum* cells was observed only for PNP concentrations of 0.02 and 0.1 g/L, with a higher yield of biomass observed for 0.02 g/L. For PNP concentrations of 0.3 and 0.5 g/L, a decrease in optical density was observed over time, indicating loss of biomass.



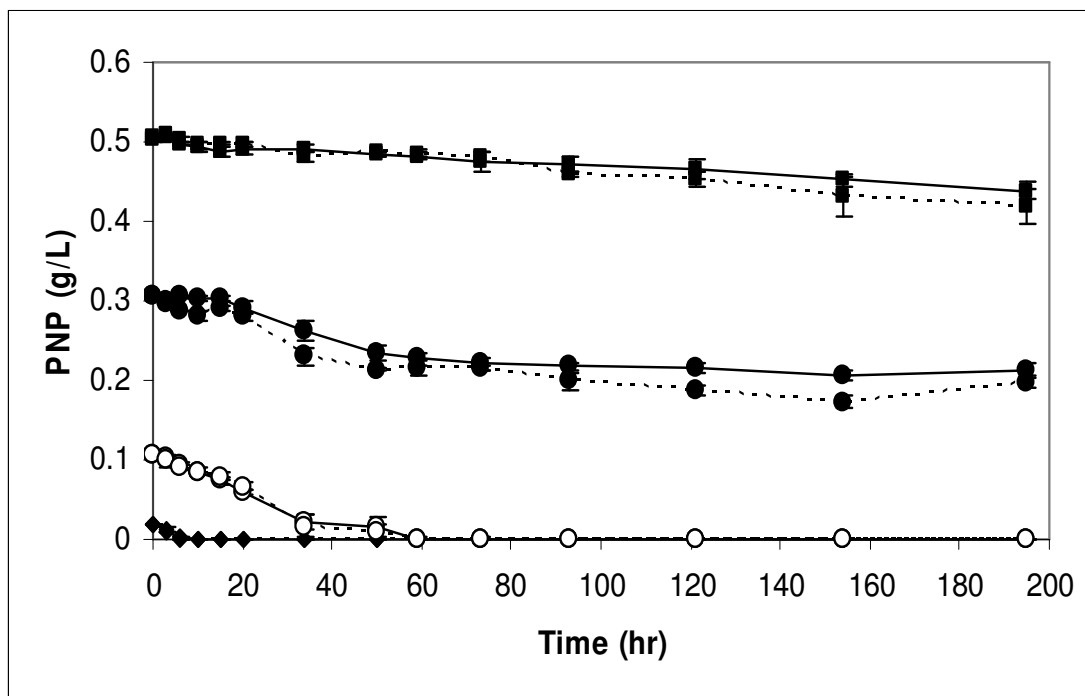


Figure III-12. Effect of both carbon source and PNP concentration on PNP degradation rates by freely suspended cells of *S. chlorophenolicum*. Final PNP concentration, (♦) 0.02 g/L; (○) 0.1 g/L; (●) 0.3 g/L; (■) 0.5 g/L. Glutamate concentration (—) 5 g/L; (---) 10 g/L. Reaction conditions: working volume, 100 mL; degradation medium, ATCC 1687; initial pH, 7.4; growth stage for addition of PNP, early exponential phase; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

Leung et al. (1997) found that the degradation of PNP by *S. chlorophenolicum* UG30 depended on its initial concentration. PNP was transformed at an initial concentration as high as 0.1 g/L and the optimal concentration for its degradation was 0.043 g/L, above which the degradation rate decreased. More than half of the PNP remained after a period of 60 hours when it was given at 0.1 g/L. In the present study, *S. chlorophenolicum* ATCC 53874 completely degraded PNP in less than 60 hours when it was given at the same concentration. It appears that PNP degradation by *S. chlorophenolicum* ATCC 53874 was faster than *S. chlorophenolicum* UG30; however, degradation conditions could have been different for each strain.

PNP was not completely degraded at concentrations of 0.3 and 0.5 g/L and no growth was observed. For lower PNP concentrations, PNP degradation was complete and a higher yield of biomass was obtained with the lowest PNP concentration. These results suggest that high PNP concentrations may inhibit the cells, resulting in cellular damage, death and consequently lack of activity. Since there was no *S. chlorophenicum* growth, the fact that some PNP was degraded indicates that there was some residual enzyme activity.

#### 4.2.5 Repeated Addition of PNP to Cultures

The potential for continuous use of *S. chlorophenicum* for degradation of PNP was evaluated. Cells were grown to early exponential phase, and then PNP was added at concentration of 0.1 g/L. When degradation was complete, PNP was added for second time at a concentration of 0.1, 0.3 or 0.5 g/L. Results are shown in Figure III-13. For the initial addition of PNP, complete degradation was observed in 20 hours, and continued growth of *S. chlorophenicum* was observed over this time. After the second addition of PNP, no growth or degradation was observed for any PNP concentration up to 144 hours. In fact, there was a slightly decrease in absorbance for all concentrations after the second addition of PNP, indicating that cells were dying.

As previously shown in section 4.2.2, the degradation of PNP by *S. chlorophenicum* appears to be proportional to growth. When PNP is added to the culture for first time, PNP is easily degraded since the cells are at the early exponential phase and have all the necessary nutrients to grow. When PNP is added to the culture for second time, the cells have reached the stationary phase, nutrients are not longer available; therefore there is no *S. chlorophenicum* growth and PNP degradation.

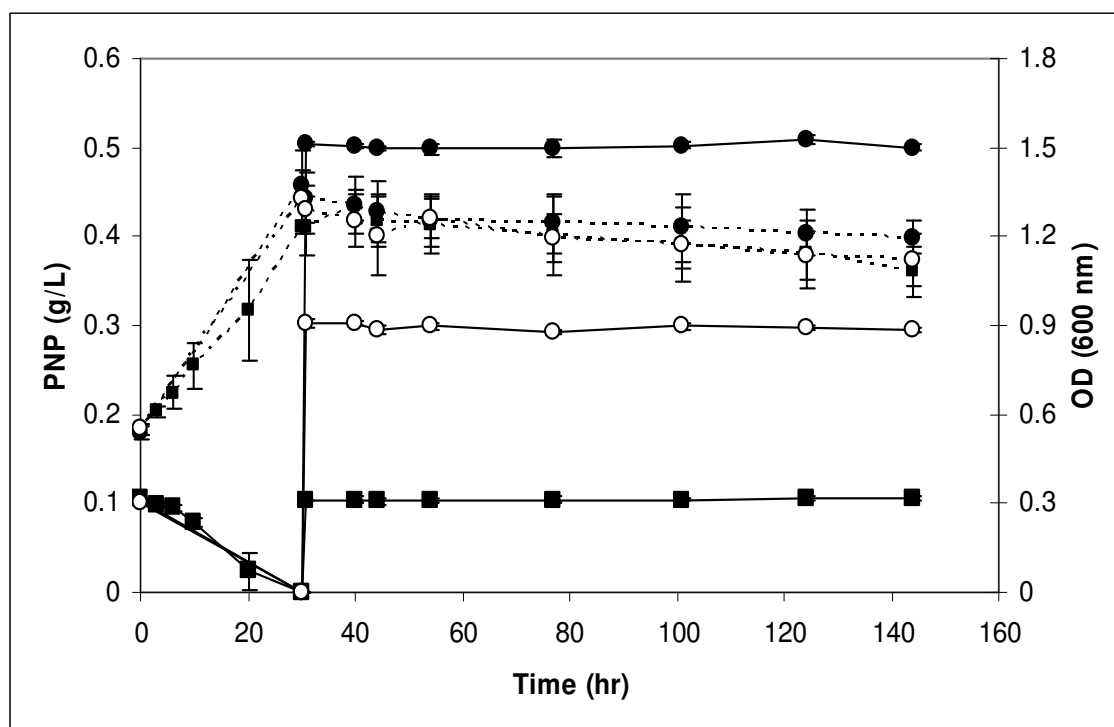


Figure III-13. Effect of freely suspended cells of *S. chlorophenolicum* on PNP degradation rates. Final PNP concentration, (■) 0.1 g/L; (○) 0.3 g/L; (●) 0.5 g/L; (—) PNP concentration; (---) growth. Reaction conditions: working volume, 100 mL; degradation medium, ATCC medium 1687; initial pH, 8.0; growth stage for addition of PNP, early exponential phase; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

Topp et al. (1988) transferred PCP-induced glutamate grown *Flavobacterium* sp. cells to mineral salts medium containing PCP with and without glutamate. Without glutamate they observed that regardless of prior induction of PCP degradation, the transfer resulted in cellular damage and long lags preceding PCP removal. The addition of glutamate promoted full recovery of activity. They suggested that the availability of a carbon source could facilitate PCP removal by attenuating the toxicity and contributing to the production and maintenance of PCP-degrading biomass. The present study suggests that for repeated use of *S. chlorophenolicum*, each time PNP is added to the medium, nutrients should also be added

and perhaps pH adjusted to 8.0. As was found by Topp et al. (1988), the availability of glutamate might promote continued activity.

#### 4.2.6 Summary of *S. chlorophenolicum* studies

Modifications of ATCC medium 1687 were evaluated to determine if the biomass yield could be increased. Increases in the concentrations of glutamate and FeSO<sub>4</sub> and the addition of glycerol gave linear growth behavior and no increase in biomass yield. Increasing ATCC medium 1687 component concentrations 10-fold completely inhibited growth. Possibly, osmotic pressure was too high or dissolved salts could have been inhibitory to membrane function.

Growth of *S. chlorophenolicum* in modified ATCC medium 1687 plus either tryptone or yeast extract considerably increased biomass compared to ATCC medium 1687. However, no PNP degradation was observed, suggesting that *S. chlorophenolicum* utilizes either tryptone or yeast extract rather than PNP when both yeast extract or tryptone and PNP are present. Both tryptone and yeast extract are very good sources of carbon and nitrogen and may demand less energy to be degraded than PNP.

The ability of *Sphingomonas* sp. to degrade PNP has been shown. However up to now no study has reported the degradation of PNP by *S. chlorophenolicum* ATCC 53874 without a lag period at concentrations up to 0.1 g/L. This fact is remarkable. It suggests that the bacterium does not need to induce metabolic pathways in order to assimilate PNP. This is environmentally important since treatment of PNP wastes or contaminated sites do not need pretreatment, reducing remediation time. Additional studies are needed to address problems such as toxicity of high PNP concentrations.

Cassidy et al. (1999) found that the concentration of PNP being degraded by UG30 could be increased as much as three times by encapsulating the cells. O'Reilly and Crawford (1989) reported that *S. chlorophenolicum* ATCC 39723 cells immobilized in polyurethane foam

mineralized higher PCP concentrations compared to free cells and that immobilized cells maintained degradation activity for up to 5 months. These studies demonstrate the important beneficial and protective effects of immobilization or encapsulation of microorganisms used to metabolize toxic compounds (Cassidy et al., 1999). Also, the sphingolipids contained by all *Sphingomonas* sp. may act as surfactants and improve the bioavailability of hydrophobic compounds (Nohynek et al., 1995). As new knowledge is generated on this interesting genus of bacteria, they may find more applications for environmental remediation of PNP and others compounds (Cassidy et al., 1999).

## CHAPTER IV

### BIODEGRADATION OF METHYL PARATHION USING A BINARY CONSORTIUM OF ENGINEERED *E. COLI* AND A NATIVE *S.* *CHLOROPHENOLICUM*

#### 1. Overview

The rapid growth in industrial and agricultural activities and advances in synthetic chemistry have led to contamination of the environment with hazardous compounds (Xu et al., 1999 and Chauhan et al., 2000). Among those contaminants are organophosphate (OP) compounds used as pesticides or chemical warfare agents (Caldwell, 1991). The OP methyl parathion (MP) is a highly toxic insecticide (Hertel, 1993). MP inhibits the activity of cholinesterase and may cause a delayed neurotoxicity (Cho, 2001). The hydrolysis products of MP are p-nitrophenol (PNP) and dimethylthiophosphate (DMTP) (Shimazu et al., 2001). PNP is toxic and a suspected carcinogen (Walker and Keasling, 2002), but there is no evidence of acute toxicity of DMTP (Orme and Kegley, 2004). This chapter describes the development of a process for remediation of MP using a binary consortium consisting of a genetically engineered *E. coli* to hydrolyze MP to PNP and DMTP and a native *S. chlorophenicum* to degrade PNP. Since the reaction medium developed for hydrolysis by *E. coli* was different than the medium used for *S. chlorophenicum*, identification of a suitable medium for both organisms was required. The MP hydrolysis medium used for *E. coli* did not support PNP degradation, but the PNP degradation medium did support degradation for both MP and PNP; therefore, medium chosen for the consortium was ATCC 1687 at pH 8.0.

Because MP had to be dissolved in an organic solvent for dispersion in the reaction medium, the effect of organic solvent on both MP and PNP was evaluated. Solvents evaluated were acetonitrile, methanol, xylene, ethanol and chloroform. No MP degradation was observed

with xylene and only slight degradation with chloroform. MP degradation was observed when it was dissolved in methanol, ethanol or acetonitrile. PNP degradation was observed only when a medium concentration of MP at 0.1 g/L was used with methanol, ethanol or acetonitrile at a solvent concentration of 1% in the medium.

## 2. Introduction

The proliferation of xenobiotic compounds in recent years has led to concerns over their contamination of the environment. These compounds are used as pesticides, solvents, explosives and in numerous other applications. In particular, their use as pesticides has increased as a result of increases in agricultural applications (Shimazu et al., 2001). Among the pesticides, OPs are extensively used in plant protection despite their high toxicity (Zhongli et al., 2001). In the United States, OP pesticides account for about 8% of the pesticides and about 50% of the total insecticides used. Annual applications exceed 75 million pounds. OP pesticides include malathion, diazinon, chlorpyrifos, guthion, malathion, MP and many others. There are 39 OP pesticides currently registered for use in the United States by the Environmental Protection Agency (EPA) (Bravo et al., 2002). Another class of OP compounds is chemical warfare agents. It is estimated that the United States stockpiles of these OPs amount to  $2.5 \times 10^4$  tons while the Soviet Union stockpiles amounts to  $4 \times 10^4$  tons (Caldwell, 1991).

The toxicity of OPs is primarily associated with inhibition of cholinesterase in the exposed organism (Cho, 2001). Cholinesterase is the enzyme responsible for deacetylation of the neurotransmitter acetylcholine (Bravo et al., 2002). The consequence of high level exposure to these neurotoxins is acetylcholine accumulation, which interferes with muscular responses, leading to the possibility of death (Shimazu et al., 2001). In addition, some OP compounds may cause induced delayed neuropathy (Cho, 2001). These toxic effects are not only observed in insects but also in wildlife and humans when exposed to high levels (Bravo et al., 2002). As many as 800,000 individuals may be affected annually by pesticide poisoning

(Caldwell, 1991). OP pesticides are the leading cause of pesticide poisoning in the United States. Contamination might occur from either occupational or accidental exposure by direct contact with air, food or water (Caldwell, 1991). Currently, the primary methods approved for disposal of toxic wastes are incineration and chemical treatment, but these methods are costly and often create new environmental problems. Incineration releases byproducts which may pose significant environmental hazards. The most common option for agricultural wastes has been disposal either on the soil surface or in settling tanks; however, this has produced some highly contaminated soils that have contributed to groundwater and well-water contamination (Caldwell, 1991).

MP is an OP insecticide used to control aphids, boll weevils, and mites on cotton, soybeans, wheat, alfalfa, rice, lettuce, onion, sugarbeets, and artichokes (Garcia et al., 2003). Because MP is a potent inhibitor of cholinesterase, its use has been restricted in the United States and in some other countries (Rani and Lalithakumari, 1994). Also, because of these health concerns, the EPA recently has cancelled the use of MP in many of the most significant food crops in the United States (Garcia et al., 2003). Pure MP is soluble in ethanol, chloroform, aliphatic solvents and slightly soluble in light petroleum. Technical MP used in preparing insecticide solutions contains 80% active ingredient, 16.7% xylene, and 3.3% inert ingredients (Hertel, 1993). Enzymatic hydrolysis of MP results in the formation of dimethylthiophosphate (DMTP) and PNP byproducts (Shimazu et al., 2001). As a result, PNP and other nitrophenols may accumulate in the soil or enter rivers and groundwater resources (Bhushan et al., 2000). PNP causes deleterious effects to plant, animal and human health. High levels of exposure to PNP may result in methemoglobin formation, liver and kidney damage, anemia, skin and eye irritation, and systemic poisoning (Bhatti et al., 2002). PNP is suspected to be a carcinogen and, therefore, a priority pollutant for the EPA (Walker and Keasling, 2002). There is no indication that DMTP causes acute toxicity, is carcinogenic, or has caused groundwater contamination. It is not a cholinesterase inhibitor (Orme and Kegley, 2004).



With the discovery in the 1960s that many soil microorganisms are capable of enzymatically degrading pesticides (Caldwell, 1991), the use of biological processes to degrade hazardous materials and xenobiotic compounds became a viable possibility. Biodegradation of toxic xenobiotics poses a double challenge to microorganisms. Since xenobiotics are not naturally occurring compounds, metabolic pathways to achieve degradation may not be available. Furthermore, in order to degrade a xenobiotic compound, the organism must strategically avoid or mitigate its toxicity and possibly that of some of its metabolic products as well (Xu et al., 1999). Because of all these factors, bioremediation processes are slow, decomposition products might be difficult to identify, and complete conversion to benign products might not be achieved.

In 1960, an organophosphate hydrolase (OPH) was discovered in sheep blood serum. Since then OPH activity has been detected in the soil bacteria *Pseudomonas diminuta* and *Flavobacterium sp* (Caldwell, 1991). OPH possesses broad substrate specificity and will tolerate significant differences in the structure of its substrates. It has been shown to effectively catalyze the hydrolysis of a wide range of OP pesticides (Shimazu et al., 2001) as well as several organophosphofluoridates, namely sarin and soman (Caldwell, 1991). The capability of OPH to hydrolyze many of the OP pesticides and chemical warfare agents could be exploited in the detoxification of contaminated systems (Caldwell, 1991 and Zongli et al., 2001). In particular, the enzymatic hydrolysis of MP reduces its toxicity by nearly 120-fold (Shimazu et al., 2001). Once the nitrophenyl group in MP was separated from phosphorus, the remaining metabolites lost the capacity to induce genotoxic effect (Cho, 2001). Also, hydrolytic cleavage of the OP bond receives considerable attention because it renders the molecule biologically inactive (Rani and Lalithakumari 1994). The OP-degrading gene (*opd*) of *Pseudomonas diminuta* that encodes the broad-spectrum OPH enzyme, has been extensively studied. Enzyme properties have been modified by genetic engineering techniques (Dave et al., 1993) resulting in recombinant *Escherichia coli* strains which express modified OPH (Hong, 1997). OP pesticides are not very soluble in water; therefore, they require an organic solvent to achieve higher concentrations in aqueous solutions. Previous OP

hydrolysis studies with OPH necessitated the use of at least 10% of methanol to improve solubility (Caldwell, 1991).

Though the degradation of both MP and PNP have been well studied and characterized by pure cultures, the simultaneous degradation of both compounds using a binary consortium consisting of a genetically engineered and a native species of bacteria has not been reported. To efficiently eliminate the toxicity of MP and its degradation product PNP, populations of cells for which the metabolic pathways for degradation are coupled in a single reaction would be desirable. To investigate this approach, studies to understand the interaction between recombinant OPH<sup>+</sup> *Escherichia coli* and native *S. chlorophenolicum* ATCC 53874 were needed. In this study the conditions for the remediation of both MP and PNP are described. This approach could lead to systems providing less costly treatment with conversion to benign products.

### **3. Materials and Methods**

#### **3.1 Bacterial Strains**

Degradation of MP was accomplished using a recombinant OPH<sup>+</sup> *E. coli* and *S. chlorophenolicum* ATCC 53874. *S. chlorophenolicum* ATCC 53874 was selected because previous studies showed that it degrades PNP more efficiently than *Burkholderia cepacia* ATCC 29354. *S. chlorophenolicum* ATCC 53874 was purchased from ATCC.

For the recombinant OPH<sup>+</sup> *E. coli*, standard recombinant DNA techniques were employed in the construction of plasmid and phage vectors for *E. coli* as described in chapter II.

### 3.2 Biomass Preparation

For hydrolysis of MP, an isolated colony of transformed *E. coli* was cultured, grown and harvested as described for MP hydrolysis. The resulting biomass was used for experiments. For degradation of PNP, an isolated colony of *S. chlorophenicum* ATCC 53874 was cultured and grown using ATCC medium 1687 with an initial pH of 8.0 and addition of PNP during the early exponential phase as described in the previous chapter.

### 3.3 Experimental Design

MP crystal (99.5% purity) was used without further purification. PNP was produced by hydrolysis in these studies.

Because the previous pure culture studies used different reaction media, a common medium had to be identified. MP hydrolysis by OPH<sup>+</sup> *E. coli* was evaluated using the PNP degradation medium, and PNP degradation by *S. chlorophenicum* ATCC 53874 was evaluated using the MP hydrolysis medium. Media were supplemented with 0.02 mM FeSO<sub>4</sub>, and final the pH was adjusted to 8.0 with NaOH, the optimum for both OPH<sup>+</sup> cell activity and PNP degradation.

Since MP solubility in water is very low, it was dissolved in an organic solvent (10g MP/L organic solvent) before adding to aqueous media. Shake flask experiments were conducted to determine the effect of organic solvent on the activity of freely suspended bacteria for both MP and PNP degradation. The effect of organic solvent was evaluated by dissolving MP in acetonitrile, methanol, xylene, ethanol or chloroform to give a final concentration from 0.1 to 0.2 g/L using a concentration of organic solvent from 1 to 10%. Methanol and ethanol were selected because they are common organic solvents and they are easily biodegraded. Acetonitrile was evaluated because is used as a commercial solvent for MP. Xylene was of particular interest since it is an ingredient in technical MP at a concentration of 16.7% (Hertel,

1993). Chloroform was evaluated because is a good solvent for MP (Hertel, 1993). The organic solvent supporting the highest degradation rate was selected. Each culture was added at a proportion of 1 to 1 w/w.

### **3.4 Reaction System**

Experiments for degradation of MP were conducted under batch reactor conditions in 250-mL Erlenmeyer flasks with a working volume of 100 mL. Reactions were carried out at 30 °C with rotary shaking at 200 rpm. Experiments were conducted in triplicate. Samples (1 mL) were taken from the bulk phase at intervals during each experiment for determination of MP, PNP, NC, and HQ concentrations.

### **3.5 Analytical Methods**

The growth phase of *S. chlorophenicum* ATCC 53874 in ATCC medium 1687 was determined by measuring the optical density at 600 nm using a spectrophotometer (Spectronic 20D<sup>+</sup>, Milton Roy, Rochester, NY). A 1.0 mL sample was placed in a polystyrene 1.5 mL cuvette to measure absorbance.

Degradation of MP in the bulk solution was determined by following the concentrations of MP and PNP. MP concentration was determined as described in Chapter II. Concentrations of PNP and its metabolites were determined as described in Chapter III. Samples (1 mL) were taken from the bulk phase at intervals during each experiment for analysis.

## 4. Results and Discussion

### 4.1 Medium Selection

Medium selection studies were conducted to determine an acceptable medium for the consortium. The reaction medium use for hydrolysis of MP by OPH<sup>+</sup> *E. coli*, CHES buffer, did not produce either PNP degradation or cell growth over a period of 190 hours (Fig. IV-1). However, the recombinant *E. coli* rapidly hydrolyzed MP in ATCC medium 1687 at pH 8.0 (Fig IV-2). *E. coli* growth was not observed, probably because of the high concentration of acetonitrile. It was also observed that the hydrolysis rate of MP in ATCC medium 1687 (1.15  $\mu\text{mol}/\text{min g biomass}$ ) was about half the rate in CHES buffer (2.76  $\mu\text{mol}/\text{min}\cdot\text{g biomass}$ ). Although experiments in ATCC medium 1687 were conducted at a lower agitation rate than those in CHES buffer (200 rpm vs. 300 rpm), the decrease in specific hydrolysis rate likely was caused by the different medium composition.

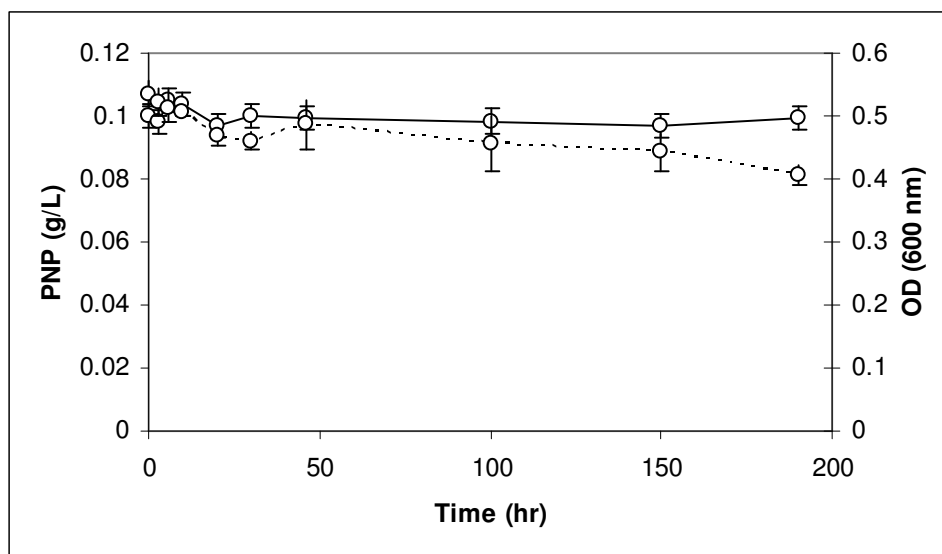


Figure IV-1. Degradation of PNP by *S. chlorophenolicum* in MP hydrolysis medium. (—) PNP concentration; (---) growth. Reaction conditions: Initial concentration, 0.1 g/L; initial pH, 8.0; initial cells optical density, 0.5; medium, 100 mM CHES buffer; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

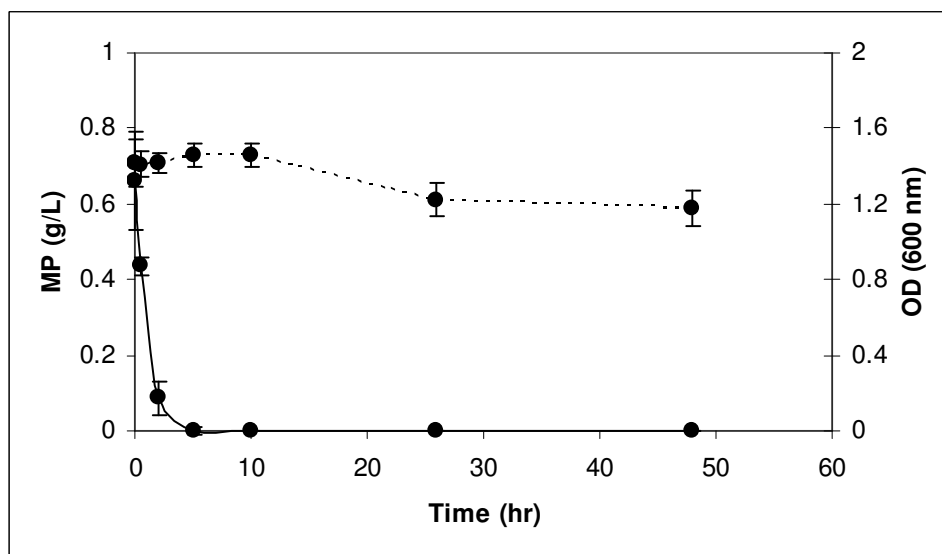


Figure IV-2. Degradation of MP by recombinant OPH<sup>+</sup> *E. coli* in PNP degradation medium. (—) MP concentration; (---) growth. Reaction conditions: Initial concentration, 1 g/L; initial pH, 8.0; medium, ATCC 1687; biomass concentration, 25 g/L organic solvent, 10% acetonitrile; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

## 4.2 Organic Solvent Selection

Because MP solubility in water is very low, an organic solvent is required to prepare media containing MP. The studies reported in chapter II on the effect of acetonitrile concentration on MP hydrolysis showed that the optimum concentration was 10%. No solvent was required for the *S. chlorophenicum* studies; therefore, several organic solvents were evaluated for their effect on the activity of freely suspended cells of *E. coli* and *S. chlorophenicum*.

Results are shown in Figure IV-3. No MP degradation was observed when MP was dissolved in xylene, and only slight degradation was observed in chloroform. No production of PNP with either solvent was detected by HPLC analysis. Both xylene and chloroform were detrimental to OPH<sup>+</sup> *E. coli* cells under the experimental conditions evaluated. Since PNP was not produced, the effect of these organic solvents was not evaluated on PNP degradation by *S. chlorophenicum*.

MP degradation was observed when it was dissolved in methanol, ethanol or acetonitrile (Fig. IV-3). The degradation rates for these three organic solvents were about the same based on production of PNP. MP was not detected by HPLC analysis since its initial concentration was very low and the reaction between MP and OPH<sup>+</sup> *E. coli* was quite rapid. Once MP hydrolysis was completed, PNP was no longer produced and its concentration remained constant up to 144 hours indicating that PNP was not degraded by *S. chlorophenicum* under these conditions. In addition, no cell growth was observed.

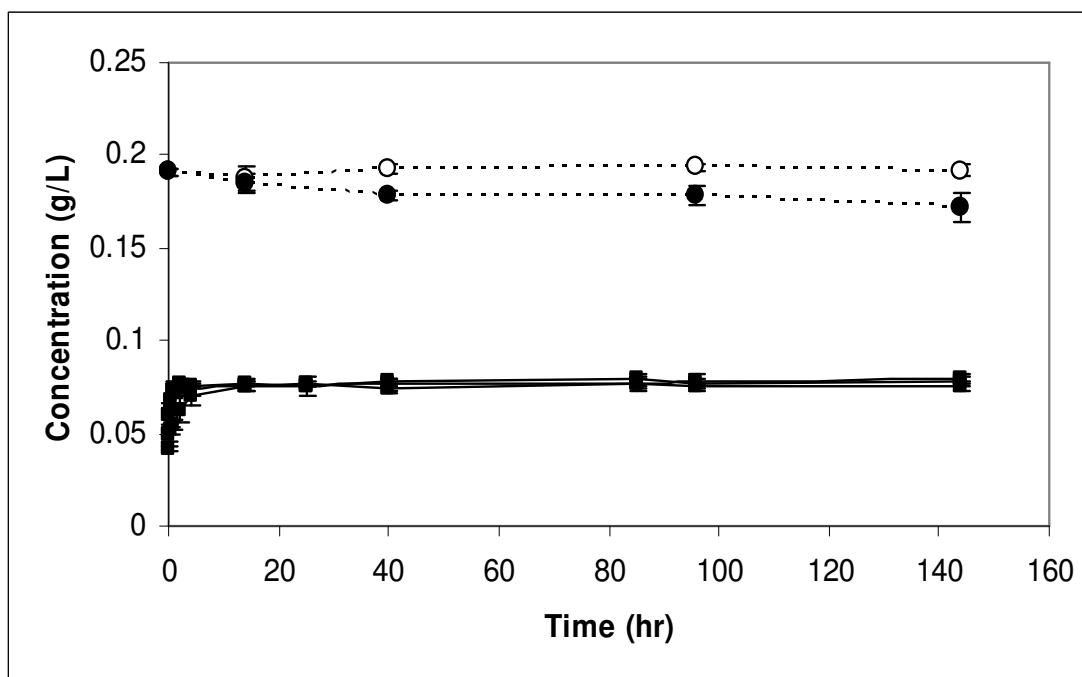


Figure IV-3. Degradation of MP and PNP with 10% organic solvent. (—) PNP; (---) MP (○) xylene; (●) chloroform; (■) methanol, ethanol and acetonitrile. Reaction conditions: Recombinant OPH<sup>+</sup> *E. coli* and *S. chlorophenolicum* in ATCC medium 1687; initial MP concentration, 0.2 g/L; initial pH, 8.0; initial cell optical density, 0.5; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

Results from experiments using a concentration of 2% of organic solvent and an initial MP concentration of 0.2 g/L are shown in Figure IV-4. With the lower concentration of organic solvent MP hydrolysis was considerably slower, taking over 40 hours to complete. In addition, MP hydrolysis showed a lag phase of 5 hours. The results for MP hydrolysis were not unexpected since hydrolysis was slower with 2.5% acetonitrile compared to 10%. There did not appear to be any degradation of PNP. However, the initial concentration of PNP did not account for the initial disappearance of MP as was reported in Chapter II. In this experiment, the PNP unaccounted for was much higher than reported in Chapter II.



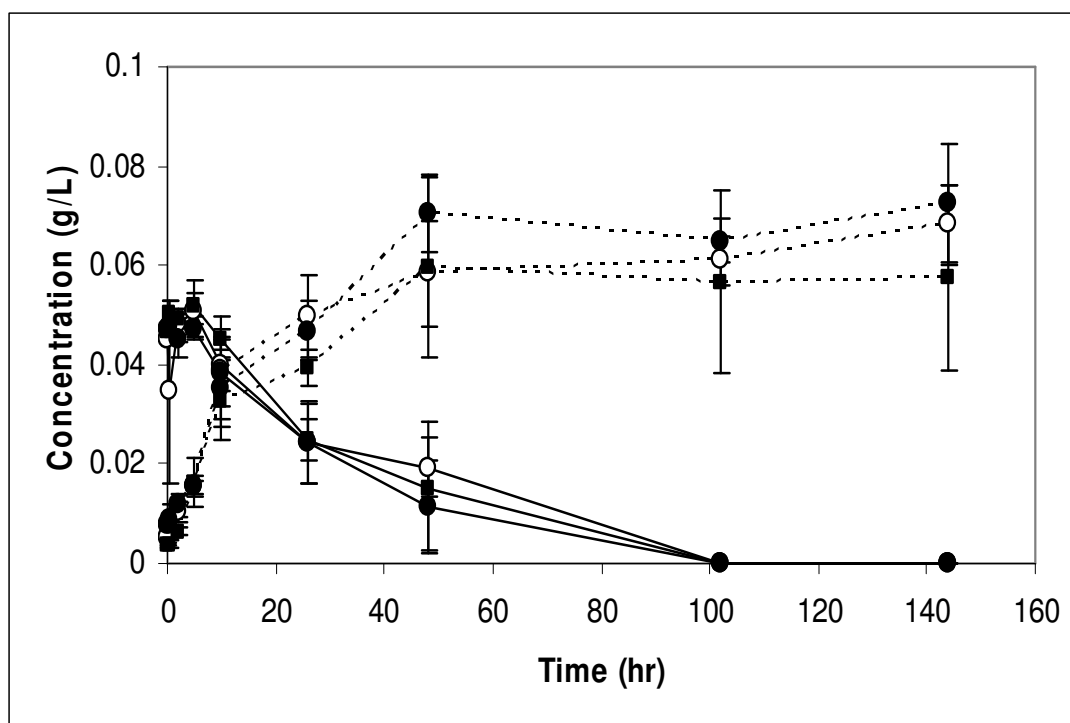


Figure IV-4. Degradation of MP and PNP with 2% organic solvent. (—) MP; (---) PNP (○) methanol; (●) acetonitrile; (■) ethanol. Reaction conditions: Recombinant OPH<sup>+</sup> *E. coli* and *S. chlorophenolicum* in ATCC medium 1687; initial MP concentration, 0.2 g/L; initial pH, 8.0; initial cell optical density, 0.5; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

Slower hydrolysis of MP is not necessarily undesirable since slow production of PNP might prevent its inhibition of degradation by *S. chlorophenolicum*. However, no disappearance of PNP was observed for the period of time evaluated.

Results from experiments using a concentration of 1% of organic solvent and an initial MP concentration of 0.1 g/L are shown in Figure IV-5. As shown previously (Fig. IV-2), MP hydrolysis was quite rapid so that no MP peak was detected. Both MP and PNP degradation were found when methanol, ethanol, or acetonitrile were used as organic solvent. It appears there was no lag in the degradation of PNP when methanol or ethanol was used as solvent.

The highest biomass yields were observed in methanol and ethanol, with acetonitrile lower; therefore, PNP degradation using acetonitrile was lower.

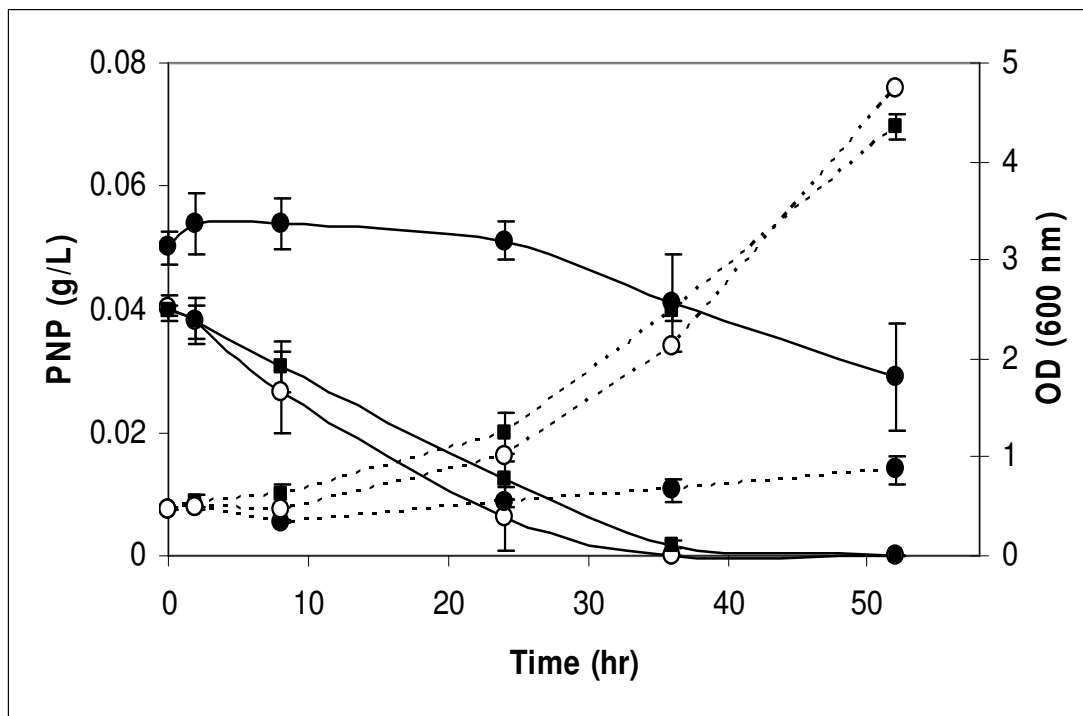


Figure IV-5. Degradation of MP and PNP with 1% organic solvent. (—) PNP; (---) growth (○) methanol; (●) acetonitrile; (■) ethanol. Reaction conditions: Recombinant OPH<sup>+</sup> *E. coli* and *S. chlorophenolicum* in ATCC medium 1687; initial MP concentration, 0.1 g/L; initial pH, 8.0; initial cell optical density, 0.5; temperature, 30 °C; agitation rate, 200 rpm; shake flasks. Vertical bars represent twice the standard deviation of the means for triplicate experiments.

The product of the degradation of PNP under these experimental conditions was investigated by HPLC analysis. As found in the degradation of PNP by *S. chlorophenolicum* and in the absence of organic solvent, the product is a peak eluted at 2.4 min that is best resolved at 360 nm. The peak area appeared to increase as the PNP area decreased; but during initial stages of degradation, the peak area was below the minimum for calculation by the HPLC software so that peak area was calculated only when more than half the PNP had disappeared.

The MP hydrolysis rate was lower using 2% organic solvent compared to 10% (Fig IV-3 and IV-4) when starting with 0.2 g/L MP. Although lower organic solvent concentrations might be expected to be sufficient for solubilizing lower MP concentrations, the hydrolysis reaction was faster using 10% solvent. This indicates that the organic solvent affects the reaction through other mechanisms besides increasing solubility.

Results from experiments using a concentration of 2% organic solvent and an initial MP concentration of 0.2 g/L showed a lower degradation rate than using 10% organic solvent. The hydrolysis showed a lag time and more PNP was unaccounted for. Previous studies have shown that part of the lost of PNP was due to absorption by OPH<sup>+</sup> *E. coli*; however, the amount of biomass used in this experiment was lower so less PNP should be absorbed. The organic solvent concentration affects both the MP hydrolysis rate and solubility but is not expected to affect the solubility of PNP. It is possible that MP was not completely dissolved so that some was lost by centrifugation during sample preparation.

No PNP degradation was observed using concentrations of organic solvent of 2 or 10% and a MP concentration of 0.2 g/L. The lack of PNP degradation is not due to toxicity by MP since it was not present in the degradation medium for most of the time period evaluated. It appeared that either the organic solvent itself or its concentration was inhibitory to the bacterium. However, results of experiments using organic solvent at a concentration of 1% and MP concentration of 0.2 g/L did not show PNP degradation. Both MP and PNP degradation were found using 1% methanol, ethanol or acetonitrile and a MP concentration of 0.1 g/L. Because the bacterium only degraded PNP when the organic solvent was 1%, it appears that organic solvent inhibited PNP degradation capability.

The findings from this study indicate that bioremediation of MP and PNP by a binary consortium was effective but only for low concentrations of MP and PNP. Results from degradation of both PNP and MP by the bacterial consortium suggest that each bacterium

may degrade the compounds more efficiently when they performed separately than when they perform as a binary consortium.

## CHAPTER V

### CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of this work was to develop and study a defined bacterial consortium for the degradation of methyl parathion (MP), with emphasis on hydrolysis of MP and degradation of p-nitrophenol (PNP), one of its hydrolysis products. MP was selected for the study because its degradation is fairly well defined. A genetically modified *E. coli* was selected to provide the capability for hydrolysis of MP since it contained the *opd* gene for organophosphate hydrolase (OPH). Additional organisms were needed to degrade the hydrolysis products, and *S. chlorophenicum* was selected for its ability to degrade PNP.

Three studies were completed in order to address the overall goal. The outcome of each study is discussed below including the significance of conclusions reached and topics recommended for further research.

The objective of the first study was to demonstrate the potential for using non-growing OPH<sup>+</sup> *E. coli* cells for biodegradation of MP at significantly higher rates than currently are obtained with native organisms. In this study, the maximum biodegradation rate was found to be  $69 \pm 3$   $\mu\text{mol/L}\cdot\text{min}$ , which was 135 times higher than reported for a native OPH<sup>+</sup> organism (0.51  $\mu\text{mol/L}\cdot\text{min}$  reported by Ramanathan and Lalithakumari, 1999). The results of this study corroborate the potential for using non-growing OPH<sup>+</sup> *E. coli* cells for biodegradation of MP at significantly higher rates than currently are obtained with native organisms.

These experiments also confirmed the need for an organic solvent to enhance the hydrolysis of MP by OPH<sup>+</sup>. The organic solvent not only facilitates the hydrolysis reaction but is necessary for solubilization of MP. For this study the organic solvent used was acetonitrile. Acetonitrile was found to be inhibitory to the hydrolysis reaction at a concentration of 25%.

Preliminary studies were carried out using methanol as organic solvent, but it appeared that MP was unstable in methanol. Further research should be conducted to evaluate the stability of MP or other OPs in methanol. Considering that the primary goal of this study was to develop technology for remediation, the use of a readily metabolizable organic solvent would be desirable since the use of other types of organic solvents could be detrimental to the environment.

The significance of this research was the rapid elimination of the toxicity of MP by OPH<sup>+</sup> *E. coli*. The maximum degradation rate was found to be 69  $\mu\text{mol/L}\cdot\text{min}$ . The MP degradation rate was higher than that found by Kim et al. (2002) for degradation of coumaphos in cattle dip waste by the same organism (13.6  $\mu\text{mol/L}\cdot\text{min}$ ). In addition, the optimum biomass concentration was 25 g/L compared to 70 g/L required for optimum coumaphos degradation. OPH<sup>+</sup> *E. coli* can be used for remediation of OPs when and where environmental conditions demand immediate action. In such cases, the use of native organisms may not provide a quick solution since their degradation rates are either slow or they cannot degrade the compound when it is present at high concentrations.

Hydrolysis of MP using OPH reduces its toxicity by 120-fold (Shimazu et al. 2001), but one of the hydrolysis products (PNP) is also toxic. The objective of the second study was to find an organism capable of degrading PNP. *B. cepacia* and *S. chlorophenicum* were evaluated, and only *S. chlorophenicum* degraded PNP under conditions studied.

*S. chlorophenicum* was capable of degrading PNP without induction when glutamate was included in the medium. Avoiding preexposure of the bacterium to the compound allows direct treatment of PNP wastes which, in turn, reduces the remediation process time. The bacterium efficiently degraded concentrations up to 0.1 g/L. To overcome the limitation of low degradation concentrations, significant efforts were made to increase the yield of biomass with PNP-degrading capability. Attempts resulted more in failure than in success. Complex growing media seemed to inhibit the degradation. Varying the components of ATCC

medium 1687 did not show positive results. Further research is needed to enhance the potential for PNP degradation. Since the culture has to be growing in a primary carbon source to degrade PNP, it remains to be studied whether continuous feeding of glutamate would allow degradation of greater amounts of PNP.

The third part of this study attempted to degrade both MP and PNP by a defined bacterial consortium. Results from first study demonstrated the need for an organic solvent for MP degradation by OPH<sup>+</sup> *E. coli*. Results from second study showed that *S. chlorophenolicum* had to be growing in a primary carbon source for PNP degradation to take place. The findings from degradation of both MP and PNP by the binary consortium showed that it is possible to degrade them, but only for lower concentrations than when the compounds were degraded by each bacterium separately. Although degradation was observed, the contribution of each bacterium remains to be determined.

These results support the conclusion that the conditions required for degradation of MP by OPH<sup>+</sup> *E. coli* were detrimental to degradation of PNP by *S. chlorophenolicum*. Therefore, for the development of remediation systems using bacterial consortia, similar conditions for the organisms to function will enhance the potential for success.

For a rapid elimination of toxicity as it may be required in case of accidental spills, genetically engineered organisms may be desirable since they can degrade high concentrations of hazardous compounds at high degradation rates. Future research should address development of a genetically modified bacterial consortium that allows degradation of different compounds at similar rates. The organisms are modified to produce the enzymes of interest, no pathways need to be induced, lag phases could be avoided, high concentrations could be degraded, degradation could take place under similar conditions and toxicity of metabolic products could be eliminated. For MP, a bacterial consortium having OPH<sup>+</sup> for MP hydrolysis and either monooxygenase or dioxygenase for PNP degradation would be

desirable. This remediation technology could be applied not just for MP, but for a variety of OPs for which metabolic products include PNP.

The overall outcome of this study confirms the possibility of using microorganisms to degrade hazardous materials. MP toxicity was eliminated as well as that of PNP. The results from this thesis will contribute to the development of remediation technology for waste treatment, which in turn will help to provide a healthier environment.



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## APENDIX A

### **PREPARATION OF BIOMASS FOR BIODEGRADATION OF METHYL PARATHION USING A BINARY CONSORTIUM OF ENGINEERED *E. COLI* AND A NATIVE *S. CHLOROPHENOLICUM***

*S. chlorophenolicum* cells were grown in ATCC medium 1687 (pH 8.0) to an OD of 0.5. Then OPH<sup>+</sup> *E. coli* cells were added to the medium to give an OD of 0.5. Recombinant *E. coli* was grown and harvested as previously described. To determine the amount of *E. coli* to add to the *S. chlorophenolicum* grown cells glutamate medium, different amounts of *E. coli* biomass were added to the experimental volume to obtain a final OD of 0.5. Therefore each microorganisms *S. chlorophenolicum* and *E. coli* were added to a final OD of 0.5. *S. chlorophenolicum* was grown to the early exponential phase and *E. coli* was harvested at the late exponential phase.

## VITA

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