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Isolation And Purification Of An Organophosphate Degrading Enzyme

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Isolation and Purification of an Organophosphate Degrading Enzyme

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Dedication

This thesis is dedicated to my husband, Mark York, my two children, Michaela Landvater and Amber Warcup, and to my parents, Don and Cindy Warcup, for all their love, prayers, and support. Also, this thesis is dedicated to my mentor, Dr. White for all her love, support, and encouragement. Finally, this thesis is dedicated to my faith in God. Without God, I never would have been able to overcome the obstacles along the way.

Biographical Sketch

Shannon W. Landvater was born March 3, 1973, in Greensboro, North Carolina. She received her Associate of Applied Science degree in Biotechnology from Alamance Community College in 1993. She received the Bachelor of Science degree in Biology from NC A&T State University in 2009. She received a Hazmat Training Certificate (40 Hours) in 2009. She is a candidate for the Master of Science degree in Biology. She is also a Woodland E. Hall scholar, and an I-BLEND scholar. She received an Advanced Waste Management Certificate in 2012. She also received a grant titled "Encapsulation of Organophosphate Degrading Enzymes in Tri-Block Copolymer Vesicles" from USA Construction Engineering Research Laboratory (CERL) in 2010. She was named Graduate Student of the Year in 2011. She has also worked in research at Flexcell International Corp. which produces biotechnology products for cellular biomechanics, and at Carolina Biological which offers science supplies and materials for use in the science classroom.

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List of Abbreviations

AChE	Acetylcholinesterase
CSM	Carbon-deficient Minimal Medium
DDT	Dichlorodiphenyltrichloroethane
DHFR	Dihydrofolate reductase
GCSM	Glycerol Carbon-deficient Minimal Medium
HRP	Horse Radish Peroxidase
IDA	Iminodiacetic Acid
IMAC	Immobilized-Metal Affinity Chromatography
IPTG	Isopropyl-β-D-thiogalactoside
Ni-NTA	Nickel-Nitrilotriacetic Acid
NTA	Nitrilotriacetic Acid
OP	Organophosphate
opd	Organophosphorus degrading gene
OPH	Organophosphorus hydrolase
PBAP	Psuedomonas bathycetes agar plates
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOC	Super Optical Broth with Catabolic repressor

Abstract

Organophosphates (OP) are a class of pesticide widely used in agriculture and are found in a number of insecticides and chemical warfare agents. OPs are extremely dangerous in that they inhibit cholinesterase function, which disrupts proper activity of the nervous system. OPs are also harmful due to their broad target range and toxicity for nontarget species (Horne, Qiu, Ollis, Russell, & Oakeshott, 2006) . Furthermore, following the introduction and wide spread use of pesticides, great environmental concerns have arisen. Some of the methods used for removing OP residues are impractical and costly, or are themselves environmentally hazardous (Horne et al., 2006). Despite these concerns, pesticides have been considered valuablel tools in agriculture given that they kill potential disease causing organisms, control insects, weeds, and other pests, and help reduce crop loss.

Recently, the potential use of environmentally safe enzymes for the decontamination of pesticides and chemical warfare agents has become the focus of many studies. Therefore, the objective of this study was to clone, functionally express, and examine the catalytic activity of the organophosphagte degrading enzyme organophosphorus hydrolase (OPH). A 1,098 bp DNA fragment containing *opd*, the gene encoding OPH, was successfully amplified by Polymerase Chain Reaction from *Flavobacterium sp*. Opd was then cloned into an expression vector and inserted into E. coli by electrophoration. Expression of OPH in the *E. coli* was verified using a colony blot SDS-PAGE. OPH was isolated and a functional assay to demonstrate the catalytic activity of the protein revealed that the protein successfully degraded the substrate paraoxon.

CHAPTER 1

Introduction

Organophosphates (OPs) are a class of pesticide widely used in agriculture and are found in some pesticides and chemical warfare agents. OPs are extremely dangerous in that they inhibit cholinesterase activity, which disrupts proper activity of the nervous system. OPs have a broad target range and are highly toxic to nontarget species (Horne et al., 2006). The toxic effects of OPs on invertebrates, vertebrates and wildlife are well documented (Singh & Walker, 2006).

Some of the methods used for removing OP residues are impractical and costly or are themselves environmentally hazardous (Horne et al., 2006). For example, bleach is very effective in decontaminating pesticides. However, this method is corrosive and results in hazardous waste (Cheng, DeFrank, & Rastogi, 1999). Another example is DS2, a decontaminating agent, which has previously been used to decontaminate chemical warfare agents. This method is also corrosive and produces hazardous waste. In light of the positive benefits of OP use in agriculture, identification of non-hazardous substances to control the accidental spread of pesticides has become desirable. Therefore, the potential use of enzymes for the decontamination of pesticides and chemical warfare agents has become the focus of many studies. Enzyme decontamination is being researched as a possible rapid removal of chemical warfare agents and pesticides. This method has also been shown to be environmentally safe and non-corrosive. Several soil bacterial species have been examined for their organophosphate degrading ability.

Although proven to be extremely dangerous, OP use has increased, especially in developing countries. Specifically, the ability to kill potential disease causing organisms and aid

in the control of insects and other pests has made these chemicals popular tools in agriculture. Furthermore, pesticides control the growth of weeds and help reduce crop loss. An additional benefit of OP use is that these chemicals do not remain in the soil for long periods of time and are therefore considered biodegradable.

In light of the positive benefits of OP use in agriculture, identification of non-hazardous substances to control the accidental spread of pesticides has become desirable. Therefore, the potential use of enzymes for the decontamination of pesticides and chemical warfare agents has become the focus of many studies. One of the earliest and most studied species known to degrade OPs is *Flavobacterium species*. Previous studies have reported that current applications that use enzyme-based methods are limited by poor long-term enzyme stability and low reactivity over a broad range of temperatures (Theriot, Du, Tove, & Grunden, 2010). Therefore, possible modifications of specific enzymes are being examined so that these enzymes may remain stable and functional under harsh conditions. These studies have revealed that encapsulation of the enzyme aids in the stability and function of them.

1.1 Statement of the Problem

OPs widely used in agriculture and are found in insecticides and chemical warfare agents such as Sarin. OPs function by inhibiting cholinesterase activity, thereby altering the normal function of the nervous system. OPs are especially dangerous due to their ability to inhibit acetylcholinesterase, broad target range, and high toxicity for nontarget species (Horne et al., 2006). Current methods of removing OP residues from the environment are often impractical, costly and may be environmentally hazardous (Horne, Sutherland, Harcourt, Russell, & Oakeshott, 2002).

1.2 Statement of Purpose and Hypothesis Tested

Recently, many studies have focused on the encapsulation of OP degrading enzymes, because they are environmentally safe and practical to use as a method of detoxification of pesticides and chemical warfare agents. However, most enzymes are very sensitive to pH, temperature, dilution effects, and other environmental factors which can cause them to undergo conformational changes, or denaturation, which may induce a loss of activity.

The *objectives* of this study were as follows:

1. To clone *opd* from *Flavobacterium species*.
2. To functionally express and purify the OPH enzyme.
3. To test the catalytic activity and functionality of OPH.

CHAPTER 2

Literature Review

2.1 History of Pesticides

According to Grube et al., in 2006 and 2008, approximately 5.2 billion pounds of pesticides were used world wide (Grube, Donaldson, Kiely, & Wu, 2011). In 2006 and 2007 the U.S. used approximately 1.1 billion pounds of pesticides, accounting for approximately 22% of the world's total usage (Grube et al., 2011). For conventional pesticides which are used in the agricultural sector as well as in industry, commercial, governmental and the home and garden sectors, the U.S. used at total of 857 million pounds, with the agricultural sector accounting for 80% of the conventional pesticide use total (Grube et al., 2011). Pesticides are also found in majority of U.S. households with 78 million out of the 105.5 million households indicating that they use some form of pesticide (Grube et al., 2011). Currently, there are more than 1,055 active ingredients registered as pesticides. Over 16,000 pesticide products are currently being marketed in the United States (Blue Morning Farm, 2011).

A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, mitigating, or controlling any pest, including vectors of human or animal disease. Additionally, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies are also included (Salako, Sholeye, & Dairo, 2012). The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport (Salako et al., 2012).

Many products used in homes are classified as pesticides. For example, cockroach sprays and baits, insect repellent for personal use, flea and tick sprays, powders, pet collars, rat and other rodent poisons, kitchen, laundry and bath disinfectants and sanitizers, products that kill mold and mildew, some weed killers, and swimming pool chemicals are all considered pesticides ("Pesticides and Wyoming Homeowners"). Pesticides by their very nature create some risk of harm. However, they are helpful to society in that they can kill potential disease causing organisms and control insects, weeds, and other pests. Pesticides help to minimize damage and therefore loss of agricultural products by controlling crop pests. They also help to control and prevent insects that can cause human and animal disease and epidemics.

When a pesticide is dispersed into the environment, only approximately 5% of the chemical reaches its intended destination. The other 95% is distributed in the environment and can contaminate the water, soil, and the air. Pesticides can also affect unintended plants, such as weeds, and could cause resistance to overgrowth preventatives.

Synthetic organophosphorus compounds have been used as plasticizers, air fuel ingredients and chemical warfare agents (Singh, 2009). The first known pesticide, Sulfur, was used by the Mesopotamians about 4,500 years ago. As early as the 15th century, mercury, arsenic and lead were being introduced to crops to kill pests. By the 17th century an extraction from tobacco plants, nicotine sulfate, was being used as an insecticide. By the 19th century the roots of tropical vegetables were used to derive pesticides. As of the 1950s arsenic was being used as one of the main ingredients in pesticides.

The Environmental Protection Agency (EPA) was established in 1970. By 1972 the EPA implemented amendments to the pesticide law. However, pesticide use continued to increase, especially in developing countries. Since there has been wide spread use of pesticides, great

environmental concerns have arisen around their use (Appendix F). Pesticides are broad based-compounds that are very effective, however they are highly toxic to humans. Pesticides have different routes of exposures, such as inhalation, ingestion, or dermal absorption. Pesticides pose serious to fatal health hazards, such as asthma, birth defects and deaths.

Silent Spring was a best-selling book about biological magnification written by Rachel Carson. She wrote this book when it was discovered that DDT (Dichlorodiphenyltrichloroethane) was preventing fish and birds from reproducing. Now DDT is banned in the U.S. but it is still used in some developing countries to prevent tropical diseases such as malaria. Although a very effective and dangerous pesticide, DDT was eventually replaced by OPs and carbamates in the U.S. by 1975.

2.1.1 Organophosphates. OPs are one of the most popular pesticides used in agriculture (Appendix E). As a result of excessive and continuous use, many terrestrial and aquatic ecosystems across the world have been contaminated with OP compounds (Singh & Walker 2006). In addition, chemical warfare agents, such as Sarin gas, are also OPs (Appendix E).

All OP compounds have similar chemical structure which includes a phosphorus linked to either an oxygen or a sulfur by a double bond (Figure 1). OPs also have similar mechanisms of toxicity (Appendix C). Hydrolysis of one of the phosphodiester bonds reduces the toxicity of the OP.

OPs are acetylcholinesterase (AChE) inhibitors(Weise, Kreienkamp, Raba, Pedak, Aaviksaar, & Hucho 1990). They inhibit the breakdown of acetylcholine by acetylcholinesterase, enabling its accumulation at nerve synapses and neuromuscular junctions. The acetylcholine continues to act because of its inability to be hydrolised to choline and acetyl CoA (Appendix D).

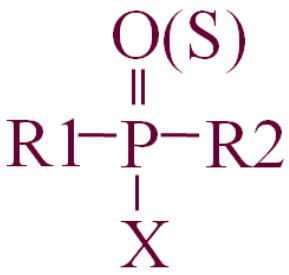


Figure 1. Organophosphate structure.

Two subsites, anionic and esteratic sites comprise the active site of AChE (Weise et al., 1990). AChE is essential in humans, animals, and insects for normal function of the central nervous system. Under normal conditions, AChE hydrolyzes the acetylcholine neurotransmitter in the synaptic membrane to prevent its accumulation. As a result choline is released and acetylated enzyme is formed (Upadhyay, 2012). The excess choline is then transported back into the nerve ending to be converted to acetylcholine and then storage. The degradation of acetylcholine results in a decrease in the cell, and ultimately a decrease in nerve impulses.

Walker & Asher (2005) state that in the cell, OPs function by binding to the active site of AChE, and therefore inactivating the enzyme. The OP affinity for the AChE active site is very strong and inactivation of AChE is irreversible. This leads to an accumulation of Ach in the peripheral and central nervous system which results in cholinergic manifestations. The symptoms of OP poisoning depend on the severity of exposure to OPs. Symptoms include increased production of mucus, chest tightness, shortness of breath and diarrhea.

2.2 History of Nerve Agents

There are two main groups of nerve agents. One is the G-agents and the other is the V-agents. G-agents such as sarin, soman, and tabun are nonpersistent and cause casualties primarily by inhalation (Upadhyay, 2012). The V-agents such as VX persist in the environment

and can therefore cause casualties by both inhalation and absorption through the skin (Upadhyay, 2012). These nerve agents are also OP compounds and are categorized as chemical warfare agents.

It is estimated that approximately 200,000 tons of OPs are stored worldwide and approximately 30,000 tons of that is stored in the United States (Singh, 2009). The first nerve agent ("Tabun" or "GA") for military use was made in Germany in 1936 by Dr. Gerhard Schrader (Chundler, 2012). Another nerve agent, "sarin" or "GB," was produced in 1938 and "Soman" or "GD" was made in 1944 (Chundler, 2012). Although it appeared that the Germans did not use these nerve agents during World War II, it has been estimated that the Germans had stockpiled tons of both Tabun and Sarin. After the War, both the United States and Russia continued producing and stockpiling these nerve agents. Moreover, the nerve agent "VX," was produced in England in the 1950s.

Nerve agents are specifically dangerous chemicals due to the difficulty of detection. They are usually colorless and may have no odor or a faint sweet smell. They are especially dangerous to populations located in low-lying areas because these chemicals are denser than air and settle towards the ground. Many nerve agents can be easily released using bombs, missiles, spray tanks, rockets and land mines. For example, the nerve gas, Sarin, was used in 1995 by members of the Aum Shinrikyo cult as a chemical warfare agent (Chamberlain, 2002). Twelve people were killed and many others wounded when they released the nerve gas in the Tokyo subway (Chamberlain, 2002). Nerve agents act in the same manner as pesticides in that they bind to the AChE molecule, inactivating the enzyme and blocking its function. Therefore, acetylcholine accumulates within the nerves and neuromuscular junctions (Appendix D).

2.3 Decontamination of Pesticides and Nerve Agents

A major problem using OPs as pesticides is that occasionally a spill will occur on a farm and equipment used to disperse the insecticide is contaminated with the OP. Pesticidesmay also be accidentally spilled into the surrounding environment. Due to the toxic nature of the chemicals, the spill must be decontaminated and the equipment cleaned before further use. In addition, stockpiling nerve gases and producing insecticides poses a problem when they are no longer needed. There are several chemical means of destroying these agents; however, these chemicals can be toxic as well and not environmentally friendly (Chamberlain, 2002).

2.4 Organophosphate Degrading Bacteria

Microbial degradation of pesticides and nerve agents offer an economical and safe way of detoxification. For selected situations, microbial processes have considerable advantages over other technologies in that microbial processes can yield precise products, function at low concentrations of solute, and require relatively low levels of technology for construction and maintenance (Mulbry & Karns, 1989). In nature, microorganisms have been found to evolve degradative traits as a result of continuous or repeated exposure to xenobiotic chemicals such as OPs (Chaudhry, 1988). Numerous pesticides have become less effective due to the enhanced microbial degradation of many chemicals.

Some bacteria quickly degrade OPs, therefore OP has a short half-life in the environment. Because of this, OPs are considered to be biodegradable and are increasingly being used. More than 100 OP pesticides are in use worldwide, accounting for ~38% of total pesticide usage (Organophosphate (Post note 12) Parliamentary). Bacteria can use OPs as an energy source because they possess AChE, therefore OPs do not affect the bacteria. Several phylogenetically distinct bacteria that can degrade OP by co-metabolism, or use OPs as a source of carbon, phosphorus or nitrogen, have been isolated from different parts of the world (Singh, 2009).

2.4.1 *F. species, P. haloplanktis, B. diminuta,* and *A. radiobactor* genomic DNA.

Flavobacterium sp. ATCC 27551 was the first bacterium determined to degrade OP compounds, and was isolated in 1973 from a soil sample in the Philippines (Singh, 2009). The rice patty from which the bacterium was found had been treated with the OP diazinon. Flavobacterium sp produces an organophosphorus hydrolyzing enzyme (OPH), which is encoded by the opd gene. OPH is located in the membrane of the Flavobacterium sp. OPH is a 35kDa, zinc-containing homodimeric protein that has been shown to be membrane associated. The OPH protein is the most widely characterized of the phosphotriesterases and has been shown to be effective in degrading a range of OP esters (Karns, Muldoon, Mulbry, Derbyshire, & Kearney, 1987). An identical opd gene is also found in the bacterium Brevundimonas diminuta (ATCC# 11568), which was isolated from an enrichment culture in the Unites States. Pseudoalteromonas haloplanktis (ATCC# 23821), contains an opaa gene that encodes an OPAA enzyme. This enzyme is a prolidase which hydrolyzes a wide range of G-type chemical warfare agents. Agrobacterium radiobacater, which was first isolated from Australian soil, contains the opdA gene which encodes the OPDA enzyme. This enzyme as well as the OPH enzyme may have evolved from pre-existing enzymes. ATCC states that Agrobacterium radiobacater is a known plant pathogen and a permit is required for purchase, therefore the Agrobacterium radiobacater genomic DNA was used instead.

2.5 Encapsulation of Organophosphate enzymes

Most enzymes are very sensitive to pH, temperature, dilution effects, and other environmental factors which can cause them to undergo conformational changes, or denaturation, which may induce a loss of activity. Therefore their stabilization may be required to maintain functional activity. One possible method for stabilizing an enzyme is to encapsulate

the protein into nanometer sized vesicles. This method protects the enzyme from denaturation due to environmental factors such as pH, temperature, dilution effects and external agents such as proteases. Unfortunately, many biotechnical applications of enzymes require environments rich in proteolytic enzymes and concentrations favouring destabilization, resulting in subsequent rapid loss in activity (Nasseau, Boublik, Meier, Winterhalter, & Fournier, 2001). Ramanthan, Luckorift, Sarsenova, Wild, Ramanculov, Olsen & Simonian (2009) were able to demonstrate a rapid method for enzyme immobilization directly on a waveguide surface by encapsulation in a silica matrix. OPH was used as a model enzyme to demonstrate the utility of lysozyme-mediated silica formation for enzyme stabilization. Researchers were able to show that silica-encapsulated OPH retained its catalytic activity for nearly 60 days with a detection limit of paraoxon of ~35 μM.

In 2007, Kern stated that decontamination strategies fall into at least two formats, personal protection and environmental decontamination. This study also discussed established enzyme-based countermeasures, describing such diverse applications as decontaminating foams for surface remediation, encapsulating enzyme with liposome for in vivo therapy, enzyme attachments to surfaces for biosensors and development of a corn expression system for large-scale enzyme production. However, there are advantages and disadvantages for enzymatic detoxification (Table 1).

Russell, Berberich, Drevon & Koepsel (2003) discussed the decontamination methods and biomaterials that have the potential to be decontamination materials of chemical warfare agents. Nardin, Theoni, Widmer, Winterhalter, and Meier (2000) focused on a new kind of nanoreactor, ABA-triblock copolymer vesicles to miniaturize processes down to the nanometer level (Figure 2).

Table 1.

Advantages and Disadvantages for enzymatic detoxification.

Advantages[24]	Disadvantages[24]
Environmentally benign	High degree of specificity
High level of specificity and activity	Few enzymes currently available
Mild reaction conditions	Brief catalytic life
Minimal side reactions	Environmental sensitivity
Light-weight	
"Enzyme cocktail" for broad coverage	

Reprinted from Ong, Kate K., *Encapsulation of Organophosphorus Acid Anhydrolase (OPAA)in Nanostructured Materials for the Detection and Decontamination of Chemical Warfare Agents*. Ph. D. Drexel University.

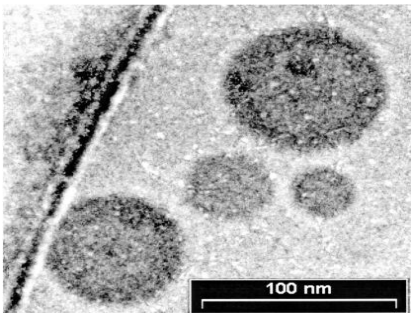


Figure 2. Cryo-transmission electron micrograph of polymerized triblock copolymer vesicles. Reprinted from Nanoreactors based on (polymerized) ABA-triblock copolymer vesicles," by Nardin et al., 2000, *Chemical Communications, 56:15,* p. 1433. Copyright 2000 by the Royal Society of Chemistry.

Van Slooten, Boerman, Romoren, Kedar, Crommelin & Storm (2001) were investigating liposomes sustained release systems for human interferon-γ: biopharmaceutical aspects. Marty, Sode & Karube (1992) were interested in biosensors for detection of organophosphates and carbamates. Allinson & LeJeune (2002) successfully developed a prototype application that can be used to clean and detoxify point-source OP spills, equipment contamination, or unwanted organophosphate application solutions. The prototype is designed for maximum utility and withstands environmental influences such as elevated temperature, extreme pH, and absence of

enzyme cofactors. The OPH enzyme was incorporated into highly absorbent polymer foams using a proprietary technique. In particular, they developed enzyme-containing foams for absorbency and high enzyme activity. Enzyme polymer foams were tested against neat and commercial grade chlorpyrifos, diazinon, methyl parathion, paraoxon, parathion, and pirimiphos-methyl. They found that in addition to increased activity and stability in extreme pH environments, their enzyme polymers impart significant buffer capacity to pesticide solutions. In conclusion, Allinson & LeJeune (2002) reported that enzyme polymer foams are effective for neutralization and detoxification of OP pesticide applications in the field. Polymerized enzyme was more stable over native enzyme when exposed to environmental influences such as elevated temperature, extreme pH, and absence of cofactors. The enzyme-containing foams successfully removed and detoxified greater than 99% of organophosphate pesticide contamination from simulated equipment and point-source spill cleanup.

CHAPTER 3

Materials and Methods

The QIAexpress System was chosen for cloning and expression because it allows for easy cloning into a vector that will allow high expression of a functional target protein.

3.1 The QIAexpress System

The QIAexpress system is based on the selectivity and affinity of the nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices for biomolecules which have been tagged with six consecutive histidine residues (Appendix B; QIAexpressionist, 2003). High-level expression of 6xHis-tagged proteins in *E. coli* using pQE vectors is based on the T5 promoter transcription-translation system. The pQE plasmid belongs to the pDS family of plasmids. This low-copy plasmid has an optimized promoter-operator element consisting of phage T5 promoter and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the T5 promoter, a 6xHis-tag coding sequence either 5' or 3' to the cloning region, a multiple cloning site and translational stop codons in all reading frames for preparation of expression constructs.

3.1.1 Regulation of expression. High levels of the *lac* repressor protein can efficiently regulate and repress the extremely high transcription rate initiated by the T5 promoter. The QIAexpress System uses an *E. coli* host strain that uses a *lac* repressor gene in *trans* to the gene to be expressed. This host strain contains the low-copy plasmid pREP4 which confers kanamycin resistance and constitutively expresses the *lac* repressor protein encoded by the *lac* I gene. There are multiple copies of the pREP4 plasmid present in the host cells which ensures the production of high levels of the *lac* protein expression. The pREP4 plasmid is maintained in *E. coli* in the presence of kanamycin at a concentration of 25µg/ml.

Isopropyl-β-D-thiogalactoside (IPTG) binds to the *lac* repressor protein and inactivates it. This rapidly induces the expression of the recombinant protein encoded by the pQE vector. The host cell's RNA polymerase can transcribe the sequence downstream from the promoter once the lac repressor is inactivated. These transcripts can then be translated into the recombinant protein. The QIAexpress *E. coli* strain M15[pREP4] contains the pREP4 plasmid.

3.1.2 *E. coli* host strain. *E. coli* strain M15[pREP4] permits high-level expression and does not contain a chromosomal copy of the *lacl*^{q} mutation. This mutation allows for the production of enough *lac* repressor to efficiently block transcription. Therefore, pREP4 must be maintained within the *E.coli* strain M15[pREP4] by selection for kanamycin resistance. The pREP4 plasmid contains a gene that confers kanamycin resistance. The M15[pREP4] strain is sensitive to Streptomycin (100 µg/ml), Rifampicin (50 µg/ml), and Nalidixic Acid (20 µg/ml). Therefore, this strain has no mutations in rpsL, rpoB, and gyrA. The M15[pREP4] strain cannot metabolize Lactose (Lac^{-1}) or Mannitol (Mtl^{-1}). The M15[pREP4] strain has the phenotype NaIS, StrS, RifS, Thi–,Lac–, Ara+, Gal+, Mtl–, F–, RecA+, Uvr+, Lon+.

3.1.3 6x His tag. The 6xHis tag facilitates binding to the Ni-NTA. The tag can be placed at the N or C-terminus of the protein, and in this experiment it was placed at the C-terminus (Figure 3).

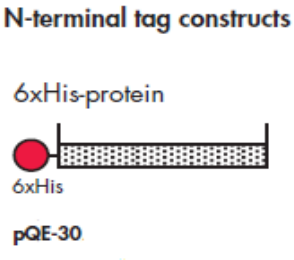


Figure 3. QIAexpress constructs. Recombinant QIA*express* constructs based on the pQE vectors can be produced by placing the 6xHis tag at the C or N-terminus of the construct.

The 6xHis tag does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell because it is poorly immunogenic, and at pH 8.0 the tag is small and uncharged. The 6xHis tag, in most cases, does not interfere with the structure or function of the purified protein. It also allows for the immobilization of the protein on chelating surfaces such as Ni-NTA. Anti·His Antibodies are used for detection as well and can detect any 6xHis-tagged protein expressed with pQE vectors.

The pQE-30 vector allows for the C-terminus 6xHis-tagged protein of interest (Figure 4). When using C-terminal tags, the insert must be cloned in frame with both the ATG start codon and the 3' 6xHis coding sequence, and only full-length proteins are purified.

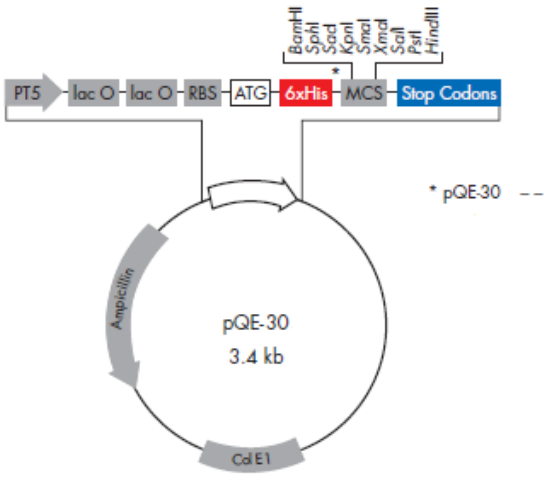


Figure 4. pQE vectors for N-terminal 6xHis tag constructs. PT5: T5 promoter, lac O: lac operator, RBS: ribosome-binding site, ATG: start codon, 6xHis: 6xHis tag sequence, MCS: multiple cloning site with restriction sites indicated, Stop Codons: stop codons in all three reading frames, origin of replication, Ampicillin: ampicillin resistance gene.

3.1.4 Ni-NTA technology. The first chromatography used to purify proteins was immobilized-metal affinity chromatography (IMAC) in 1975 which used the chelating ligand iminodiacetic acid (IDA; Appendix A) (Porath, Carlsson, Olsson, & Belfrage, 1975). IDA was

charged with metal ions such as $Zn^{2+,}$ Cu^{2+}, or $Ni^{2+,}$ and then used to purify a variety of different proteins (Sulkowski, 1985). IDA cannot tightly bind metal ions and only has three meal-chelating sites. This weak binding can lead to ion leaching upon loading with strongly chelating proteins during wash steps. This can result in impure products, low yield, and metal-ion contamination of isolated proteins.

Nitrilotriacetic acid (NTA) is a tetradentate chelating adsorbent that overcomes these problems. NTA binds metal ions far more stable than other chelating resins because it occupies four to six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag (Appendix B). NTA retains the ions under a wide variety of conditions, especially under stringent wash conditions. This allows NTA matrices to bind 6xHis-tagged proteins more tightly than IDA matrices.

3.1.5 Cloning with pQE vectors using restriction enzymes. The pQE30 vector chosen for cloning is linearized using the appropriate restriction enzymes according to the enzyme's manufacturer's recommended buffer and incubation conditions (Figure 5). Following the digestion, the vector is gel-purified prior to ligation in order to remove residual supercoiled and nicked plasmid. The insert is prepared using the same restriction enzymes, buffer, and incubation conditions as the pQE-30 vector. The pQE-30 vector and construct can be maintained in the E. coli strain M15[pREP4] in the presence of ampicillin at a concentration of 100 µg/ml. The ligation of the prepared insert and vector is carried out using T4 DNA ligase.

3.1.6 Purification under native conditions. It is more difficult to predict the amount of soluble protein present in the lysate under native conditions. To purify significant amount of 6xHis-tagged protein, a 50-to 100-fold concentration is recommended. A culture volume of 100 ml was determined for this experiment. Under native conditions there is a higher potential for

binding background contaminants so low concentrations of imidazole in the lysis and wash buffers are recommended. The imidazole ring, which is part of the structure of histidine, binds to the nickel ions immobilized by the NTA groups on the matrix.

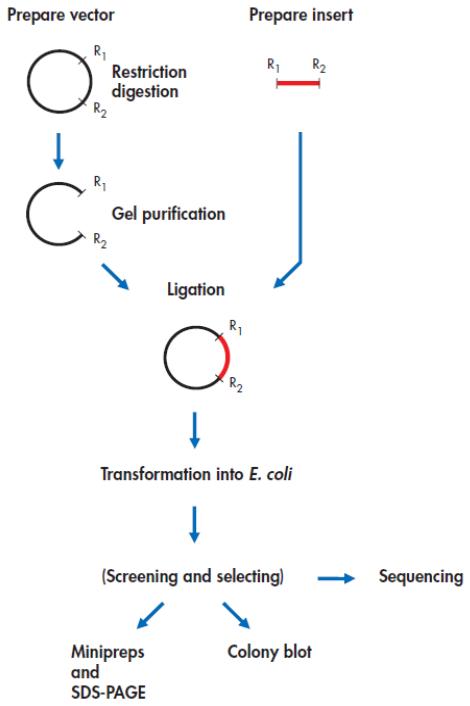


Figure 5. Construction of QIAexpress expression vectors. R1, R2: restriction sites.

This binding also disrupts the binding of dispersed histidine residues in nontagged background proteins. The 6xHis-tagged proteins can bind to the Ni-NTA groups on the matrix with an affinity much greater than that of antibody-antigen interactions and does not depend on the three-dimensional structure of the protein. Any nonspecific binding of proteins to the NTA resin itself can be easily washed away under relatively stringent conditions that do not affect the binding of 6xHis-tagged proteins (Figure 6).

3.2 Bacteria Strains and Media

The *opd* (organophosphorus degrading) gene encodes the organophosphorus hydrolase protein, and is found in *Flavobacterium species*. The bacterium was obtained from ATCC (ATCC # 27551). This strain was maintained by subculture on Wakimoto agar plates (WAG).

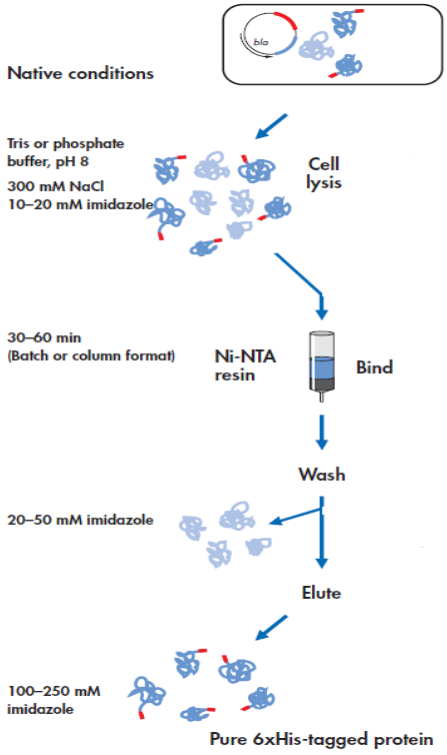


Figure 6. Purification of 6x His-tagged proteins under native conditions.

The strain was grown at 30°C for 18-24 hours. Frozen stocks were prepared by streaking bacteria for isolation on WAG and growing under conditions listed above. A single colony was selected and streaked for lawn growth. The bacteria were then transferred to *Flavobacterium sp.* freeze media (0.5g $Ca(NO_3)_2$, 2.0g Na_2HPO_4, 5g Peptone, 15g Sucrose, 0.5g $FeSO_4$, 15g Agar, and 1L of H_2O) with 10% glycerol and stored at -80°C.

The *opaA* (organophosphorus acid anhydrolase) gene encodes the metalloprotease oligopeptidase A, and can be found in the bacterium *Pseudoalteromonas haloplanktis*. The

bacterium was obtained from ATCC (ATCC # 23821). This strain was maintained on Pseudomonas bathycetes agar plates (PBAP). The strain was grown at 26°C for 18-24 hours. Frozen stocks were prepared by streaking bacteria for isolation on PBAP and growing under conditions described above. A single colony was selected and streaked for lawn growth. The bacteria were then transferred to *Pseudoalteromonas haloplanktis* freeze media (3g Yeast extract, 24g NaCl, 10g Proteose Peptone, 0.7g KCl, 5.3g $MgCl_2$, 7g $MgSO_4$, and 1L of H_2O) with 10% glycerol and stored at -80°C.

The *opdA* gene encodes the phophotriesterase protein, and is found in *Rhizobium radiobacter* The genomic DNA was obtained from ATCC (ATCC # 33970D-5). The DNA was reconstituted according to ATCC instructions. Briefly, the lyophilized genomic DNA was resuspended in 250µl of molecular grade water and incubated overnight at 4^{o}C followed by a one hour incubation at 65^{o}C. The DNA was resuspended to a final concentration of 20µg/µl and stored at 4^{o}C.

3.3 Amplification and Sequencing of *opd, opaA* and *opdA* in Bacteria Strains

3.3.1 Amplification of *opd*. Primers were designed to amplify the *opd* open reading frame, with the addition of a SacI and HindIII restriction site embedded at the beginning of the primer sequence. The addition of four base pairs was added to ensure correct digestion at the restriction sites. Briefly, primer opd forward and opd reverse were designed using the genome sequence of the *opd* (GenBank). PCR was performed using opd forward and opd reverse at 100pmon each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200 µM each]); Promega, Madison, WI) and whole *Flavobacterium species* cells as DNA template according to manufacturer's instruction. The PCR was performed under the following conditions: a single denaturation at 95°C for 2 min and 40 cycles, each consisting of 1 min denaturation at 95°C,

annealing at 59℃ for 1 min, and extension at 72℃ for 2 min. Equal volumes of each sample was loaded onto an 8% agarose gel and subjected to electrophoresis. A 1,098 bp fragment of DNA was amplified from several colonies of *Flavobacterium species* (Figure 8).

3.3.2 Amplification of *opaA*. Primers were designed to amplify the *opaA* open reading frame, with the addition of a SacI and SalI restriction site embedded at the beginning of the primer sequence. The addition of four base pairs was added to ensure correct digestion at the restriction sites. Briefly, primer opaA forward and opaA reverse were designed using the genome sequence of the *opdA* (GenBank). PCR was performed using opaA forward and opaA reverse at 100pmon each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200 µM each]); Promega, Madison, WI) and whole *Pseudoalteromonas haloplanktis* cells as DNA template according to manufacturer's instruction. The PCR was performed under the following conditions: a single denaturation at 95°C for 2 min and 40 cycles, each consisting of 1 min denaturation at 95℃, annealing at 59°C for 1 min, and extension at 72℃ for 2 min. Equal volumes of each sample was loaded onto an 8% agarose gel and subjected to electrophoresis. A 1,320 bp fragment of DNA was amplified from several colonies of *Pseudoalteromonas haloplanktis* (Figure 8).

3.3.3 Amplification of *opdA*. Primers were designed to amplify the *opdA* open reading frame (Table 1). Briefly, primer opdA forward and opdA reverse were designed using the genome sequence of the *opdA* (GenBank). PCR was performed using opdA forward and opdA reverse at 100pmon each, Master Mix (1X Go-taq DNA polymerase, dNTPs [200 µM each]); Promega, Madison, WI) and *Rhizobium radiobacter* genomic DNA as the template according to manufacturer's instruction. The PCR was performed under the following conditions: a single denaturation at 95℃ for 2 min and 40 cycles, each consisting of 1 min denaturation at 95°C,

annealing at 50℃ for 1 min, and extension at 72℃ for 2 min. Equal volumes of each sample was loaded onto an 8% agarose gel and subjected to electrophoresis. A 1,155 bp fragment of DNA was amplified from several samples of *Rhizobium radiobacter* genomic DNA (Figure 9)

Table 2.

List of specific primers used in this study.

Name	Oligo Sequence	Source (Reference)
opd forward	GCGC**GAGCTC**ATGCAAACGAGAAGGGTTG TGCTCAAG	This study
opd reverse	GCGC**AAGCTT**TCATGACGCCCGCAAGGT	This study
opaA forward	GCGC**GAGCTC**ATGGAAAAATTAGCCGTTTT ATACGC	This study
opaA reverse	GCGC**GTCGAC**TTAGTAATAAATTACGCGTC ATATTTTCTAGGCTATCTTC	This study
opdA forward	ATGCAAACGAGAAGAGATGCACTTAAGTC	This study
opdA reverse	CAGTTCTATGGCTTGCTACT	This study

3.4 Sequencing of *opd*

Following PCR amplification of *opd*, DNA sequencing was performed. To prepare samples for sequencing, the PCR products were purified using the DNA Wizard Genomic Preparation Kit (Promega, Madison, WI). Next, approximately 30 ng of the purified DNA was mixed with 100 pmol of either opd forward or opd reverse in a fresh tube. Samples were submitted to Eurofins MWG Operon (www.operon.com/products/sequencing/) for sequence determination. Since high quality sequence data could not be obtained using primers *opd forward* and *opd reverse*, primers *opdintreversertop (opdirt), opdintforwardbot (opdifb), opdreverseplasmid (opdrp), and opdforwardplasmid (opdfp)* were designed and used in

subsequent PCR and sequencing reactions (Table 2). Primers were designed 79 bp upstream of the *opd* within the pQE-30 plasmid (*opdforwardplasmid*; TAGCTTCCTTAGCTCCTGA) and 137 bp downstream of the *opd* within the pQE-30 plasmid (*opdreverseplasmid*; TAGCTTCCTTAGCTCCTGA). An internal reverse primer was designed 576 bp within the *opd* (*opdreversertop;* GAAGAACTGTGTGAGTTCCT). An internal forward primer was also designed 596 bp within the *opd* (*opdintforwardbot*; CTGCGTGAGATTCAATATGGC) (Figure 7). An approximate 1098 nucleotide fragment was obtained from the *Flavobacterium sp*.strain (Figure 8).

Table 3.

List of newly designed primers used in this study.

Name	**Oligo Sequence**	**Source (Reference)**
Opdirt	GAAGAACTGTGTGAGTTCCT	This study
Opdifb	CTGCGTGAGATTCAATATGGC	This study
Opdrp	TAGCTTCCTTAGCTCCTGA	This study
Opdfp	GTGAGCGGATAACAATTTCACACAG	This study

3.5 Sequencing of *opaA* and *opdA*

Due to several unsuccessful attempts to clone *opaA* and *opdA* into the *E. coli* M15[pREP4] strain and time restraints, the sequencing of *opaA* and *opdA* was not completed. Further use of *opaA* and *opdA* was discontinued. *Opd* became the focus of the research.

3.6 Sequence Translation

Using the Biology Workbench program (http://workbench.sdsc.edu/) and the deduced nucleotide sequence with Finch TV, the longest open reading frame was determined. The

longest open reading frame using the sequence for *opd* obtained from GenBank website (http://www.ncbi.nlm.nih.gov/genbank/) under Accession No. AY766084.1 was obtained. The deduced nucleotide sequence results that were translated into the amino acid sequence were compared to the *opd* amino acid sequence obtained from GenBank under Accession No. AY766084.1.

3.7 Cloning of *opd*

To clone *opd*, a revised cloning strategy was implemented. The pCR2.1 vector and the purified PCR product were digested with EcoRI enzyme. This allowed for EcoRI overhangs on the vector as well as the PCR product. Using the New England BioLabs Quick Ligation Kit, the pCR2.1 vector and the PCR product, both containing the EcoRI overhangs, were ligated.

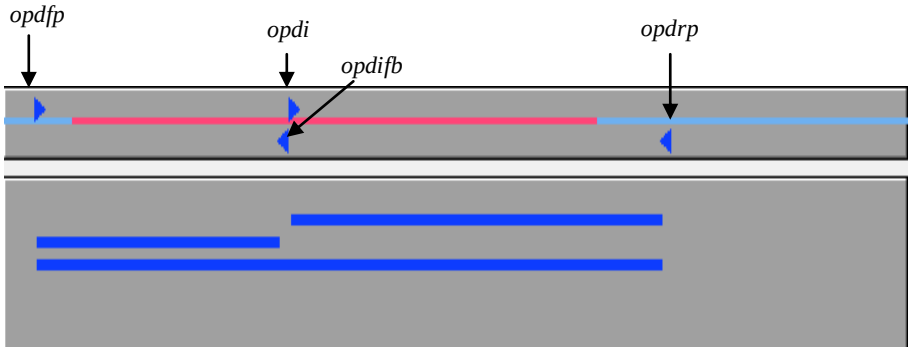


Figure 7. DNA sequence strategy using the AmplifX 1.5.4 software.

Briefly, 50ng of vector with a 3-fold molar excess of PCR product were mixed and brought to a volume of 10ul with de-ionized water. Next, 10ul of 2x Quick Ligation Buffer and 1ul of Quick T4 DNA Ligase was added and mixed. The mixture was incubated at room temperature (25^{o}C) for 5 minutes.

Following the ligation, the pCR2.1 vector containing the PCR product (*opd)* was electroporated into electrocompetent *E. coli* cells from Invitrogen. Briefly, 80ul of electrocompetent *E. coli* cells and 2ul of ligation mix were placed in a chilled 0.2cm cuvette.

The electroporation was performed under the following conditions: Voltage set at 2500 V, Resistance set at 200 Ω, and the Capacitance set at 23 μF. After electroporation, the cells were placed in SOC (super optimal broth with catabolic repressor) media for a one hour recovery. The *E. coli* cells were then plated on Luria Broth Agar plates that contained 100μg/ml Ampicillin (LB/Amp) and incubated over night at 37 ^{o}C. Single colonies were obtained and struck for lawn growth on LB/Amp plates and incubated overnight at 37 ^{o}C. The bacteria were then transferred to *E. coli* freeze media of LB/Amp with 10% glycerol and stored at -80°C.

A restriction digest was performed using the pCR2.1 vector containing the *opd* and the pQE-30 vector, and EcoRI enzyme. This allowed for the linearization of the pQE-30 vector with EcoRI overhangs. The *opd* was isolated from the pCR2.1 vector leaving the EcoRI overhangs. The *opd* and the linearized pQE-30 vector with the EcoRI overhangs were extracted from the gel and purified using the QIAGEN Gel Extraction Kit. Using the New England BioLabs Quick Ligation Kit, the pQE-30 vector and the purified *opd* both containing EcoRI overhangs, were ligated. Briefly, 50ng of vector with a 3-fold molar excess of *opd* were mixed and brought to a volume of 10ul with de-ionized water. Next, 10ul of 2x Quick Ligation Buffer and 1ul of Quick T4 DNA Ligase was added and mixed. The mixture was incubated at room temperature (25^{o}C) for 5 minutes.

Following the ligation, the pQE-30 vector containing the *opd* was electroporated into the electrocompetent *E. coli* strain M15[pREP4]. Briefly, 80ul of electrocompetent *E. coli* M15[pREP4] cells and 2ul of ligation mix were placed in a chilled 0.2cm cuvette. The electroporation was performed under the following conditions: Voltage set at 2500 V, Resistance set at 200 Ω, and the Capacitance set at 23 μF. After electroporation, the cells were placed in SOC media for a one hour recovery. The *E. coli* M15[pREP4] cells were then plated on Luria

Broth Agar plates that contained 100µg/ml Ampicillin and 25µg/ml Kanamycin (LB/Amp/Kan) and incubated over night at 37 °C. Single colonies were obtained and struck for lawn growth on LB/Amp/ Kan plates and incubated overnight at 37 °C. The bacteria were then transferred to *E. coli* M15[pREP4] freeze media of LB/Amp/Kan with 10% glycerol and stored at -80°C.

3.8 Isolation and Purification of the OPH Protein

Protein minipreps of 6xHis-tagged proteins from *E. coli* M15[pREP4] cells under native conditions was performed using the QIAexpressionist kit to isolate the OPH protein. *E. coli* M15[pREP4] cells with the pQE-30 vector containing the *opd* were grown on LB/Amp/Kan plates overnight at 37 °C. One half of the plate was resuspended in LB/Amp/Kan broth. The cells were pelleted and resuspended in lysis buffer. Lysozyme, from chicken egg white, was added to 1 mg/ml and incubated on ice for 30 minutes. The cells were lysed and centrifuged. The supernatant was added to a 50% slurry of Ni-NTA resin (10 µl resin has a capacity for 50–100 µg 6xHis-tagged protein) and mixed gently for 30 minutes at 4°C. The resin was pelleted and the supernatant was discarded. The resin was washed with a wash buffer and the protein was eluted with an elution buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the OPH purified protein was performed. The gel was a 10% acrylamide resolving gel. The gel was run at 200 constant volts for approximately 45 minutes. Next the gel was washed three times for five minutes each at room temperature. Then the gel was stained with Simply Blue Safe Stain from Invitrogen for one hour. The gel was then destained for one hour with de-ionized water. To enhance the band, the gel was soaked in a 20% sodium chloride solution over night. The gel was analyzed using the *GS-800*™ Calibrated Densitometer from Bio-Rad.

Using the QIAexpressionist kit, a colony blot was performed for the identification of clones expressing a 6xHis-tagged protein. Penta His HRP Conjugate was used which contains a horseradish peroxidase moiety that allows direct detection by chemiluminescent. Clones expressing a 6xHis-tagged protein were grown overnight at 37 ^{o}C on a LB/Amp/Kan grid plate. Expression of the OPD protein was induced by the addition of 250µM isopropyl-β-D-thiogalactoside (IPTG).

Protein concentrations were determined according to Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin and bovine gamma globulin as a standards. One liter of glycerol carbon-deficient minimal medium (GCSM) was prepared with the following composition: 0.2g $MgSO_4$, 0.08g $Ca(NO_3)_2$, 0.005g $FeSO_4$, 4.8g K_2HPO_4, 1.2g KH_2PO_4, and 1% glycerol. 10ml of GCSM media was aliquoted into two 15ml tubes. One liter of carbon-deficient minimal medium (CSM) was prepared with the following composition: 0.2g $MgSO_4$, 0.08g $Ca(NO_3)_2$, 0.005g $FeSO_4$, 4.8g K_2HPO_4, and 1.2g KH_2PO_4. 200ul of the negative control and 200ul of the positive control was added to tubes 1, and 2 containing GCSM medium, respectively. The GCSM medium was used to minimize the shock to the cells and limit cell death. The samples were incubated in a shaking 30^{o}C water bath for one hour. The cells were pelleted and resuspended in 10ml of CSM medium. 15ul of the purified OPD protein was added to 10 ml of CSM medium. Paraoxon was added to a final concentration of 100ug/ml. The tubes were then covered with foil to prevent photo-hydrolysis. The samples were incubated in a shaking 30^{o}C water bath. Samples were taken once a day for three days. Hydrolysis of paraoxon was measured spectrophotometrically by monitoring the production of *p*-nitrophenol at 405nm. *E. coli* cells without the pQE30-OPD vector were used as a negative control. The *Flavobacterium sp.* strain carrying the *opd* gene was used as a positive control.

3.9 Analysis and Alignment of *opd* Nucleotide and Protein Sequences

DNA sequences were obtained electronically from Eurofins MWG (http://www.operon.com/) in an ABI format. To view, edit and perform BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/) searches with the nucleotide sequences, FinchTV (www.geospiza.com/Products/finchtv/), a free web-based DNA sequencing chromatogram trace viewer was used. Sequence alignments were constructed with the free web-based Molecular Evolutionary Genetics Analysis (MEGA4) program (http://www.megasoftware.net/).

CHAPTER 4

Results

4.1 Amplification of *opd*

Previous studies by Mulbry and Karns (1989) demonstrated that *opd* in the Flavobacterium sp. strain could be amplified using PCR primers, designed to *opd*. Therefore, to amplify *opd* from *Flavobacterium sp*. strain, whole cells were subjected to PCR and agarose gel electrophoresis. As predicted, an approximately 1 kb DNA product containing *opd* was successfully amplified (Figure 8).

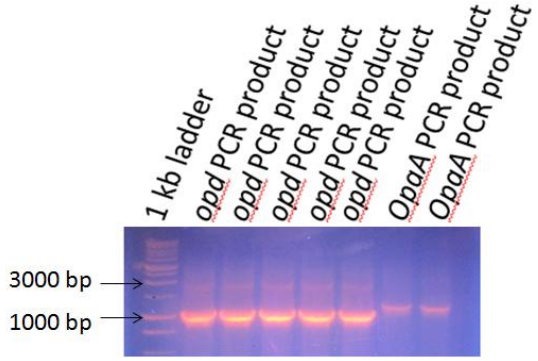


Figure 8. Amplification of *opd* and *opaA. Lane* 1, 1kb DNA molecular weight marker (Promega); lanes 2 – 6, 1098bp DNA fragments from the *Flavobacterium sp.*; and lanes 7-8, 1320bp DNA fragments from *Pseudoalteromonas haloplanktis*. An approximate 1000 bp fragment was successfully amplified from *Flavobacterium sp*. An approximate 1300 bp fragment was successfully amplified from *Pseudoalteromonas haloplanktis*.

4.2 Amplification of *opaA*

Previous studies by Horne et al. (2006) demonstrated that *opaA* in the *Pseudoalteromonas haloplanktis* strain could be amplified using PCR primers, designed to *opaA* (Figure 8). Therefore, to amplify *opaA* from *Pseudoalteromonas haloplanktis* strain, whole cells were subjected to PCR and agarose gel electrophoresis. As predicted, an approximately 1.3 kb DNA product containing *opaA* was successfully amplified (Figure 8).

4.3 Amplification of *opdA*

Previous studies by Horne et al. (2003) demonstrated that *opdA* in the *Rhizobium radiobaacter* genomic DNA could be amplified using PCR primers, designed to *opdA.* Therefore, to amplify *opdA* from *Rhizobium radiobaacter* genomic DNA, whole cells were subjected to PCR and agarose gel electrophoresis. As predicted, an approximately 1.3 kb DNA product containing *opdA* was successfully amplified (Figure 9).

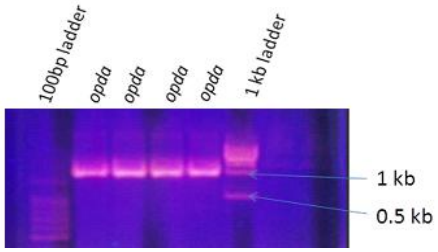


Figure 9. Amplification of *opdA.* Lane 1 is the 100bp DNA molecular marker. The expected size DNA fragments, 1155bp, were amplified from the *Rhizobium radiobacter* genomic DNA (lanes 2,3,4 and 5). Lane 6 is the 1kb DNA molecular marker.

4.4 Sequencing of *opd*

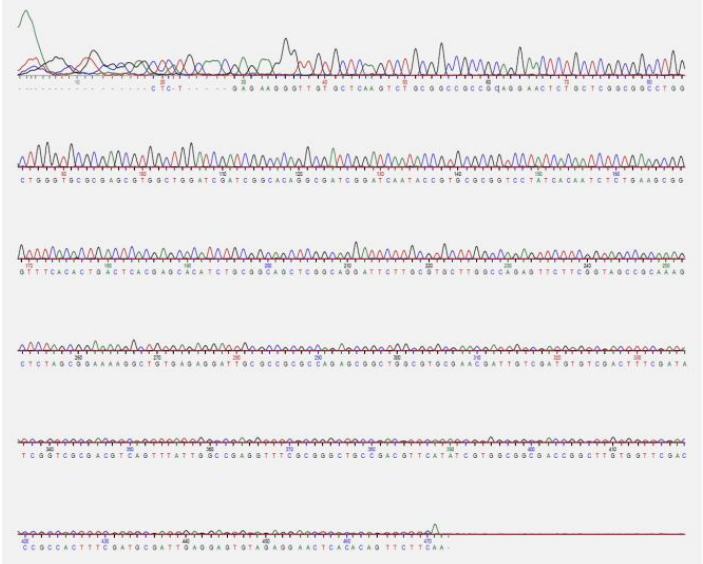
Sequencing results were obtained from Eurofins MWG operon. Examination of the deduced nucleotide sequences of the *opd* with FinchTV revealed that the 1098 base pair gene was isolated from *Flavobacterium sp.* (Figure 10).

4.5 Sequence Translation

Using the Biology Workbench program (http://workbench.sdsc.edu/) and the deduced nucleotide sequence with Finch TV, the longest open reading frame was determined. The longest open reading frame using the sequence for *opd* obtained from GenBank website (http://www.ncbi.nlm.nih.gov/genbank/) under Accession No. AY766084.1 was determined to

be identical to the longest open reading frame of the deduced nucleotide sequence with Finch TV.

(A)



(B)

atgcaaacgagaagggttgtgctcaagtctgcggccgccgcaggaactctgctcggcggcctggctgggtgcgcgagcgtggctgg
atcgatcggcacaggcgatcggatcaataccgtgcgcggtcctatcacaatctctgaagcgggtttcacactgactcacgagcaca
tctgcggcagctcggcaggattcttgcgtgcttggccagagttcttcggtagccgcaaagctctagcggaaaaggctgtgagagga
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aggaactcacacagttcttcctgcgtgagattcaatatggcatcaaagacaccggaattagggcgggcattatcaaggtcgcgacc
acaggcaaggcgacccccttcaggagttagtgttaaaggcggccgcccgggccagcttggccaccggtgttccggtaaccactca
cacggcagcaagtcagcgcgatggtgagcagcaggccgccatttttgagtccgaaggcttgagcccctcacgggtttgtattggtc
acagcgatgatactgacgatttgagctatctcaccgccctcgctgcgcgcggatacctcatcggtctagaccacatcccgcacagt
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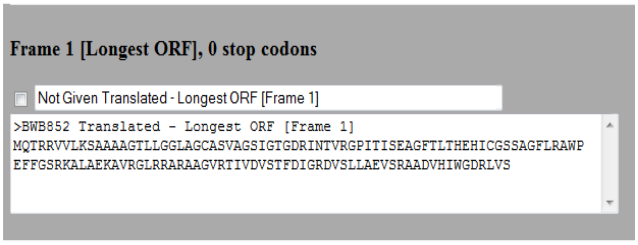
tgatggatcgcgtgaaccccgacgggatggccttcattccactgagagtgatcccattcctacgagagaagggcgtcccacaggaa
acgctggcaggcatcactgtgactaacccggcgcggttcttgtcaccgaccttgcgggcgtcatga

Figure 10. Sequencing results of *opd*. Chromatogram of *opd* sequencing results (A). Highlighted area of *opd* sequenced in this study (B).

Nucleotide sequence results were translated into the amino acid sequence and were found to be identical to the *opd* amino acid sequence obtained from GenBank under Accession No. AY766084.1.

(A)



(B)

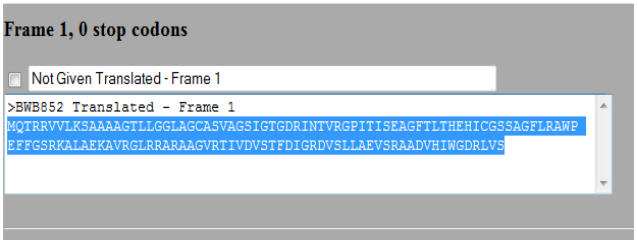


Figure 11. Longest open reading frame of *opd*. Longest open reading frame of the *opd* from genbank (A). Longest open reading frame of nucleotide sequence results with Finch TV (B).

4.6 Cloning of *opd*

Using the restriction enzyme EcoRI, the pQE-30 vector and the pCR2.1-OPD vector were digested. The pQE-30 vector was linearized and the *opd* with EcoRI overhangs was isolated from the pCR2.1-OPD vector (Figure 12).

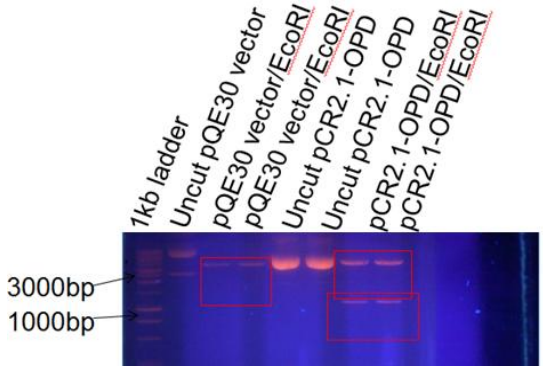


Figure 12. Digestion with EcoRI enzyme. Lane 1 is the 1kb DNA ladder. Lane 2 is uncut pQE30 vector. Linearization of pQE-30 vector (Lanes 3 and 4). Lane 5 and 6 is uncut pCR2.1-OPD. Isolation of *opd* from pCR2.1 vector (Lanes 7 and 8). The expected fragment size for the *opd* is 1098 bp, the pQE-30 vector is 3.4 Kb, and the pCR2.1 vector is 3.9Kb.

4.7 Electroporation

Following the digestion with the EcoRI enzyme, the linearized pQE-30 and the *opd* with the EcoRI overhangs were ligated together as previously described. The ligated pQE30-OPD was then electroporated into *E. coli* strain M15[pREP4] (Figure 13). The electroporation was successful as seen in Figure 13 (C).

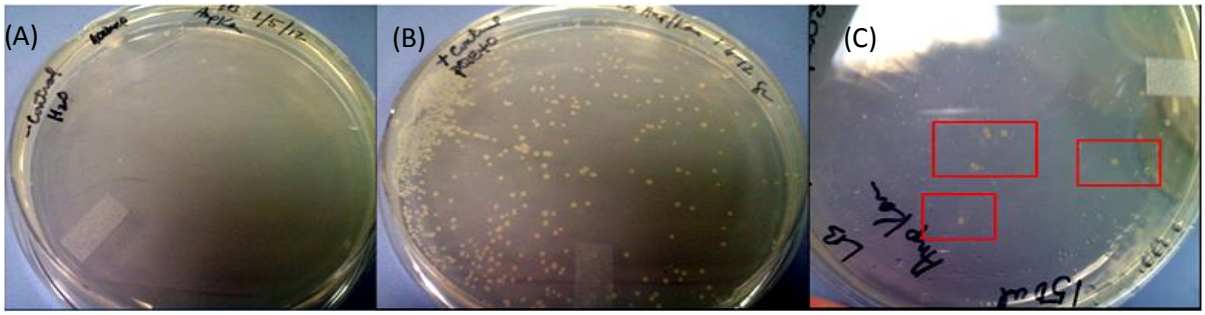


Figure 13. Electroporation of pQE30-OPD into M15[pREP4]. (A) Negative control using E.coli cells and water, (B) Positive control using M15[pREP4] containing the pQE-40 plasmid(C) Highlighted in a red box are single colonies of M15[pREP4] containing the pQE-30/*opd* plasmid.

4.8 Expression and Purification of OPH Protein

A colony blot was performed for the identification of clones expressing a 6xHis-tagged protein (Figure 15). Penta His HRP Conjugate was used which contains a horseradish peroxidase moiety that allows direct detection by chemiluminescent. Some background was detected with the negative control. However, when compared to the positive control containing the pQE40 vector expressing the DHFR protein, the dots were more intensely expressed with the positive control than with the negative control. In comparison the M15[pREP4] cells with the pQE30 vector containing the *opd*, the OPH protein appears to be expressed at a similar level as the positive control.

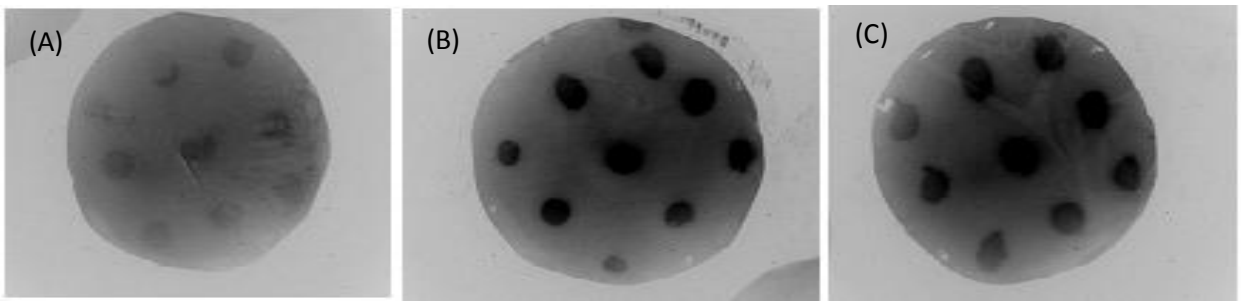


Figure 14. Colony blot of 6x His-OPH protein expression. (A) Negative control with *E. coli* cells and water, (B) Positive control with M15[pREP4] pQE-40 plasmid expressing the 6x His- DHFR protein, (C) M15[pREP4] containing the pQE-30/*opd* plasmid producing the 6x His- OPD protein.

4.9 SDS-PAGE of Purified OPH Protein

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the OPH purified protein was performed (Figure 14). There was a light band at approximately 35kDa, highlighted by the box.

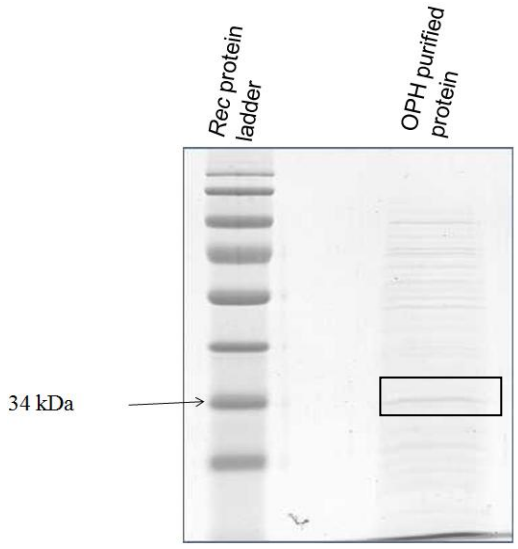


Figure 15. SDS PAGE of OPH protein. The expected OPH protein size was 35 kDa. A band at approximately 35 kDA was present.

4.10 OPH Enzymatic Assay

A preliminary enzyme assay was performed using paraoxon as the substrate (Figure 16). The bacteria and purified OPH protein were placed in CSM media which contains minimal if any

ingredients the bacteria could use as an energy source to survive. Paraoxon was added at a 100ug/ml concentration to each sample. If the bacteria were able to use the paraoxon as an energy source, then the paraoxon was degraded. If the protein was able to breakdown the paraoxon, then the paraoxon was degraded. One of the by-products of paraoxon degradation is *p*- nitrophenol which was measured at an absorbance of 405nm. The negative control was not able to utilize the paraoxon as an energy source which is consistent with the *E.coli* used; it did not contain the *opd*. This is shown by the absorbance readings remaining fairly constant. The positive control absorbance readings increased showing that *Flavobacterium species* is able to use paraoxon as an energy source and degrade paraoxon. These results are consistent with previous research in that the *Flavobacterium species* contains the *opd*. The increasing absorbance readings proved that the purified OPH protein was also able to degrade the paraoxon.

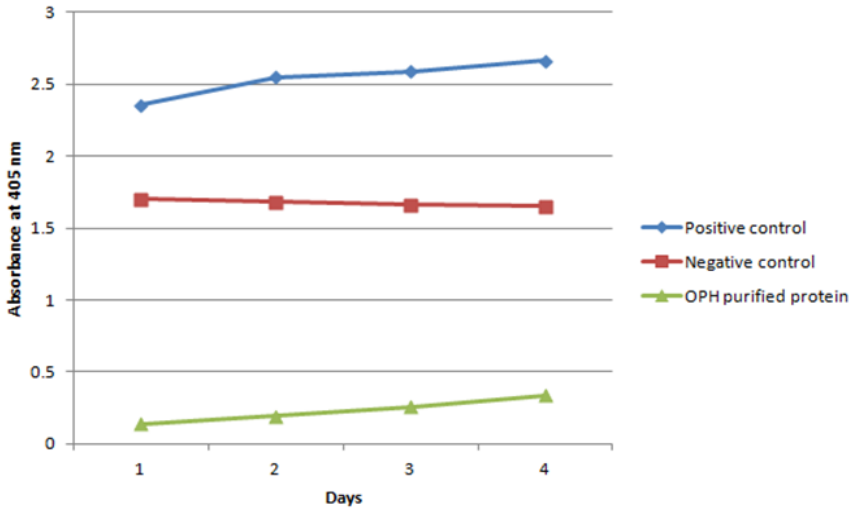


Figure 16. Degradation of paraoxon. Starting concentration of paraoxon is 100ug/ml. Absorbance readings were taken at 405nm over a period of 4 days. *Flavobacterium sp.* was used as a positive control, and *E.coli* was used as a negative control.

CHAPTER 5

Discussion and Conclusion

Organophosphates (OPs) are a class of pesticide widely used in agriculture and are found in pesticides and chemical warfare agents. OPs are extremely dangerous in that they inhibit the cholinesterase function, which disrupts proper activity of the nervous system. OPs are also dangerous because they have a broad target range and are highly toxic for nontarget species (Horne et al., 2006). OPs do not remain in the soil for long periods of time, therefore they are considered to be biodegradable. The toxic effects of OPs on invertebrates, vertebrates and wildlife are well documented (Singh & Walker, 2006). Although considered biodegradable, and proven to be extremely dangerous, OP use has increased, especially in developing countries. However, pesticides are helpful to society in that they can kill potential disease causing organisms, control insects, weeds, and other pests, and help reduce crop loss. Since there has been wide spread use of pesticides, great environmental concerns have arisen.

Some of the methods previously used for removing OP residues are costly, corrosive, result in hazardous waste, and are themselves environmentally hazardous (Horne et al., 2006; Cheng et al., 1999). Therefore, other methods of detoxifying OPs are being researched. Enzymes have become a main focus for many studies in research as a safe a practical way of removing chemical warfare agents and pesticides. Several soil bacterial species have been shown to degrade OPs, including *Flavobacterium species*. The *Flavobacterium species* contains an *opd* which encodes an OPH protein. The OPH protein is the most widely characterized of the phosphotriesterase enzymes and has been shown to be effective in degrading a range of OP esters (Karns et al., 1987).

However earlier studies have reported that current applications that use enzyme-based methods are limited by poor long-term enzyme stability and low reactivity over a broad range of temperatures (Theriot et al., 2010). The application of enzyme-based methods exposes the enzymes to harsh conditions that could possibly cause denaturation of the enzyme leading them to become non-functional. Encapsulation of the enzymes aids in their stability and function when exposed to harsh conditions.

The objectives of this study were to clone the *opd*, to functionally express and purify the OPH protein, and finally to test the catalytic activity and functionality of the OPH protein. In this report, a 1098 base pair *opd* was successfully amplified from *Flavobacterium species* by PCR (Figure 8). The 1320 base pair *opaA* was successfully amplified by from *Pseudoalteromonas haloplanktis* by PCR as well (Figure 8). As seen in Figure 9, the 1155 base pair *opdA* was also successfully amplified from the *Rhizobium radiobacter* genomic DNA.

Sequencing of the *opd*, *opaA* and *opdA* DNA fragments was attempted. The *opd* was successfully sequenced (Figure 10), however, sequencing of the *opaA* and *opdA* was not successful. The deduced nucleotide sequence of *opd* was translated into the amino acid sequence and used to determine the longest open reading frame. The longest open reading frame was determined to be identical to the open reading frame for the *opd* previously sequenced in GenBank (Figure 11). This also verified that the *opd* was effectively cloned in-frame into the pQE-30 vector.

Next, the pQE-30/*opd* was inserted into the M15[pREP4] cells by electroporation (Figure 13). An SDS-PAGE was performed to show that the OPH protein was present and being overexpressed (Figure 14). The expected OPH protein size of 35kDa was present. A colony blot verified which colonies were expressing the OPH protein (Figure 15). The M15[pREP4] cells

with the pQE-30 vector containing the *opd*, the OPH protein appeared to be expressed at a similar level as the positive control.

Finally, an enzyme assay to determine the degradation of paraoxon was performed (Figure 16). The *Flavobacterium species* was used as the positive control. *E. coli* without the *opd* was used as the negative control. Purified OPH protein was used as well. The production of *p*-nitrophenol as a by-product of the breakdown of paraoxon was measured at 405nm. The absorbance readings at 405nm indicated that the positive control and the purified OPH protein were able to use the paraoxon as an energy source. However, the negative control was not able to utilize the paraoxon and therefore the absorbance readings remained nearly constant.

In conclusion, *opd* was successfully amplified and cloned into a high-level protein expression vector. The OPH protein was purified and the catalytic function was demonstrated by the ability of OPH to degrade the organophosphate pesticide, paraoxon.

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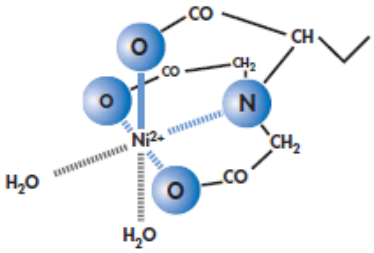
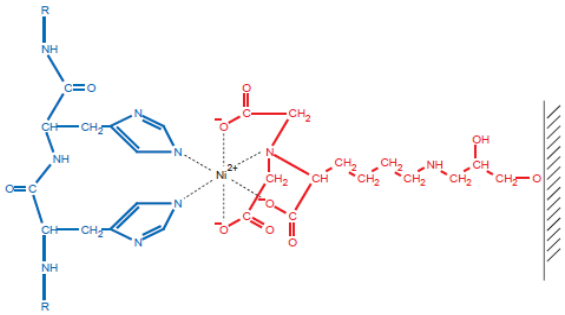
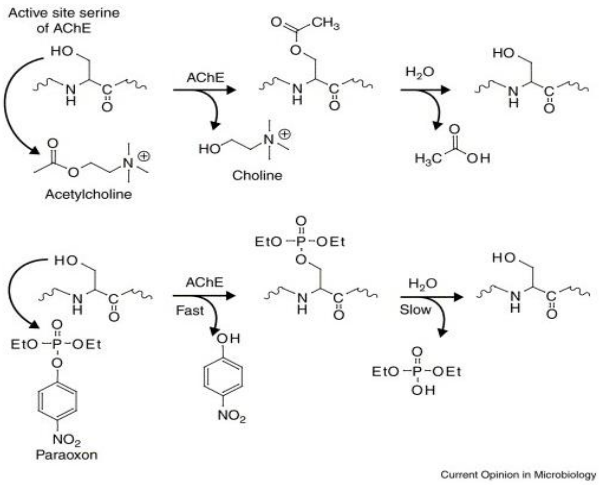
Appendix



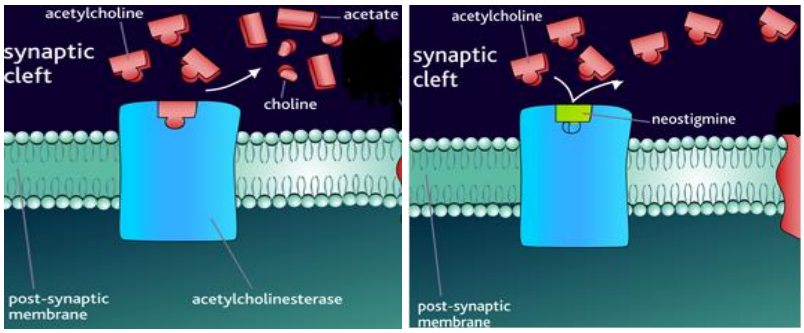
A. Interaction of metal chelate matrices with nickel ions.



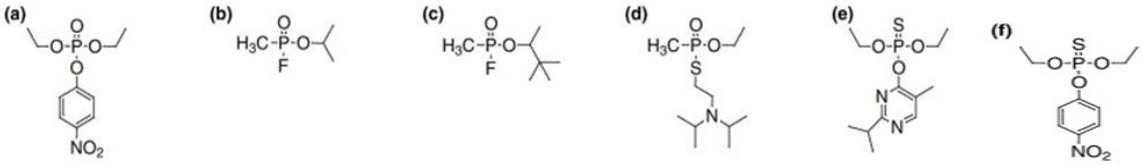
B. Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix.



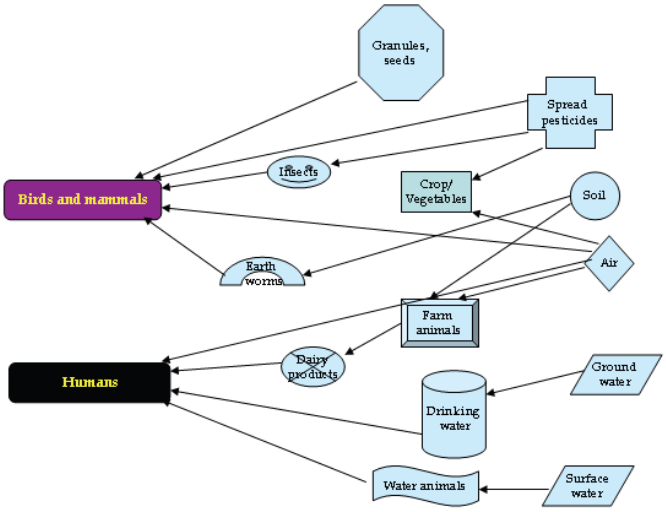
C. Organophosphate (paraoxon) mechanism of toxicity.



D. Acetylcholinesterase inhibition. (A) Normal action of acetylcholine. (B) Neostigmine represents an organophosphate which is inhibiting acetylcholinesterase, leading to its accumulation.



E. Structures of some of the compounds discussed in this thesis: (a) paraoxon; (b) sarin; (c) soman; (d) VX; (e) diazinon; (f) parathion



F. Possible routes of environmental exposure of OP Pesticides/nerve agents to humans and wildlife.