Recombinant Expression and development of enzyme-based detoxification strategies for OP-based Pesticides

M.Sc. Thesis

By

Isha Dhingra

BIOSCIENCES AND BIOMEDICAL ENGINEERING

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"RECOMBINANT EXPRESSION AND DEVELOPMENT OF ENZYME-BASED DETOXIFICATION STRATEGIES FOR OP-BASED PESTICIDES"** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my work carried out during the time period from June 2022 to May 2023 and year of joining the M.Sc. program August 2021 to May 2023 under the supervision of Prof. Prashant Kodgire, Professor, BSBE, IIT Indore, and Dr. Abhijeet Joshi, Associate Professor, BSBE, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Isha Dhingra 08/05/2023

Signature of the student with date
Isha Dhingra

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

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Isha Dhingra has successfully given his/her M.Sc. Oral Examination held on **8 May 2023**.

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Date: 08/05/2023

Signature of PSPC Member
(Dr. Sunil Kumar Boda)
Date: 08/05/2023

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Isha Dhingra

DEDICATION

This thesis is wholeheartedly dedicated to my parents, who have inspired me. They have always been my source of strength and have provided me with moral, spiritual, emotional, and financial support. I also dedicate this thesis to my brother and sister, who have stood by me through thick and thin, and my seniors, without whom this project won’t have been completed.

Abstract

Pesticides are widely used in agriculture to protect crops from pests and increase yields. However, their use has been associated with adverse environmental and health effects due to organophosphate (OP) pesticides. OP poisoning has been linked to various symptoms, including neurological disorders and death. To address these issues, there is a growing interest in developing methods for detecting pesticides and the remediation of contaminated soils and water. The present study investigates the enzymatic degradation of various OP Pesticides. Investigating the use of immobilized enzymes in bioremediation processes for the degradation of OP pesticides and the real-life application of enzymes degrading OP-based Pesticides.

The research involved discovering a new substrate being degraded by OP hydrolyzing enzymes. The enzyme was also immobilized onto alginate beads. These immobilized enzymes were then tested for their ability to degrade OP pesticides in contaminated water samples. The results showed that the immobilized enzymes effectively degraded OP pesticides, with degradation rates up to 99% using 0.2U of immobilized enzyme. The efficiency of the immobilized enzymes was found to be dependent on several factors, including the type of enzyme, the immobilization method, and the characteristics of the contaminated environment. The research also evaluated the stability and reusability of the immobilized enzymes and found that they retained their activity after several cycles of use.

Overall, the findings of this study suggest that immobilized enzymes can be a promising tool for the bioremediation of OP pesticide-contaminated environments offering several advantages, including increased stability and reusability, and can provide an environmentally friendly and cost-effective approach for removing OP pesticides. The research also highlights the need to optimize further and develop immobilized enzyme-based bioremediation strategies to clean contaminated environments effectively.

LIST OF PUBLICATIONS

From Thesis

Immobilization of OPAA-FL into Alginate Beads and its characterization for the Bioremediation of pesticide-contaminated water- Research Article (Manuscript under Preparation).

Bioremediation and detoxification of organophosphate pesticides using OP-degrading enzymes- Review Article (Manuscript under Preparation).

Apart from the thesis-

Vyas T., Parsai K., Dhingra I., Joshi A., (2023) Nano Sensors for detection of Volatile Organic Compounds. In: Advances in Smart Nanomaterials and their Applications, first edition- Book Chapter (Available Online)

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ABBREVIATIONS

AChE Acetylcholine Esterase

OPAA-FL Organophosphorus acid anhydrases

OPH Organophosphorus hydrolase

OPs Organophosphorus compounds

PTE Phosphotriesterase

CWA Chemical Warfare Agents

BSA Bovine serum albumin

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

OD Optical density

PPM Parts Per Million

LD-50 Lethal Dose-50

LOD Limit of detection

MPH Methyl parathion hydrolase

Ni NTA Nickel nitriloacetic acid

Nus-A N-utilization substance protein A

OC Organochlorine

Chapter 1

Introduction

1.1 Pesticides: A Global Threat

With the world population reaching 8 billion, an increase in hunger and food crisis is a significant concern. The increasing population has burdened the agriculture sector, wherein there is a proportional increase in crop production among the people. To increase the production of crops, there is substantial use of pesticides. Any substance used to control pests, unwanted weeds, and plant infections is known as a pesticide. Pesticides' exact mechanism of action is complicated and not entirely known, but they act by ingestion, inhalation, or contact. The overuse of pesticides has a significant consequence as only a limited portion of them meet the cause. Literature has shown that only 0.1% of pesticides applied to crops reach the target pests, and the rest of them find their way to contaminate soil, groundwater, and air [1]. **Fig. 1.1** represents the typical movement of pesticides occurring in different ways, via air in the form of droplets and water, thus contaminating natural water bodies; pesticides can also leach out into the soil, contaminating it and the crops. Their fate in the soil environment is a primary concern for scientists because they directly affect soil microorganisms and soil fertility [2]. Pesticide pollution is a significant problem in the agricultural sector, leading to yearly crop loss. This could even lower the soil's nutritional quality and the food we eat [3].

Literature has revealed that India stands in the top 5 highest pesticide-consuming countries globally. **Fig. 1.2** shows the frequency with which the pesticides are used and consumed globally. Due to their widespread use, people are continuously exposed to low levels of pesticides. There lies a concern in the mechanism of action of a pesticide, which must have no effect on nontarget microorganisms and should be biodegradable [4]**.** The increasing use of pesticides is a significant safety issue. Their toxic nature renders them a risk to human health [5] as exposure of the commoner to pesticides can occur in

different ways by air, water, soil, diets, etc. For agricultural workers, pesticide exposure via skin contact is also widespread. Direct contact with these contaminants on the skin has dire consequences; it can cause reproductive disorders, neurological diseases, respiratory disorders, diabetes, and even cancer. The type of toxic nerve agents, the duration, the mode of exposure, and the health status of an individual have an essential role in the potential consequence on the health of these pesticides [3].

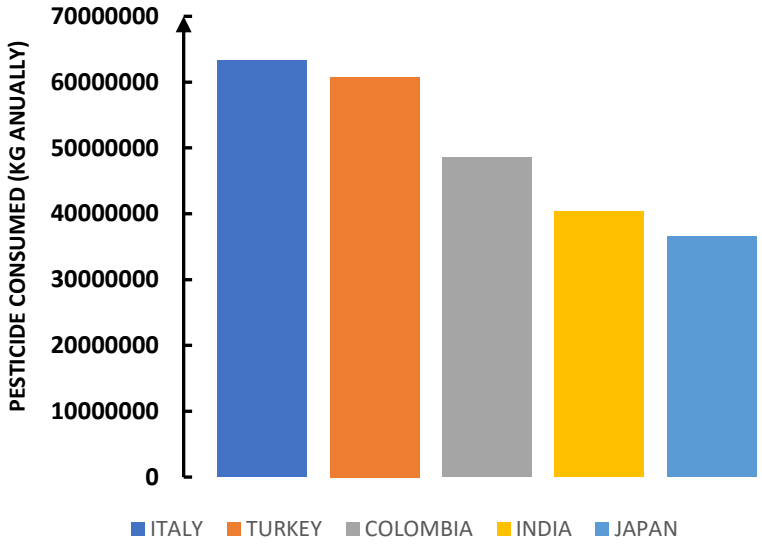


Figure 1.2. Pesticide usage globally. India is among the top 5 pesticides consuming countries (kg annually), number 4 after Italy, Turkey, and Colombia[6].

The mechanism of a pesticide's action, composition, mode of action, environmental stability, and spectrum of activity can all be used to categorize them [1]. On the above parameters, they are majorly divided into four classes- carbamates, organophosphates, organochlorines, and pyrethroids.

1.2 Why OP Pesticides?

OP Pesticides are the esters of phosphoric acid and its derivatives. They have a central Phosphorus(P) atom with a P=S or P=O bond, R1 and R2 are two side groups that are generally alkoxy (OCH3 or OC2H5) and a

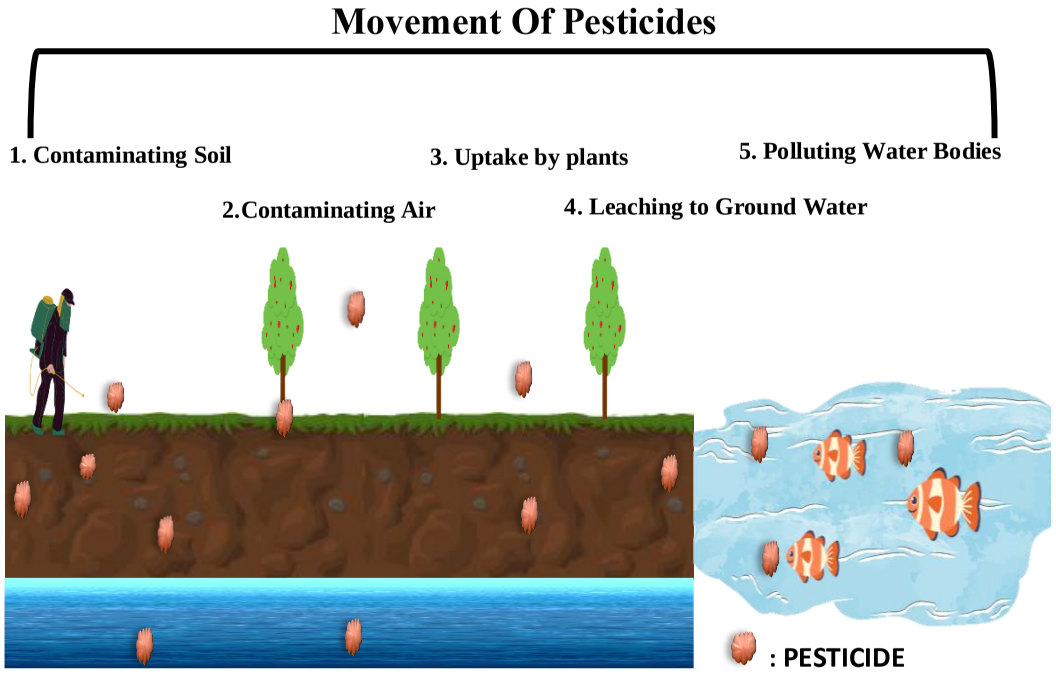


Figure 1.1. Schematic representation of the movement of pesticides in the environment

leaving group (X). This leaving group is substituted nucleophilically by the oxygen of serine esterase enzymes. Examples of commonly used Organophosphate pesticides are Paraoxon, Parathion, Malathion, Dimethoate, chlorpyrifos, and Coumaphos **Fig. 1.3.** Shows the general structure of Organophosphate Pesticides and examples of various OP-Pesticides. A single or repeated exposure to OP insecticides is possible. A single exposure is generally high-dose exposure that leads to acute OP Poisoning, whereas repeated exposure leads to chronic poisoning by long-term repeated exposure to OP Compounds. Both have toxic and harmful effects on human health [7]. Organophosphorus (OP) compound pesticide poisoning is a significant public health issue, resulting in over 300,000 fatalities and several million nonfatal cases annually.

Apart from their use as pesticides, they were also used in chemical warfare as nerve agents [6]. OP compounds are like a double-edged sword that, on one side, protect crops but, on the other side, pose a severe threat to humans and the environment. They are known to cause various symptoms depending on the time of exposure. Mild symptoms like nausea, headache, sneezing, blurry vision, and lethal complications like neurodegeneration, cancer, Paralysis,and PTSD can occur [8].

The danger posed by OP pesticides is not simply restricted to agricultural settings; these chemicals can also be present in everyday household items like cockroach spray and mosquito repellent. Additionally, the OP Pesticides may contaminate the food [7]. The presence of pesticides in food remains a significant concern. Their accumulation in the blood can lead to persistence, bio-concentration, and bio-magnification through the food chain [9].

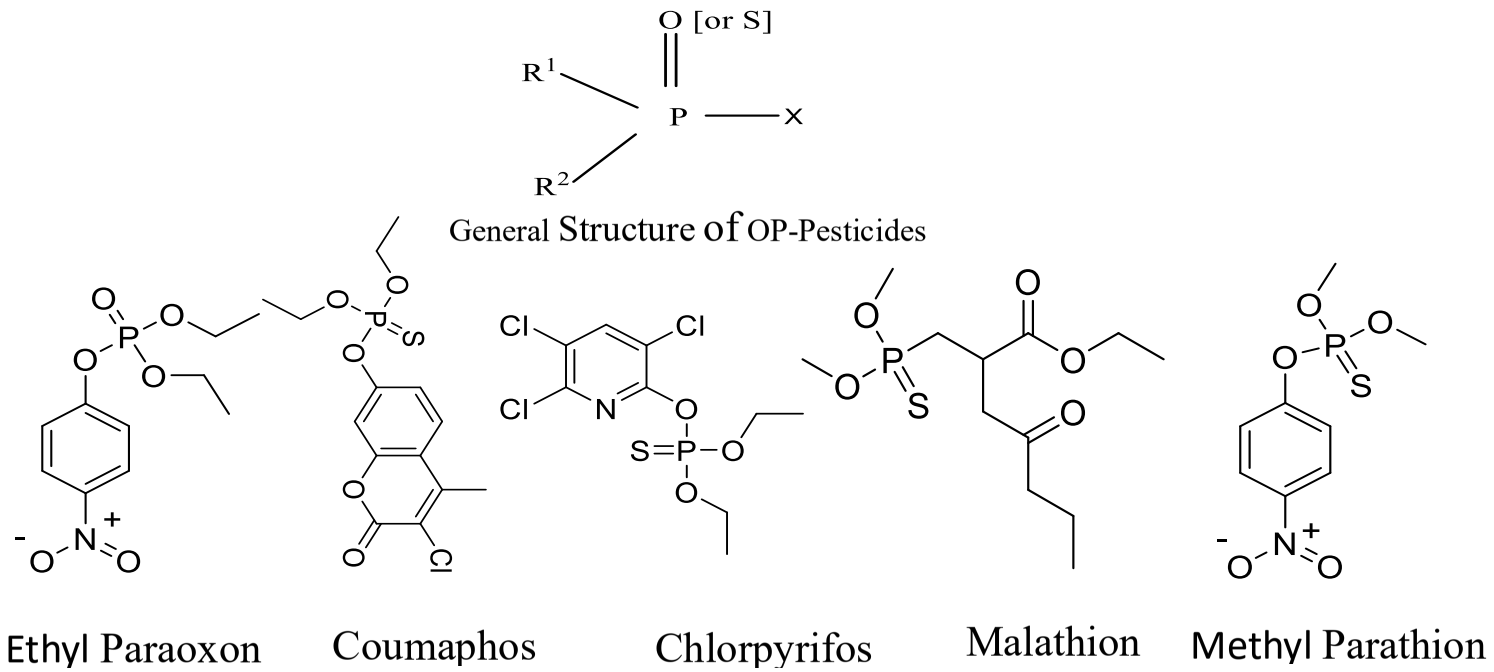


Figure 1.3. Organophosphate Pesticides. (A) The general structure of an Organophosphate Pesticide, central Phosphorus(P) atom with a P=S or P=O bond, R1, and R2 are two side groups that are generally alkoxy (OCH3 or OC2H5) and a leaving group (X). **(B)** Structures of some common OP pesticides.

Figure 1.4. Symptoms of poisoning by Organophosphate Pesticides. Symptoms of OP pesticide poisoning can vary depending on the type and amount of pesticide exposure and the individual's age, health status, and other factors.

Typical OP Poisoning is caused by producing toxic Oxon metabolites, leading to neuropathy in humans. Acetylcholinesterase (AChE) and neuropathy target esterase (NTE), two examples of target proteins phosphorylated by these metabolites, are two such proteins [10]. They can also act by binding to the nicotinic or muscarinic receptors in the Nervous System. The schematic representation of Poisoning caused by OP Pesticides is shown in **Fig 1.5**. Our nerves release acetylcholine, which is vital in signal transmission and contraction of muscles. Acetylcholine Esterase (AChE) breaks down Ach and results in muscle relaxation. OP pesticide acts by irreversibly inhibiting the action of AChE by phosphorylating them, which results in the accumulation of ACh leading to neurodegenerative diseases [11]. Organophosphate poisoning exerts a direct toxic influence on the central nervous system.

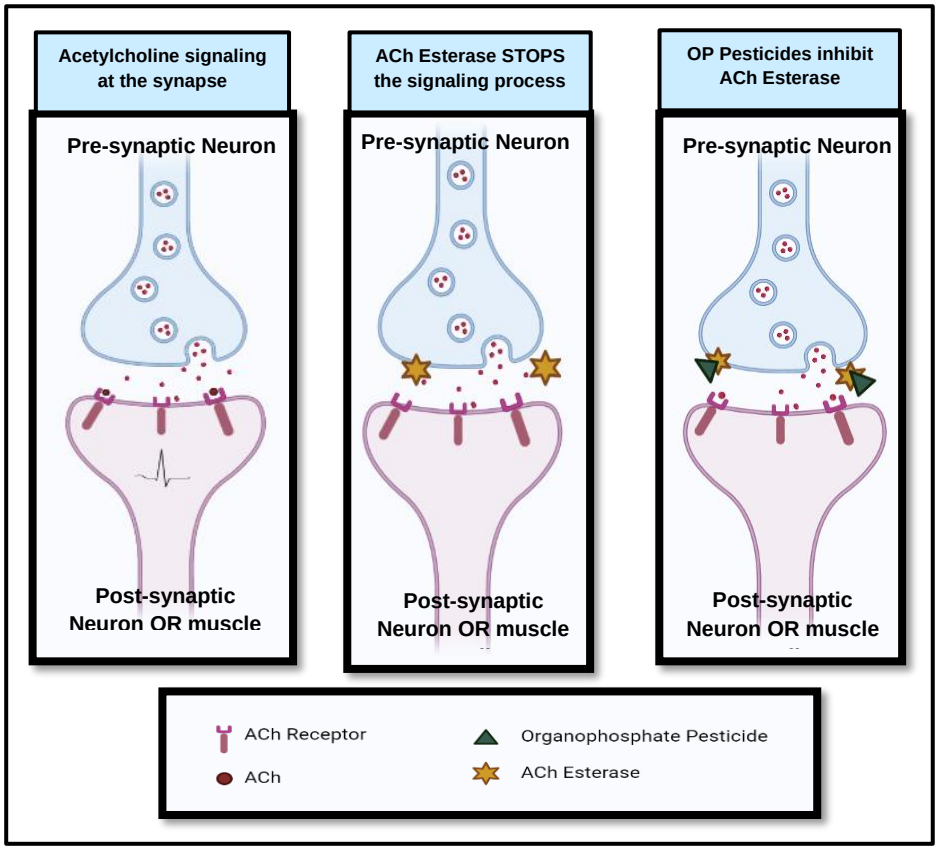


Figure 1.5. Schematic representation of the mechanism of OP Pesticide poisoning by irreversible inhibition of AChE [11]**.**

1.3 Pesticide Degradation

Despite many uses of pesticides, they pose a significant threat to human health and the environment. The presence of pesticides in the food chain is a serious concern. There is an urgent need to develop new decontamination strategies. Generally, in the environment breakdown of pesticides occurs by the indigenous microorganisms through a process named Biodegradation. Organophosphate pesticides have been shown to have fast degradation rates, but their toxicity to mammals is high. This is because OP pesticides are readily soluble in water, making them more prone to human intake [6]. Conventionally there are three methods for the degradation of pesticides:

1. **Physical Decontamination**
2. **Chemical Decontamination**
3. **Bioremediation**

1.3.1 Physical decontamination

Physical methods involve incineration, the use of sorbent materials, and washing or burying. The advantages of the physical detection of pesticides are their efficiency and ease of use. However, the major problem with physical methods is that it leads to secondary contamination, have the potential for volatile release, and might also require transport material [12].

1.3.2 Chemical decontamination

It involves the use of chemical reagents for the degradation of pesticides. Methods like hydrolysis, Oxidation, and Reduction all fall under chemical methods of decontamination. The underlying advantage of chemical methods is their ease of use, efficiency, and rapid decontamination. But chemical methods are generally expensive. The corrosive agent may damage the equipment, and it can also lead to secondary contamination.

1.3.3 Bioremediation

Bioremediation is the method of degradation of pesticides using microbes or their products. Bioremediation ability has been revealed in archaea, bacteria, algae, and fungal species. According to published research, microbial degradation of pesticides is ten times faster than physical degradation and an order of magnitude faster than chemical hydrolysis [6]. Moreover, bioremediation is a fast, environment-friendly, and economical method for removing pesticides, and there is no problem of secondary contamination associated with Bioremediation [13].

1.3.3.1 Microbial Bioremediation

Microbial bioremediation occurs by hydrolysis of P-O alkyl and aryl bonds. Microbes possess unique metabolic features in the natural environment. Microbial consortia distribute metabolic stress among various strains in the group to robustly perform the degradation of toxic compounds. Understanding the physiological, metabolic, ecological, molecular, and microbiological factors that are involved in the transformation of the pollutant is essential for microbial bioremediation.

There are reports available that suggest the degradation of OP Pesticides like malathion into non-toxic metabolites such as malathion monoacid, dimethyl phosphate, phosphorothioates, and dimethyl phosphorothioate using fungal strains like *Aspergillus, Penicillium, Rhizoctonia*. But all fungi do not have the catalytic and metabolic machinery to hydrolyze the P-O, P-S, and P-F bonds; hence research is still going on to find and modify enzymes from fungal strains that can hydrolyze OP Pesticides.

Algae are efficient and economical organisms for the bioremediation of toxic compounds, including pesticides. Bioremediation of OPs using algae has an added advantage as it also acts as the Bio-Fertilizer. A few examples of algal species used for the bioremediation of OP Pesticides are *Nostoc muscorum, Spirulina*

platensis, Chlorella vulgaris, Scenedesmus quadricuda, and *Anabaena oryzae.*

Bacterial bioremediation of pesticides is the most common method for the degradation of pesticides. *Flavobacterium* was the first bacteria isolated that could degrade OP pesticides. Extensive research is being done globally to find bacteria that can degrade OP Pesticides. Both the bio-mineralization and co-metabolism of OP pesticides by bacteria have been investigated.

A promising weapon in our arsenal for the bioremediation of organophosphate pesticides could be the partial or complete breakdown of OP pesticides by various bacterial species. However, there are several restrictions on this microbial remediation, including the number of microbes present, the ideal pH, the required temperature and nutrients, and the presence of pollutants.

In order to overcome these problems, a new system for the degradation of pesticides was developed: enzymatic Bioremediation. A cell-free enzyme system is an effective method owing to its better efficiency, specificity, efficacy, and ability to work under harsh conditions.

1.3.3.2 Enzymatic bioremediation of pesticides

Organophosphate hydrolyzing enzymes are enzymes that can hydrolyze the OP compounds to become nontoxic agents. There are reports suggestive of many OPs hydrolyzing enzymes from various organisms, including mammals, but the most potent and efficient are the ones isolated from microbes. Some of the examples of OP hydrolyzing enzymes that are of microbial origin are Organophosphorus hydrolase (OPH), Organophosphorus acid hydrolase (OPAA), Phosphotriesterase-lactonases (PLL), Methyl Parathion hydrolase (MPH), SsoPox. Mazur in 1946 first reported these enzymes, and they were later classified based on their substrate specificity.

1.4 Organophosphate Hydrolyzing Enzymes.

1.4.1 Organophosphorus acid Anhydrolase (OPAA)

Organophosphorus acid hydrolase (OPAA) is specific for the enzymatic degradation of OP compounds and G and V series nerve agents. OPAA belongs to the prolidase family and is isolated from halophilic *Alteromonas* sp.JD6.5, a gram-negative bacterium. **Fig.1.6 (A)** shows the structure of OPAA. These 517 amino acids (59 kDa) contain Mn in its active site. It is similar to PTE in stereospecificity but is slightly less efficient. OPAA is optimally operational at a pH range of 7.5-8.5 and a temperature range of about 40-55°C and requires Mn^{2+} for its activity. The substrate binding pocket of OPAA is represented by three pockets. A nucleophilic attack on either the carbonyl oxygen of the scissile peptide bond of the dipeptide [Xaa-Pro] or the phosphorus center of NA is made possible by the water molecule or hydroxide ion in the metal center, and this causes the cleavage of the OP complex. This enzyme shows higher efficiencies when expressed in *Escherichia coli* and a higher level of enzymatic activity.

1.4.2 Organophosphate Hydrolase (OPH)

Organophosphate hydrolase (OPH), usually referred to as Phosphotriesterase (PTE), was initially isolated from *Pseudomonas diminuta* (now known as *Brevundimonas diminuta* MG) and is homo-dimeric, TIM-barrel folded protein. This enzyme is encoded by Organophosphate degradation(*opd*) genes. In recent years, Organophosphate hydrolase grabbed major attention for the non-toxic bioremediation of OP nerve agents. OPH is a broad-specificity enzyme, and it can cleave P-O, P-CN, P-F, and P-S bonds. Upon hydrolysis, OPH results in the formation of two protons and one alcohol which is chromophoric. OPH uses divalent metal ions such as Zn^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+}, Ca^{2+}, and $Fe^{2+,}$ etc., for performing nucleophilic attacks by activating water molecules required for hydrolysis. **Fig. 1.6 (B)** shows the structure of OPH.

The hydrolysis reaction of organophosphate involves the core metal ion in three significant ways. By neutralizing, the metal ion prevents the leaving group from developing a negative charge. The metal ion accelerates the attack of the hydroxyl ion by increasing the polarisation of the P=O link and so enhancing the electrophilicity at the phosphorous center. The metal ion may also lower the pKa of the attached water molecule and raise the nucleophilicity of the attacking hydroxide ion. Different factors like pH, temperature, and substrate concentration affect the activity of OPH. It has been reported that OPH shows maximum activity at 37°C and a pH range of 7.5-9. **Table 1.1** shows the major differences between OPAA and OPH.

1.4.3 Other OP Hydrolyzing Enzymes

Other examples of OP hydrolyzing bacteria are Methyl Parathion Hydrolase (MPH), Paraoxonase (PON), Diisopropyl-fluorophosphatase (DFPase) **Fig. 1.6 (C, D, E).** MPH is encoded by the *mpd* gene isolated from *Plesiomonas* sp. Strain M6. It is a Zn-containing homodimer that catalyzes the degradation of OP Pesticide: Methyl Parathion. PON is a human serum enzyme that can potentially degrade paraoxon and nerve agents. It is a monomeric enzyme with Ca at its binding site. Another enzyme that can hydrolyze P-F bonds is DFPase, a 35kDa monomeric protein. DFPase is isolated from the brain of *Loligo Vulgaris* Squid.

1.5. Enzymatic Bioremediation using Immobilized Enzymes

Usually, the enzymes in the free state act on the substrate and give product, but this use of enzymes is wasteful as free enzymes cannot be recovered after the reaction. Immobilized enzymes increase the stability, reduce cost by simple recovery, and ensure reutilization of the enzyme leading to multiple catalytic cycle efficiency [14]. Various matrices or supports are used for the immobilization of enzymes which could be natural polymers (alginate, chitosan, gelatin, cellulose, starch), synthetic polymers (Diethylamino ethyl cellulose, polyvinyl chloride, polyethylene glycol), or inorganic materials (zeolites, silica, glass,

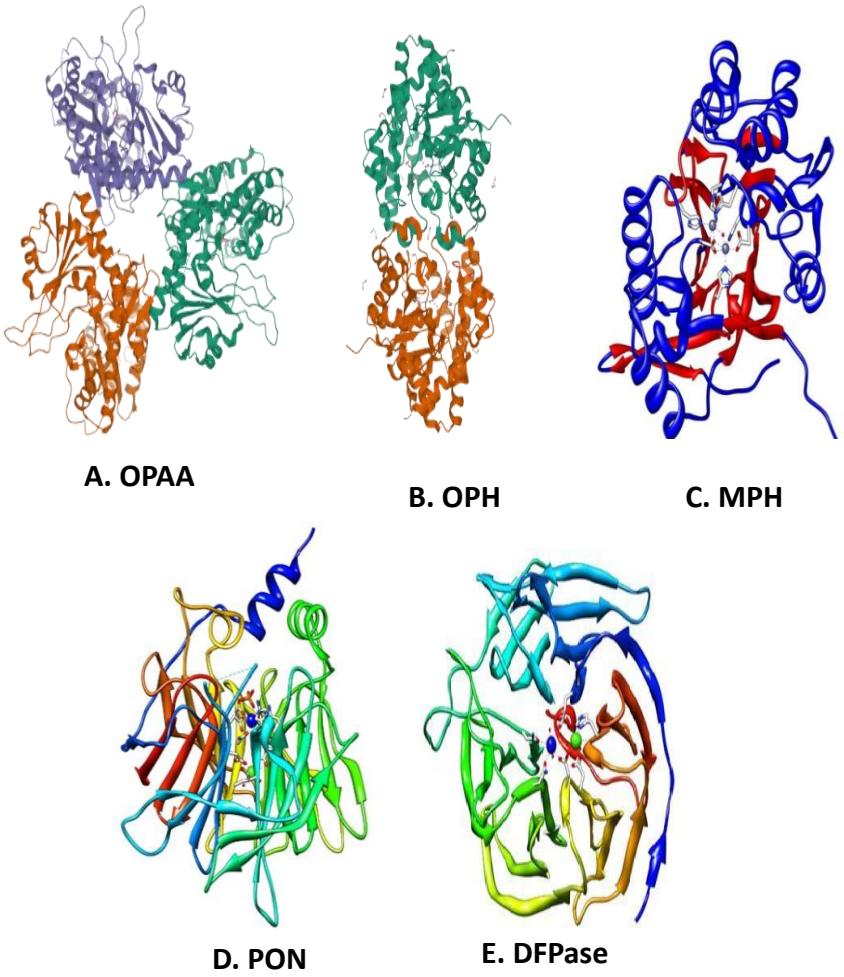


Figure 1.6. OP Hydrolyzing Enzymes A) OPAA "3L7G", B) PTE from *Pseudomonas diminuta* (OPH) "1HZY", C) MPH "1P9E", D) Serum PON "1V04", E) DFPase "1E1A":

Table1.1 Differences between OP compound degrading Enzymes: OPAA and OPH

Enzyme	Organophosphorus Hydrolase (OPH)	Organophosphorus Acid Anhydrolase (OPAA)
Organism Isolated from	*Pseudomonas diminuta, Flavobacterium, Agrobacterium radiobacter*	*Alteromonas* sp. JD6.5, *Pseudoalteromonas haloplanktis*
Bonds cleaved	P-O, P-F, P-CN, P-S	P-O, P-F, P-CN, P-S
Metal Ion	Zn	Mn
The active form of the enzyme	Dimer	Monomer
Size	37kDa	58kDa
Substrate specificity	Paraoxon, Azinophos-ethyl demethon-S, Acephate, Malathion, Phosalone, Methamidophos, Tetriso, (Sp)-EPN, DFP, VX, Soman, Mipafox, Dursban, Parathion, Coumaphos, Diazinon, Fensulfothion, Methyl parathion	Paraoxon, DFP, Soman, Sarin, Mipafox
References	[15], [16]	[16], [17]

ceramics). Enzyme immobilization occurs via different methods example, Encapsulation, Adsorption, Entrapment, Covalent Binding, and cross-Linking. Immobilization of enzymes is a regular practice in industrial setups. It combines the properties of enzymes, like selectivity stability and kinetics, with the physical and chemical properties of the carrier matrix. This results in maximizing the physical and enzymatic stability of the biocatalyst**. Table 1.2** shows different matrices used for the immobilization of different OP hydrolyzing enzymes [18]–[29]. Literature suggests that immobilization of enzyme has better application for bioremediation of pesticides.

Table 1.2 Different matrices used for immobilization of OP Hydrolyzing Enzymes

S.No	Matrix	Immobilization method	Enzyme	Pesticide	Kinetic Parameters	Efficiency	Stability	Reusability
1	Carbon nanotube paper	Covalent Binding	OPH	Methyl Paraoxon	NA	22% biodegradation	NA	NA
2	Functionalized ferric magnetic nanoparticles	Covalent Binding	OPH	Ethyl parathion	Km increased 1.8-2 times	Around 70% of immobilization	pH stability, 6.3-fold increase in comparison to free enzyme	Reused for 7 cycles, 80% activity retained after 5 cycles, activity retained for 30 days
3	Nylon 6 membranes, Nylon 11 powder, and nylon tubing	Covalent Binding	PTE	Paraoxon, Ethyl Parathion, DFP	Km increased to 5-6 times	NA	Limited activity in hydrolyzing OPs and CWA in presence of a solvent	stable for at least 20 months

4	Cellulose containing carriers	adsorption	His-OPH	Paraoxon, Parathion	NA	65-92% greater activity with the carriers	130 half-life lifthe e in soil	about 90% of original activity retained after storage for 60 days at 8°C
5	Modified cellulose microfibers	Covalent Binding	OPH	Paraoxon	Km increased to 1.8-2.06 times	Immobilization yield was 68.32% and 75.31% onto epoxy and CDI activated Cellulose respectively	More thermal and storage stability, denaturation resistance against pH	Immobilized OPH onto epoxy and CDI-modified cellulose preserved around 25% and 6% of their initial activity within one month at 25°C, Reusability for 10 batches.
6	Spores of Bacillus subtilis	Covalent attachment	OPH	paraoxon	Catalytic efficiency increased to 1.3-1.5 times	NA	Improved pH Stability	80% intact activity after 10 cycles
7	Quartz slide	Covalent attachment	OPH	paraoxon	NA	LOD 5nM	Increased pH and thermal stability	The recovery rate was 60%

8	Porous glass and porous silica beads	Covalent attachment	OPH	Parathion	Km was increased	enzyme bound was around 75-96%	stable in different solvents	NA
9	Elastin-like Polypeptide	Covalent attachment	OPH	parathion	NA	LOD 10-12nM	Stability improved in conjunction with BSA	Retained 93% stability even after 15 days
10	CBD-OPH Fusion	Conjugation	OPH	Coumaphos	Slight increase in Km	NA	NA	Retained for over 45 days
11	Elastin-OPH fusion	Conjugation	OPH	Paraoxon	10% increase in Km, 5% decrease in Kcat	67% Yield	Stability at increased salt concentrations	retain 100% activity for a span of three weeks
12	Carboxylated-Pluronic F127 Amphiphilic block copolymer	Conjugation	OPH	Paraoxon	No change in Km, Kcat increased by 1.5 times	NA	Thermal stability	Long term stability of 3 months was achieved

Chapter 2

Objectives

1. Determination of Enzymatic Degradation of Organophosphorus Pesticides using Organophosphorus acid anhydrolase (OPAA)-FL variant and recombinant Organophosphorus Hydrolase (OPH).

2. Development of Bioremediation Strategies of Organophosphorus pesticides using Immobilized OPAA-FL enzyme.

3. Real-Life Application of Organophosphorus Pesticide Degrading Enzymes; OPAA-FL and Nus-OPH.

Chapter 3

Materials and methods

3.1 Materials

Strains: The *E. coli* Rosetta cells were used as host cells for expressing OPAA-FL and Nus-OPH.

Chemicals: Luria Bertani Broth Miller (HiMedia), Kanamycin (HiMedia), Lysozyme, Tris-Cl, NaCl, Imidazole, Bis-Tris Propane buffer, CHES Buffer ethyl paraoxon (Sigma Aldrich), Coumaphos. Dimethoate, Chlorpyrifos, Malathion, Methyl Parathion, BSA, Alginate, PMSF, p-NP, IPTG, $MnCl_2$, $CoCl_2$, Glycine ($C_2H_5NO_2$), Ethylene diamine tetra acetic acid (EDTA), Sodium hydrogen phosphate(Na_2HPO_4), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydroxide (NaOH), Sodium dodecyl sulphate or Sodium lauryl sulphate (SDS), Coomassie brilliant blue G-250, β-mercaptoethanol ($HOCH_2CH_2SH$), Luria-Bertani broth (LB- broth), Kanamycin, Acrylamide (C_3H_5NO), N, N′-Methylene bisacrylamide (MBAA), Triethylenetetramine or Tetramethyl ethylene diamine (TEMED), Ammonium per sulphate ($(NH_4)_2S_2O_8$), bromophenol blue or 3′,3″,5′,5″-tetra bromophenol sulfonphthalein ($C_{19}H_{10}Br_4O_5S$), Bovine serum albumin (BSA), Isopropyl-β-D-thiogalactopyranoside ($C_9H_{18}O_5S$), Imidazole ($C_3H_4N_2$), Glycerol ($C_3H_8O_3$), Methanol (CH_3OH), Glacial acetic acid(CH_3COOH), Ethanol (C_2H_5OH), alginic acid sodium salt from brown algae or Sodium alginate, 1,3 Bis [tris(hydroxymethyl)methylamino] propane (Bis-Tris propane) $C_{11}H_{26}N_2O_6$, 2- (Cyclohexyl amino) ethane sulfonic acid (CHES) ($C_8H_{17}NO_3S$), Ni NTA Sepharose beads, Glycine.

All chemicals were of molecular biology grade and procured from different manufacturers like Sigma Aldrich Chemical Pvt. Ltd., Invitrogen Pvt. Ltd., MP Biomedical USA, HiMedia Pvt. Ltd.India, Alfa Aesar Pvt. Ltd., Sisco Research Lab Pvt. Ltd. India, and Otto. Chemie Pvt. Ltd.

3.2 Methods

3.2.1 Induction and Expression of Recombinant Protein: OPAA-FL and Nus-OPH

For the primary culture, a single colony of transformed cells was inoculated into an LB broth tube with 10 µg/ml Kanamycin (working concentration). The culture was incubated for 10-12 hours at 37 °C, 200 rpm. The required volume of culture was added from the primary culture to the secondary culture, and cells were kept at 37 °C on an incubator shaker till 0.8 the OD was attained. For induction, 0.1 mM IPTG and 0.1 mM $CoCl_2$ for Nus-OPH and 1 mM IPTG, and 1 mM $MnCl_2$ for the OPAA-FL variant were added to the secondary culture. The culture was incubated at 180 rpm, at 16 °C/16 hours Nus-OPH, and 20 °C/20 hours for the OPAA-FL variant. After induction, the cells were harvested at 10000 rpm at 4 °C for 10 minutes. The resultant pellet was washed and resuspended with 50 mM Tris-Cl (pH 8.0), 500 mM NaCl, 1 mM PMSF, and 10% glycerol.

Later, for the lysis of cells, lysozyme was added to a final concentration of 100 µg/ml. The cells were then incubated at 30°C for 20 minutes. Lysozyme treatment was followed by sonication. At last, the cell lysate was centrifuged at 14000 rpm and 4 °C for 10 minutes. The lysate was then analyzed on 8% SDS-PAGE for Nus-OPH and on 12 % SDS-PAGE for OPAA-FL.

3.2.2 Purification of Recombinant Protein: OPAA-FL and Nus-OPH

Protein Purification was performed by Ni-NTA Sepharose beads. For affinity chromatography using Ni NTA Sepharose beads, the input sample was prepared in a way that it had the composition of the equilibration buffer (500 mM NaCl, 50 mM Tris of pH 8.0, and 10 mM Imidazole). The column was washed with Autoclaved Distilled Water twice and centrifuged in a swinging bucket centrifuge at 600 rpm. This was followed by recharging the beadsusing 0.1M $NiSO_4$, followed by

centrifugation at 600 rpm for 10 minutes, and washing with D/W. The column was then equilibrated using an Equilibration Buffer (500 mM nNaCl, 50 mM Tris of pH 8.0, and 10 mM Imidazole), followed by centrifugation at 600 rpm for 10 minutes. The Input samplewas loaded to the pre-equilibrated column containing Ni NTA tagged Sepharose beads and incubated for 2 Hrs on the shaker at 100 rpm. The column was then centrifuged at 600 rpm, and the flow-through was collected. The column was then washed with Wash 1 buffer (50 mM Tris of pH 8.0, 500 mM NaCl and 50 mM Imidazole) for the OPAA-FL variant and Wash 1 and Wash 2 both (50 mM Tris-Cl of pH 8.0, 500 mM NaCl, and 90 mM Imidazole) for Nus-OPH. Elution of purified protein was done using elution buffer (50 mM Tris of pH 8.0, 500 mM NaCl and 150 mM Imidazole). All fractions were analyzed on SDS-PAGE.

3.2.3 Buffer exchange and concentration of the purified Nus-OPH and OPAA-FL-variant

The purified protein was in 500 mM NaCl, 50 mM Tris-Cl of pH 8.0, and a fixed concentration of imidazole, i.e., 150 mM for OPH, OPAA-FL variant. It was then buffer exchanged and concentratedto 500 mM NaCl, 50 mM Tris-Cl of pH 8.0 with 15 mM Imidazole for OPH, and 50 mM NaCl, 50 mM Tris-Cl of pH 8.0 with 15 mM Imidazole for OPAA-FL-variant. The purified protein was loaded (500 µL) into the amicon and centrifuged at 4000 g until the volume of the protein in the amicon was reduced to 10 times lesser (50 µL) than the loaded protein. The volume was again made up tothe initial volume using 50 mM Tris-Cl of pH 8.0 and 500 mM NaCl for OPH and 50 mM Tris-Cl of pH 8.0, and 50 mM NaCl for OPAA-FL-variant. Amicons were again centrifuged at 4000 g until the volume was reduced to 10 times lesser than the initial volume. In the collection tube, amicon was inverted and centrifuged at 1000 g for 2 minutes for the collection of the desalted and concentrated sample. The temperature was maintained at 4°C throughout the procedure. The buffer exchanged, and concentrated proteins were then collected in a fresh autoclaved microcentrifuge tube and tested for their activity against OPs.

3.2.4 Activity Assay for Recombinant Protein

3.2.4.1 Activity Assay for OPAA-FL and Nus-OPH against Ethyl Paraoxon

To determine the activity of OPAA-FL against EP, 1 µL of purified, buffer exchanged and concentrated protein, 1 mM ethyl paraoxon, 1 mM $MnCl_2$, 50 mM Bis-Tris propane buffer, pH 8.5, were added, and the final volume was made up to 250 µL with SDW. Incubation of 15 minutes at 50 °C was given to the reaction mixture. For Nus-OPH, 1 µL of purified, buffer exchanged and concentrated protein, 50 mM CHES Buffer pH 9.0, 0.1 mM $CoCl_2$, and 1 mM Ethyl Paraoxon was added, and the reaction volume was made up to 200 µL using SDW and incubated at 37 °C for 5 minutes. A control reaction was set up with similar constituents but no enzyme. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. A specific activity's value was calculated as U/mg [30], [31].

3.2.4.2 Activity of OPAA-FL against Coumaphos

To check the activity of the enzyme against Coumaphos, first a calibration curve of chlorferon was made using 0-300 µM concentration. The fluorescence was recorded using excitation wavelength of 377 nm and emission wavelength of 470 nm. Then for enzymatic assay, 1 µL of purified buffer was exchanged, and the concentrated enzyme was added to the reaction mixture containing 50 mM BTP, 0.2 mM Coumaphos, 1 mM $MnCl_2$, and SDW for OPAA-FL and, 50 mM CHES Buffer Ph 9.0, 0.1 mM $CoCl_2$, 0.2 mM Substrate and SDW for Nus-OPH. The reaction mixture was incubated at 50 °C for 15 minutes and 37 °C for 5 minutes for OPAA-FL and Nus-OPH, respectively. After that, the absorbance (377 nm) and Fluorescence (Excitation wavelength: 377 nm and emission wavelength: 470 nm) readings were taken in Micro Plate Reader, synergy H1 BioTek, to check the production and quantification of chlorferon.

3.2.5 Thin Layer Chromatography for analysis of the degraded product of Coumaphos

Activation of plate: The silica gel plate was activated by pre-running into methanol. This wasdone to remove any contamination. The plate was then dried.

Saturation of TLC chamber: The mobile phase Benzene: Ethyl Acetate (70:30) was added into the TLC chamber, which was then covered with a lid for 15 minutes before adding the spotted TLC plate. This was done to saturate the TLCchamber.

Spotting: 10 µL of Standard Coumaphos solution (10 mM), Control 1 (50 mM BTP pH 8.5, 1 mM $MnCl_2$, 1 mM Substrate, SDW), Control 2 (50 mM CHES pH 9.0, 1 mM $CoCl_2$, 1 mM Substrate, SDW), Experimental 1 (50 mM BTP pH 8.5, 1 mM $MnCl_2$, 1 mM Substrate, Enzyme, SDW), Experimental 2 (50 mM CHES pH 9.0, 1 mM $CoCl_2$, 1 mM Substrate, Enzyme, SDW) were spotted. The plate was dried for 5 minutes and was then suspended in the TLC Chamber. Capillary forces cause the solvent to rise in layers and transport the sample mixture.

Visualization of plate: The plate was visualized under UV at two wavelengths, 254 nm, and 366 nm,to check the formation of degraded product [32].

3.2.6 HPLC analysis for enzymatic degradation of Coumaphos by OPAA-FL

For enzymatic degradation of Coumaphos, first, the standard substrate and product were run on HPLC. Then to check enzymatic degradation, 1 µL of the purified enzyme was added to 50 mM BTP pH-8.5, 1 mM $MnCl_2$, and 0.2 mM Substrate, and the volume of the reaction was made up to 250 µL. This was then incubated at 50 °C for 15 minutes. A reaction with no enzyme was set as a control. The progress of the reaction was evaluated by HPLC (Prominence-i LC-2030 C (Shimadzu, Japan) instrument. A C18 (5 µm) (4.6 x 250 mm) HPLC column (Shimadzu, Japan) was used with mobile phase in isocratic mode

comprised of 80: 20 of Methanol:0.75 mM Phosphoric acid. The UV detector was set at 320 nm with a flow rate of 1 ml/min [33].

3.2.7 Kinetic Studies of Coumaphos Degradation by OPAA-FL

The kinetic parameters for OPAA-FL using Coumaphos as the substrate in concentrations of 0.05-7000 µM were carried out at 50 °C, using Bis-Tris Propane pH 8.5. The absorbance readings were calculated at 377 nm wavelength. The fluorescent estimation was done using an excitation wavelength of 377 nm and an Emission Wavelength of 460 nm. The kinetic parameters were calculated according to published literature [34].

3.2.8 To determine the impact of pH and Temperature on the degradation of Coumaphos by OPAA-FL

To determine the temperature optima for OPAA-FL with Coumaphos, the activity was determined at a temperature ranging from 20 °C-70 °C using 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, 5 µL purified OPAA-FL and the reaction volume was made up to 250 µL. For determining the optimum pH for OPAA-FL with Coumaphos, the activity was determined at 50 °C using a pH range from 6-10 in the presence of either 50 mM Sodium Phosphate Buffer (pH- 6, 6.5, 7, 7.5, 8) or 50 mM Bis-Tris Propane buffer (pH 8.5, 9,10), 1 mM $MnCl_2$, 5µL purified OPAA-FL, and the reaction volume was made up to 250 µL.

3.2.9 Immobilization of OPAA-FL variant proteins in Alginate Beads via encapsulation

For the preparation of Beads, 2% (w/v) of Sodium Alginate was used. Purified Buffer Exchanged OPAA-FL enzyme was taken and mixed with an alginate solution. As a crosslinker, 2% (w/v) $CaCl_2$ was taken. The beads were made with the help of a 5 ml syringe. Slowly with the same pressure, the solution from the needle was passed into the flask of $CaCl_2$ kept over a magnetic stirrer. As a control, a batch of beads with no protein was made. After the beads were formed, they were centrifuged at 7000 g. This was by washing with Distilled water until no

protein in the supernatant was observed, all the estimations were done using the Bradford assay.

3.2.10 To determine the impact of pH and Temperature on the activity of Immobilized OPAA-FL as compared to free OPAA-FL against Ethyl Paraoxon

To compare the temperature optima for free and immobilized OPAA-FL with Ethyl Paraoxon, the enzyme activity was determined at a temperature ranging from 20 °C-70 °C using 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, 1 µL of purified OPAA-FL and 5 Enzyme Immobilized Beads respectively and the reaction volume was made up to 250 µL. For checking the optimum pH for free and immobilized OPAA-FL with Ethyl Paraoxon, the activity was determined at 50 °C using a pH range from 6-10 in the presence of either 50 mM Sodium Phosphate Buffer (pH- 6, 6.5, 7, 7.5, 8) or 50 mM BTP (Bis-Tris Propane) buffer (pH 8.5, 9, 10), 1 mM $MnCl_2$, 1 µL of purified OPAA-FL and 5 Enzyme Immobilized Beads respectively and the reaction volume was made up to 250 µL.

3.2.11 Kinetic studies of Immobilized OPAA-FL as compared to free OPAA-FL against Ethyl Paraoxon

At 50 °C and pH 8.5, Bis-Tris Propane, the kinetic parameters for pure and immobilized OPAA-FL utilizing Ethyl Paraoxon as the substrate in concentrations of 0.1-9.0 mM. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. The enzyme kinetic parameters for ethyl paraoxon were calculated according to published literature [34].

3.2.12 Colorimetric Biosensing of Immobilized Enzyme as compared to free OPAA-FL against Ethyl Paraoxon

Colorimetric sensing of Ethyl Paraoxon using OPAA-FL was performed using Free as well as Immobilized OPAA-FL variants. Free Enzyme or sodium alginate Immobilized enzymes were incubated at 50

°C with 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, and substrate concentration in the range of 0.05-2000 µM were used. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. The Limit of Detection and Sensitivity parameters were calculated according to published literature.

3.2.13 Reusability Studies of Immobilized OPAA-FL against Ethyl Paraoxon

To check the efficiency of reusability of our Immobilized protein, a fixed number of beads were taken and incubated at 50 °C with 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, 1.0 mM substrate, and the reaction volume was made up to 250 µL for 15 minutes. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. After each cycle, the beads were washed extensively with water to remove any residual substrate and to prepare the beads for the next cycle. The washing step was done to remove any residual PNP that was left in the beads and was continued till no PNP formation occurred in the supernatant. The efficiency of reusability was determined by calculating the ratio of activity in each cycle to its original activity.

3.2.14 Storage Stability Studies of Immobilized OPAA-FL against Ethyl Paraoxon

The storage stability of immobilized beads was calculated to check their long-term viability. For this, a fixed number of beads were taken and incubated at 50 °C with 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, and 1 mM substrate, and the reaction volume was made up to 250 µL for 15 minutes. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. This reading was taken as Day 0 reading. Then at fixed time intervals, the same number of fresh beads were used, and enzyme activity was calculated using the method described above. The % retained activity was determined by calculating the ratio of

immobilized enzyme activity after storage for a specific time interval to the original activity.

3.2.15 Effect of different Solvents on the activity of Free vs. Immobilized Enzyme

To study the effect of different solvents on the activity of free and immobilized OPAA-FL with Ethyl Paraoxon, the enzyme activity was determined at a temperature of 50 °C using 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, 1 µL of purified OPAA-FL and 5 Enzyme Immobilized Beads respectively and the reaction volume was made up to 250 µL using 1% SDS, 10%Methanol, 10% Ethanol, 10% Hexane, and 20% NaCl.

3.2.16 Degradation studies of pesticide Ethyl Paraoxon contaminated water using Immobilized OPAA-FL and Free OPAA-FL

For performing the preliminary degradation studies of Ethyl Paraoxon using an immobilized enzyme, 0.003, 0.01, and 0.15 Units of Free enzyme and equivalent units of beads were taken. The free and Immobilized enzymes were incubated at 50 °C with 50mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, and substrate concentration 500 µM (137ppm). Following specific time intervals, aliquots were taken, and the amount of PNP formed at 410 nm was measured to calculate the increase in absorbance. The absorbance readings were used to determine the concentration (ppm) of PNP formed using the PNP calibration Curve. % Hydrolysis at different time intervals was calculated as the ratio of ppm of PNP formed to the ppm of Ethyl Paraoxon added or by quantifying the amount of substrate degraded.

Then the degradation pattern was studied in HPLC (Prominence-i LC-2030 C (Shimadzu, Japan)) instrument. A C18 (5 µm) (4.6 x 250 mm) HPLC column (Shimadzu, Japan) was used with mobile phase in isocratic mode comprised of 70: 30 of Methanol: Milli Q water. The UV detector was set at 254 nm with a flow rate of 1 ml/min. A calibration curve was also developed for EP for the quantification of hydrolysis.

3.2.17 Preparation of Calibration Curves for Ethyl Paraoxon and spike Recovery Experiment

Colorimetric Biosensing of ethyl paraoxon was done by using Purified OPAA-FL with 50mM BTP buffer pH 8.5, 1 mM $MnCl_2$. The OPAA-Fl activity was calculated by exposing different concentrations of EP (0.005-1 mM). The reaction mixture (250 µL) was incubated for 15 minutes at 50 °C. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity.

Analytical Parameters of Calibration Curves

Linearity: Linearity is a function of values that can be represented graphically as a straightline. It can be defined as the capability to show "Results that are directly proportional to theconcentration of the analyte in the sample." (Regression Line)

Limit of Detection or LOD: It is described as the smallest amount of analyte in the sample that can be detected, albeit it is not always measured as a precise value.

$$LOD = 3.3 \times \frac{standard\ deviation\ of\ lowest\ point}{slope}$$

Limit of Quantification or LOQ: It is described as the smallest amount of analyte in the sample that can be accurately quantified and measured quantitatively.

$$\mathbf{LOQ = 3*LOD}$$

Sensitivity: It is the slope of the curve and is defined as the minimum detectable response which is generated by the change in concentration

.

Resolution: It is the minimum concentration difference that can be detected by the analytical method.

$$\boldsymbol{Resolution = \frac{standard\ deviation\ of\ lowest\ point}{slope}}$$

3.2.18 Spike and Recovery Studies

Spike and recovery studies are done to check the accuracy of the calibration curve. For this purpose, a known concentration of Ethyl Paraoxon is added to the enzymatic reaction, and absorbance is measured. The observed absorbance is used to predict the concentration of Ethyl Paraoxon using the calibration curve. The predicted concentration is then compared with the actual concentration, and recovery is checked. The recovery percentage is indicative of the accuracy and error of the calibration curve. From the stock of Ethyl Paraoxon, dilutions of 0.05 mM, 0.2 mM, 0.5 mM, 0.9 mM, and 1 mM were prepared. A 250 µL sample for every concentration was prepared in tap water taken from Simrol, Madhya Pradesh, India. The reaction was incubated at 50 °C for 15 minutes. The data was then observed for calculating % recovery.

3.2.19 Decontamination Studies on Textiles

For decontamination studies, cotton clothes were cut into squares (2 X 2 cm^2) and were autoclaved. This cloth was then contaminated with 250 µmol ethyl paraoxon. 50 µg of the enzyme was dissolved in 10 ml of different solutions: Buffer, 1% SDS, 20% NaCl, Tap Water, and 0.5% Soap Solution. After 20 minutes, the pesticide-contaminated cloth was incubated in each of the above-mentioned solutions at 50 °C. Aliquots of each solution were taken at desired time intervals to check the production of PNP at 410 nm. A control sample for each solution was prepared in which no enzyme was added to check paraoxon self-hydrolysis [35].

Chapter 4

Determination of Enzymatic Degradation of OP Pesticides using recombinant Organophosphorus hydrolyzing enzymes OPAA-FL and Nus-OPH

4.1. Introduction

Pesticides are any substance that is used to control any form of pests, unwanted weeds, and plant infections. With increasing urbanization, there is a high load on the agricultural sector which further increases the use of pesticides. Increasing use of pesticide is a major health concern as it is not only harmful to the environment but also cause serious health complications in human beings. They are majorly divided into four classes- carbamates, organophosphates, organochlorines, and pyrethroids. OP Pesticides are the esters of phosphoric acid and its derivatives. They have a central Phosphorus (P) atom with a P=S or P=O bond, R1 and R2 are two side groups that are generally alkoxy (OCH3 or OC2H5) and a leaving group (X). This leaving group is substituted nucleophilically by the oxygen of serine esterase enzymes. Examples of commonly used Organophosphate pesticides are Paraoxon, Parathion, Malathion, Dimethoate, chlorpyrifos, and Coumaphos. The typical OP Poisoning is caused by the production of toxic Oxon metabolites which leads to neuropathy in humans. These metabolites act by phosphorylating target proteins like acetylcholinesterase (AChE) and neuropathy target esterase (NTE) [10]. They can also act by binding to the nicotinic or muscarinic receptors in the Nervous System. Our nerves release a chemical called acetylcholine, which is important in signal transmission; contraction of muscles. Acetylcholine Esterase (AChE) breaks down ACh and results in muscle relaxation. OP pesticide acts by irreversibly inhibiting the action of AChE by phosphorylating them, which results in the accumulation of ACh, leading to neurodegenerative diseases [11]. Organophosphate hydrolyzing enzymes are enzymes that can hydrolyze the OP compounds to become nontoxic agents. There are reports suggestive of many OPS hydrolyzing enzymes from various

organisms, including mammals, but the most potent and efficient are the ones isolated from microbes. Some of the examples of OP hydrolyzing enzymes that are of microbial origin are Organophosphorus hydrolase (OPH), Organophosphorus acid hydrolase (OPAA), Phosphotriesterase-like lactonases (PLL), Methyl Parathion hydrolase (MPH), SsoPox.

In this study, a variant OPAA-FL and Nus-OPH were expressed in *E. coli* Rosetta (DE3) and purified to study the enzymatic degradation of OP Pesticides. These were further used to check the enzymatic degradation of different OP Pesticides. Another substrate -Coumaphos, was identified, which could be degraded by the expressed recombinant enzymes. Coumaphos is an organophosphate insecticide used to control a wide range of pests, including insects, mites, and ticks, in agricultural and residential settings. It is commonly used in farming to control pests on crops such as corn, cotton, and fruit trees. However, it is a highly toxic substance that can negatively affect humans and the environment. It is classified as a Group D carcinogen by the US Environmental Protection Agency (EPA), meaning it is not classifiable as human carcinogenicity based on insufficient evidence. Coumaphos can cause various health problems, including headaches, nausea, dizziness, and respiratory problems. Long-term exposure to coumaphos can lead to more severe health effects, such as neurological disorders and cancer. The study further involves studying the kinetic parameters for the degradation of Coumaphos by OPAA-FL.

4.2 Results and Discussion

4.2.1 Induction and Expression of Recombinant Protein: OPAA-FL and Nus-OPH

E. coli Rosetta (DE3) cells were transformed with OPAA-FL and Nus-OPH variant expression vector. The cells were induced with 1mM IPTG for OPAA-FL and 0.1 mM IPTG for Nus-OPH. 1 mM $MnCl_2$ and 0.1 mM $CoCl_2$ were added as co-factor for OPAA-FL and Nus-OPH, respectively. The induced cells were harvested and lysed using 50 mM Tris pH 8.0, 500 mM NaCl, and 10% glycerol for both OPAA-FL and Nus-OPH. Separation of supernatant and pellet fraction was done from

the cell lysates in order to check the amount of protein present in the soluble fraction. The results of the expression of OPAA-FL and Nus-OPH are shown in **Fig. 4.1.** Much of the expression of the 50 kDa OPAA-FL protein was found in the soluble fraction (**Fig. 4.1 (A),** lane 5). Similarly, the 93 kDa protein Nus-OPH is also expressed in the soluble fraction (**Fig. 4.1**, **(B)** lane 4).

4.2.2 Purification of Recombinant Protein: OPAA-FL and Nus-OPH

Since both OPAA-FL and Nus-OPH contain N-terminal His-tag, affinity purification was performed using Ni-NTA resin. The pre-equilibrated column was loaded with an input sample prepared in an equilibration buffer containing 50 mM Tris-Cl pH 8.0, 500 mM NaCl, and 10 mM Imidazole for both OPAA-FL and Nus-OPH. Subsequently, the column was washed with wash buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, and 50 mM imidazole for OPAA-FL and 50 mM and 90 mM imidazole for Nus-OPH. The samples were eluted using elution buffer comprising of 50 mM tris pH 8.0, 500 mM NaCl, 150 mM, and 300 mM imidazole for both OPAA-FL and Nus-OPH. The samples were collected and analyzed using SDS-PAGE. **Fig. 4.2** shows that both OPAA-FL and Nus-OPH were purified with more than 95% purity.

4.2.3 Activity Assay for OPAA-FL and Nus-OPH against Ethyl Paraoxon

To determine whether the purified enzyme is active or not, both OPAA-FL and Nus-OPH variant enzyme assay was performed with Ethyl Paraoxon. Ethyl Paraoxon in the presence of enzyme hydrolyzes to form p-Nitrophenol and Diethyl phosphoric acid, as shown in **Fig. 4.3** PNP is a yellow color compound and is used for colorimetric estimation at 410 nm. To perform the assay, 1 µL of purified, buffer exchanged and concentrated protein, 1 mM ethyl paraoxon, 1 mM $MnCl_2$, 50 mM Bis-Tris propane buffer, pH 8.5, was added, and the final volume was made

up to 250 µL with SDW. Incubation was done for 15 minutes at 50 °C for 15 minutes For Nus-OPH 1 µL of purified, buffer exchanged and concentrated protein, 50mM CHES Buffer pH 9.0, 0.1 mM $CoCl_2$, 1 mM Ethyl Paraoxon were added, and the reaction volume was made up to 200 µL using SDW and incubated at 37 °C for 5 minutes. A control reaction was setup with similar constituents but no enzyme. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. UV-Vis spectrum is shown in **Fig. 4.4**. Specific activity was calculated as U/mg (micromoles of the substrate hydrolyzed per milligram of protein).

Specific activities of OPAA-FL and Nus-OPH against Ethyl Paraoxon are mentioned in **Fig 4.5**. High activity in induced cell lysate was observed as compared to the uninduced. The purified Buffer-Exchanged and concentrated protein showed the highest activity against EP for both proteins. For OPAA-FL, the specific activity in uninduced cell fraction was 0.04 U/mg. In the induced fraction, it was 1.04 U/mg, while for purified protein, it was 5.20 U/mg. For Nus-OPH, the specific activity in uninduced cell fraction was 1.725 U/mg. In the induced fraction, it was 14.54 U/mg, while for purified protein, it was 75.37 U/mg.

4.2.4. Activity of OPAA-FL against Coumaphos

To check whether the purified enzymes are enzymatically active for other OP compounds or not, an assay of OPAA-FL and Nus-OPH against Coumaphos was done. Coumaphos is an organophosphate pesticide that is used to control a variety of pests, such as insects, mites, and ticks, in crops, livestock, and poultry. Coumaphos is a highly toxic substance and can pose significant risks to human health and the environment. Exposure to this pesticide can cause a range of health problems, and in severe cases, it can lead to seizures, coma, and even death. Coumaphos degrades into fluorescent by-products, Chlorferon and DETP.

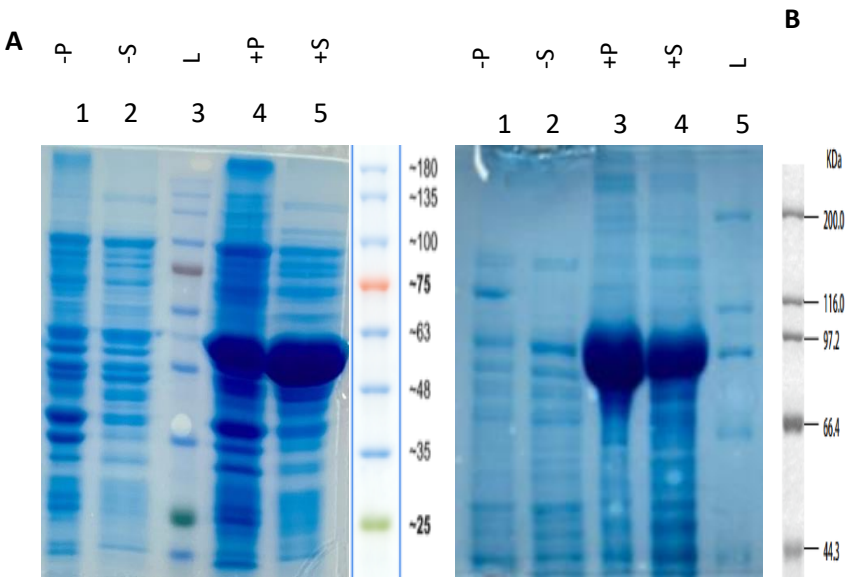


Figure 4.1. Induction and Expression of OPAA-FL and Nus-OPH: A) The induction of OPAA-FL was done at 16 °C for 16 hours by 1 mM IPTG; Lane 1- Uninduced Pellet, Lane 2- Uninduced Supernatant, Lane 3-Protein Marker, Lane 4- Induced Pellet, Lane 5- Induced Supernatant. B) The induction of Nus-OPH was done at 20 °C for 20 hours by 0.1 mM IPTG; Lane 1- Uninduced Pellet, Lane 2- Uninduced Supernatant, Lane 3- Induced Pellet, Lane 4- Induced Supernatant, Lane 5- Protein Marker.

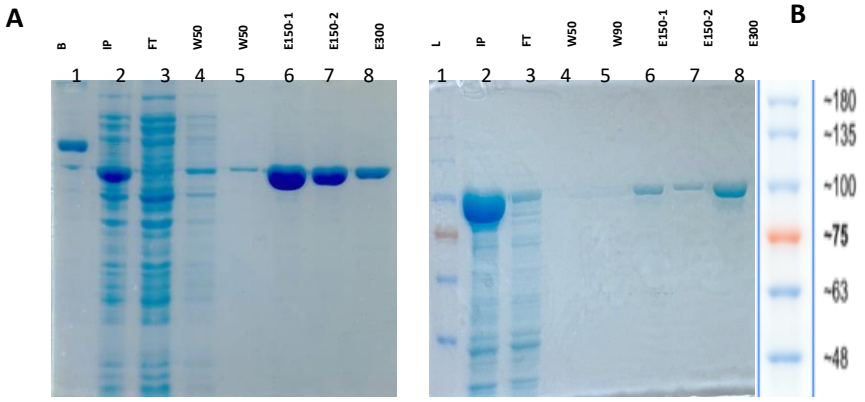


Figure 4.2. Purification of OPAA-FL and Nus-OPH: A) The purification of OPAA-FL was done by Ni-NTA Beads, Lane 1- BSA, Lane 2- Input Sample, Lane 3- Flow Through, Lane 4- Wash 1(50 mM Imidazole), Lane 5- Wash 2 (50 mM Imidazole), Lane 6- Elution 1 (150 mM Imidazole), Lane 7- Elution 2 (150 mM Imidazole), Lane 8- Elution 3 (300 mM Imidazole). B) The purification of Nus-OPH was done by Ni-NTA Beads, Lane 1-Protein Marker, Lane 2- Input Sample, Lane 3- Flow Through, Lane 4- Wash 1(50 mM Imidazole), Lane 5- Wash 2 (90 mM Imidazole), Lane 6- Elution 1 (150 mM Imidazole), Lane 7- Elution 2 (150 mM Imidazole), Lane 8-Elution 3 (300 mM Imidazole).

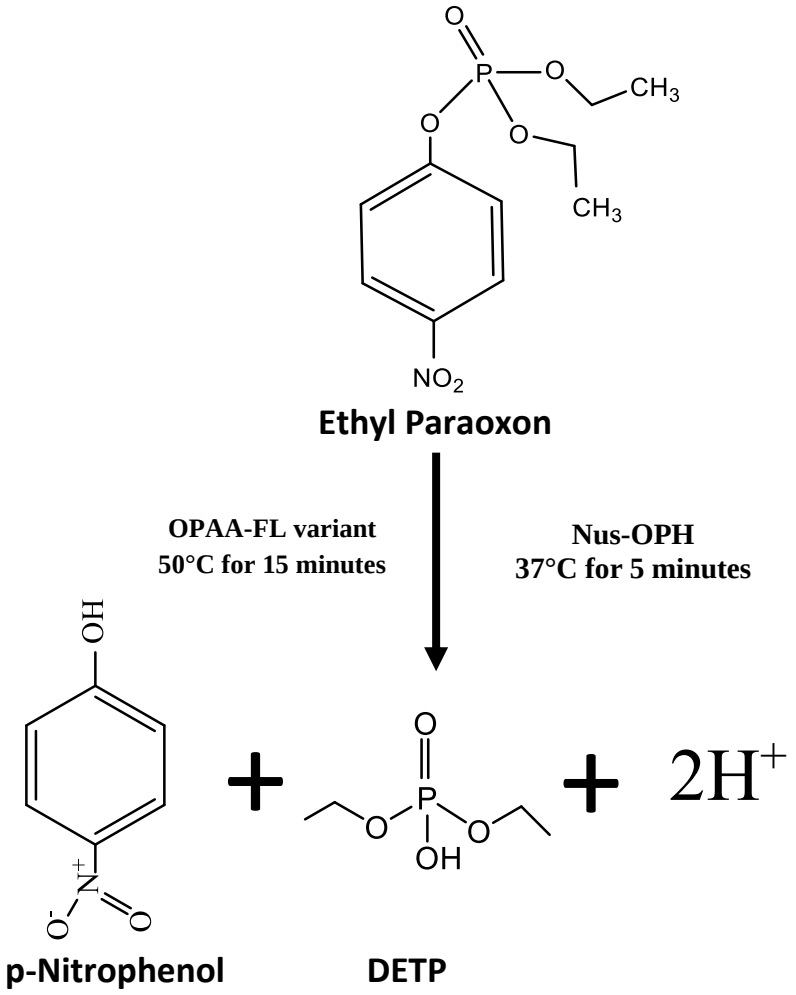


Figure 4.3. The hydrolysis reaction of OP compound Ethyl Paraoxon leads to the formation of p-Nitrophenol and DETP, important for colorimetric and fluorometric estimation, respectively.

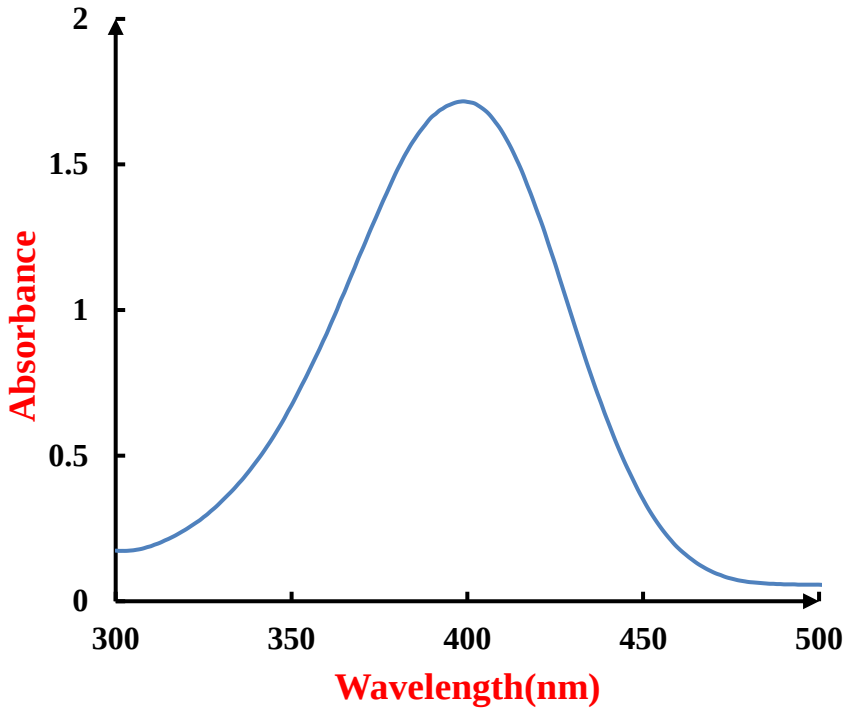


Figure 4.4. The UV-Vis spectrum of p-nitrophenol at 410 nm.

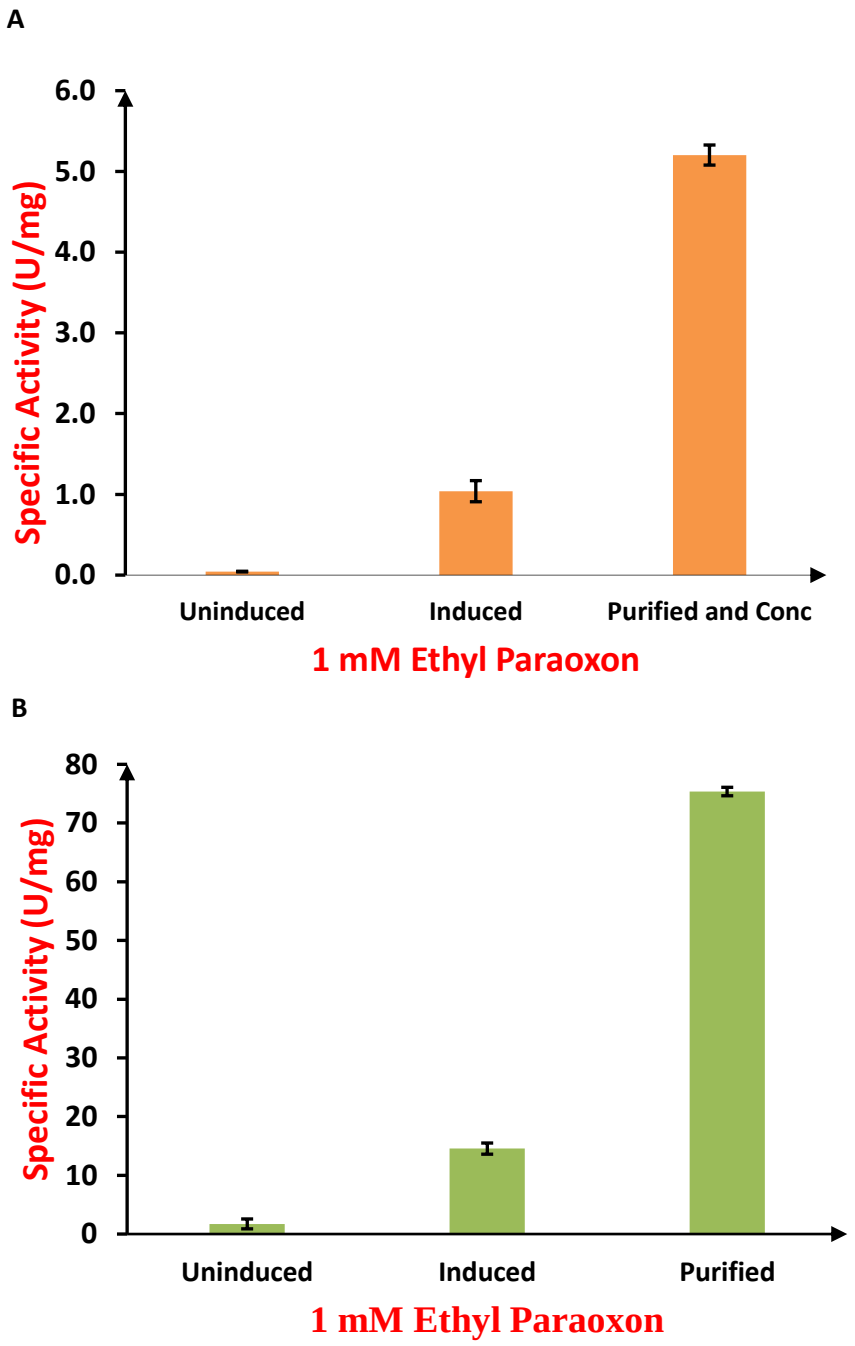


Figure 4.5. Colorimetric activity assay of OPAA-FL and Nus-OPH against Ethyl Paraoxon. A) OPAA-FL and B) Nus-OPH with 1mM substrate; Ethyl Paraoxon. The error bars show the standard deviation, and the results are the average of two identical samples.

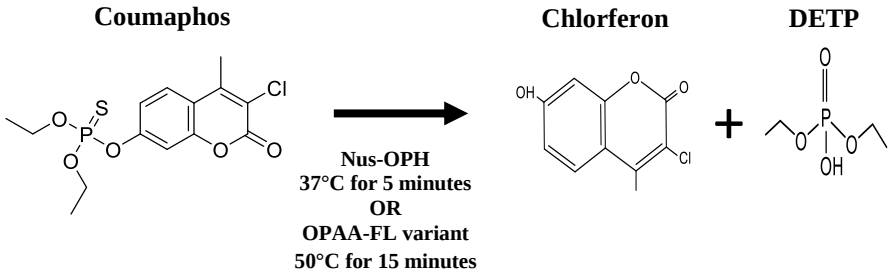


Figure 4.6. Hydrolysis Reaction of Coumaphos. Upon treatment with OP hydrolyzing enzymes, Coumaphos degrades to form a fluorescent compound chlorferon.

The first enzyme chosen for studying the degradation of Coumaphos was OPAA-FL. To determine whether degraded product chlorferon is formed or not, the excitation and emission spectra were determined as shown in **Fig. 4.7** by using 1 µL of purified, buffer exchanged and concentrated enzyme OPAA-FL, 50 mM BTP, 0.2 mM Substrate,1 mM $MnCl_2$, and SDW. The reaction mixture was incubated for 15 minutes at 50 °C. Now to determine the enzyme activity of OPAA-FL and Nus-OPH against Coumaphos, 1 µL of purified buffer exchanged, and the concentrated enzyme was added to the reaction mixture containing 50 mM BTP, 0.2 mM Substrate,1 mM $MnCl_2$, and SDW. The reaction mixture was incubated at 50 °C for 15 minutes for OPAA-FL, and 1 µL of purified, buffer exchanged, and concentrated protein, 50 mM CHES Buffer pH 9.0, 0.1 mM $CoCl_2$, 0.2 mM substrate were added, and the reaction volume was made up to 200 µL using SDW and incubated at 37 °C for 5 minutes. Enzyme activity was calculated by measuring the absorbance and fluorescence of Chlorferon at excitation 377 nm and emission wavelength 470 nm, as shown in **Fig. 4.8** The purified protein shows exceptionally high RFU at emission wavelength 470 nm, excited at 377 nm. This was indicative of Coumaphos degradation.

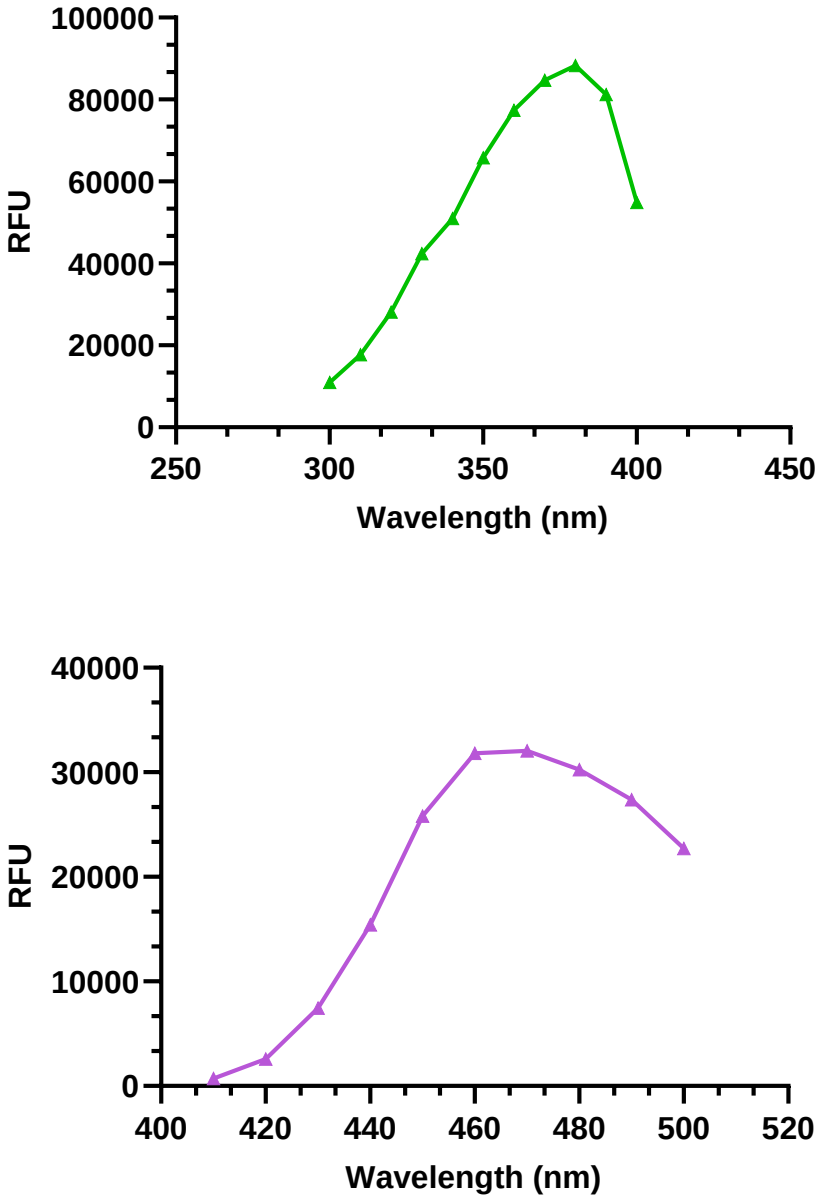


Figure 4.7 Excitation and Emission spectra for the Coumaphos degraded product. The excitation wavelength was found to be 377 nm and the emission wavelength was found to be 470 nm. These wavelengths were like what is mentioned in literature indicating that Coumaphos degradation by OPAA-FL and Nus-OPH is resulting in the formation of chlorferon.

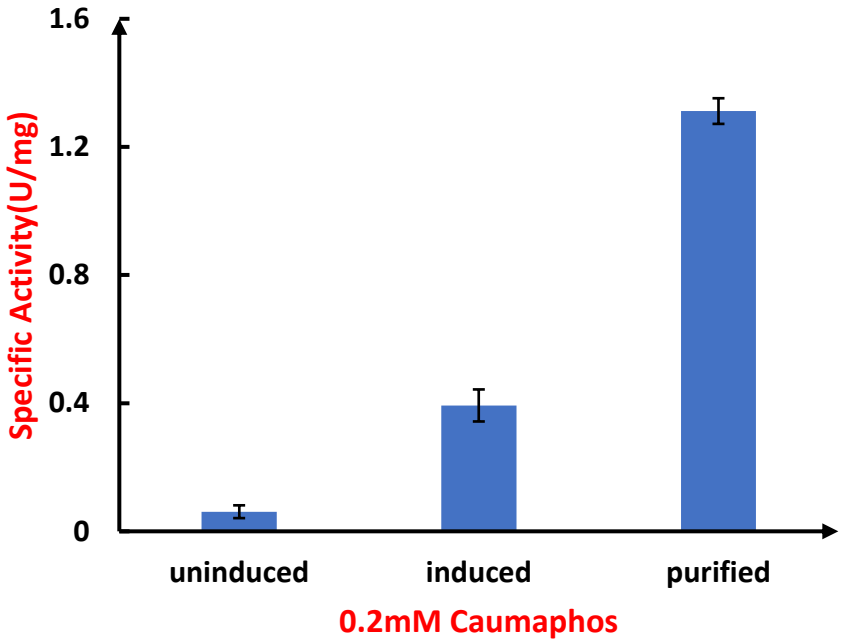


Figure 4.8. Activity assay of OPAA-FL against Coumaphos. OPAA-FL. The error bars show the standard deviation, and the results are the average of two identical samples.

4.2.5 Thin Layer Chromatography for analysis of the degraded product of Coumaphos

To confirm Coumaphos degradation by OPAA-FL and Nus-OPH, a few confirmatory tests were performed. The first confirmatory experiment performed was Thin Layer Chromatography, where a mixture of components can be separated between a stationary phase and a liquid mobile phase. The aim was to check the production of the fluorescent compound chlorferon upon excitation of a TLC plate at far UV wavelength that is similar to the excitation wavelength of Chlorferon. **Fig. 4.9** depicts the result of the TLC experiment performed using Mobile Phase: Benzene: Ethyl Acetate (70:30). Lane 1, 2, 4, 5, 7, and 8 in **Fig. 4.9** A shows a spot that is indicative of substrate Coumaphos since it is absent in lane 3and 6 of the same as they contain only enzyme. An intense fluorescent spot was seen in lanes 4 and 8 in **Fig. 4.9 B** when excited at far UV, indicative of chlorferon, as these spots are the result of enzyme reaction performed for the hydrolysis of Coumaphos by OPAA-FL and Nus-OPH.

4.2.6. HPLC analysis for Enzymatic Degradation of Coumaphos by OPAA-FL

HPLC (Prominence-i LC-2030 C (Shimadzu, Japan)) instrument was used in the detection of Coumaphos degradation. Using a C18 column and a mobile phase comprised 80: 20 of Methanol:0.75mM Phosphoric acid. The UV detector was set at 320nm with a flow rate of 1.0 ml/min. Retention time (RT) of standard Coumaphos was found at 6.6 minutes **Fig. 4.10(A)** and 3.4 minutes for Standard product Chlorferon **Fig. 4.10(B)** with 320nm detection. **Fig. 4.10(C)** shows an enzymatic reaction. The control reaction has a peak at RT 6.6, indicative of the presence of substrate, while the experimental reaction, which has an enzyme, shows a peak at RT 3.4, suggestive of product formation and hence degradation of coumaphos.

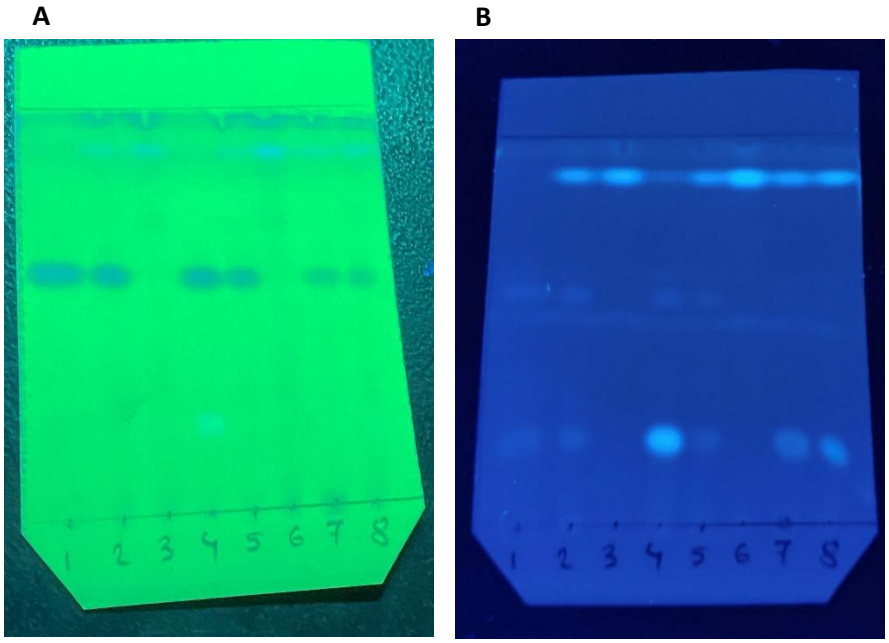


Figure 4.9. TLC for the activity of OPAA-FL and Nus-OPH against Coumaphos depicting the formation of the product after enzymatic activity at (A): UV and (B) Far UV {Mobile Phase: Benzene: Ethyl Acetate (70:30)}, lane 1: Standard Compound, lane 2: Control 1 (Only Substrate), lane 3: Control 2 (Only Nus-OPH), lane 4: Experimental (Nus-OPH+ Substrate), lane 5: Experimental (Nus-OPH), lane 6: Control 3 (only OPAA-FL), lane 7: Experimental (OPAA-FL+ Substrate), lane 8: Experimental (OPAA-FL+Substrate.

4.2.7. Kinetic Studies of Coumaphos Degradation by OPAA-FL

Now, to check whether the degradation of Coumaphos by OPAA-FL follows Michelis-Menten Kinetics or not. The degradation was determined at substrate concentrations 0.05-3000 µM. The reaction was carried out in the presence of 50mM BTP at 50 °C. **Fig. 4.11** and **4.12** shows the characteristic curve of enzyme kinetics. It was observed that the reaction reached saturation at around 200 µM Substrate concentration. This might be due to the precipitation of substrate at higher concentrations. Hence all the further experiments were performed using 0.2 mM or 200 µM substrate.

4.2.8. To determine the impact of pH and Temperature on the Degradation of Coumaphos by OPAA-FL

The study observed slight activity with Coumaphos as the substrate, and the effect of pH and Temperature was determined. To determine the temperature optima for OPAA-FL with Coumaphos, the activity was determined at a temperature ranging from 20 °C-70 °C using 50 mM Bis-Tris Propane buffer pH 8.5, 1 mM $MnCl_2$, 5 µL purified OPAA-FL, and the reaction volume was made up to 250 µL. The %Intensity increased gradually till 50 °C and then decreased; thus, the experiment indicated that 50 °C was the optimum temperature to conduct enzyme activity assay of Coumaphos with OPAA-FL **(Fig. 4.13 A).** The optimum pH determination for OPAA-FL with Coumaphos was done using a pH range from 6-10 in the presence of either 50 mM Sodium Phosphate Buffer (pH- 6, 6.5, 7, 7.5, 8) or 50 mM Bis-Tris Propane buffer (pH 8.5, 9,10), 1 mM MnCl2, 5 µL purified OPAA-FL, and the reaction volume was made upto 250 µL. This was incubated at 50 °C for 15 minutes. The experiment suggested optimum pH for the activity of Coumaphos with OPAA-FL was 8.5. **(Fig. 4.13 B)**

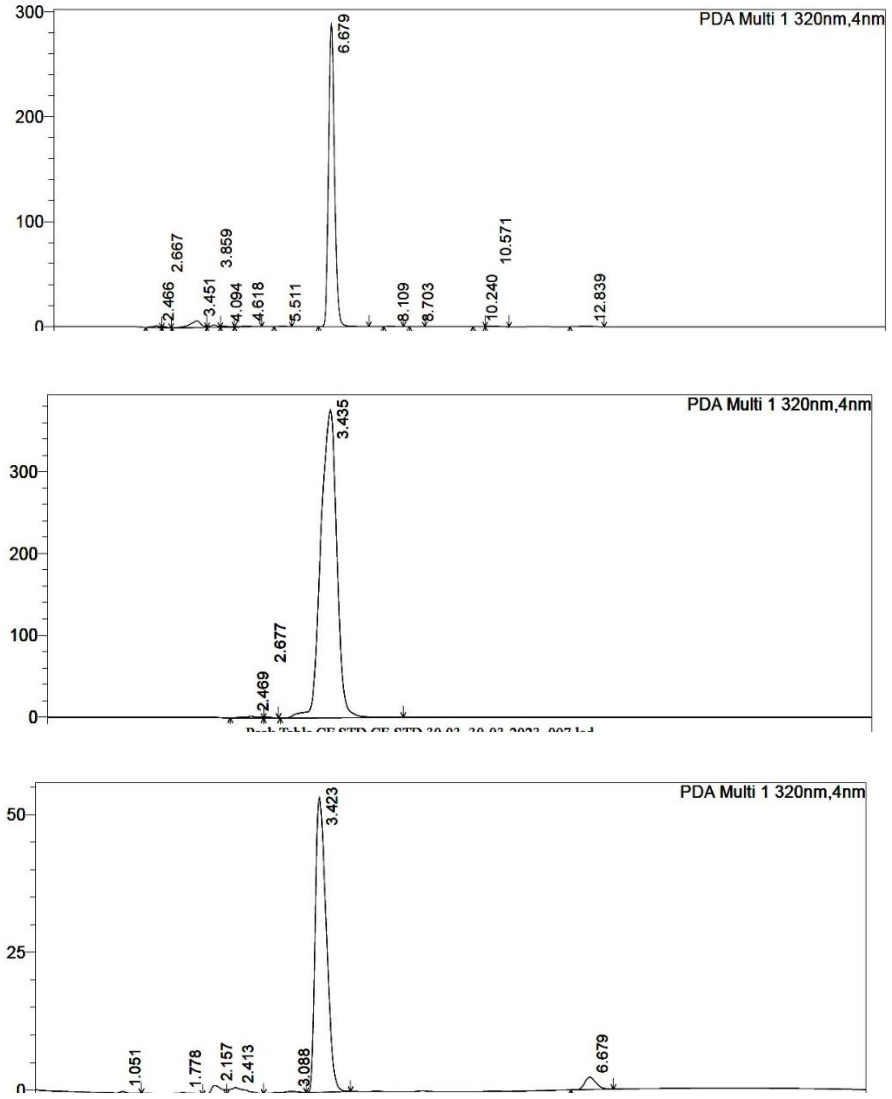


Fig. 4.10 HPLC Results for Reaction of coumaphos degradation using OPAA-FL (A) HPLC Retention time 6.6 minutes for standard substrate Coumaphos. **(B)** HPLC Retention time 3.4 minutes for standard product Chlorferon. **(C)** Enzymatic degradation of coumaphos using OPAA-FL showing the production of the product (Rt-3.4 minutes).

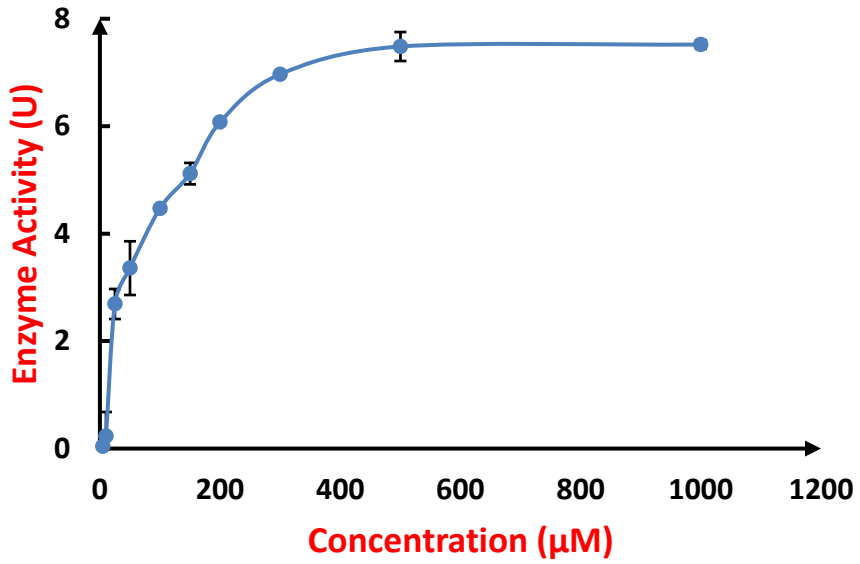


Fig. 4.11. The Michaelis-Menten graph at different concentrations of Substrate: Coumaphos, showing attainment of saturation at around 0.2 mM coumaphos concentration.

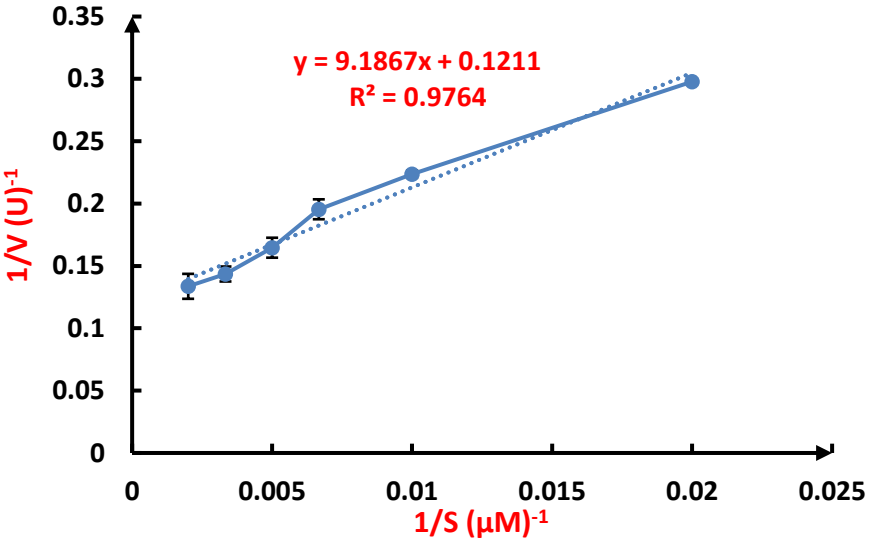


Fig. 4.12. Lineweaver Burk plot for OPAA-FL Variant against Ethyl Paraoxon. The error bars show the standard deviation, and the results are the average of two identical samples.

Table 4.1. Kinetic Parameters for OPAA-FL with Coumaphos

Sample	Coumaphos (μM)	V_{max} (U)	K_m (μM)	K_{cat} $(min)^{-1}$	K_{cat}/K_m $(mM^{-1}$ $min^{-1})$
Purified OPAA-FL	50-500	8.26	75.81	$1 * 10^4$	145.2

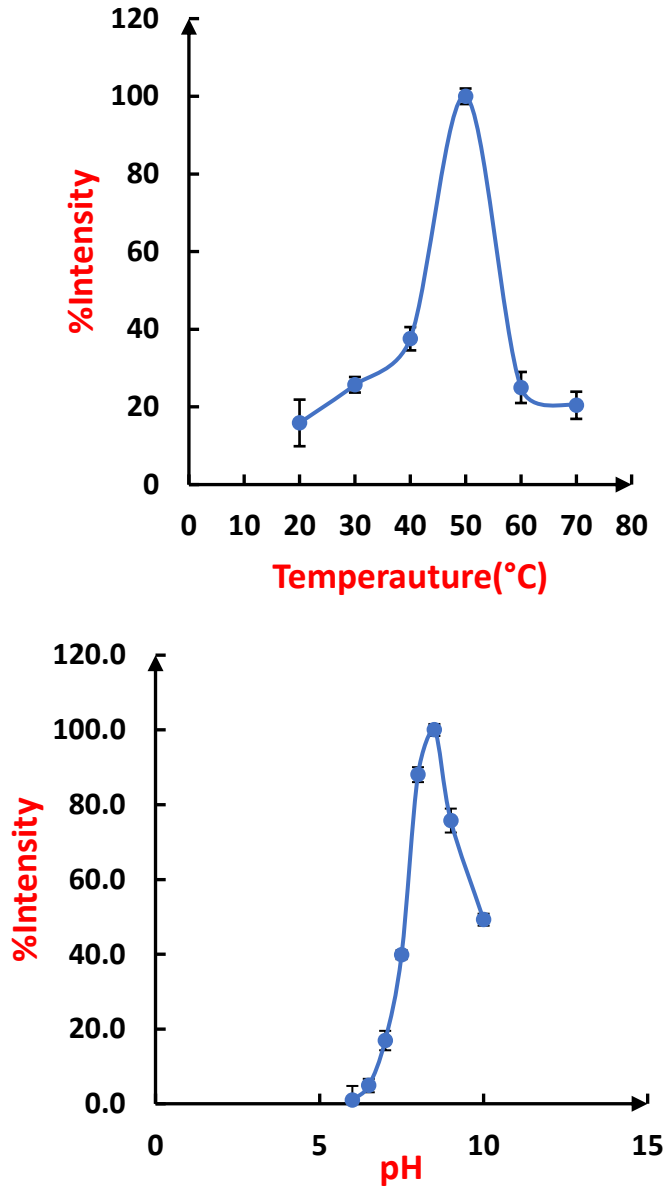


Figure 4.13. Impact of temperature and Ph on the OPAA-FL activity against Coumaphos. (A) Effect of Temperature on the activity of OPAA-FL with Coumaphos. (B) Effect of pH on the activity of OPAA-FL with Coumaphos. The error bars show the standard deviation, and the results are the average of two identical samples.

4.3 Summary

Enzymatic degradation of pesticides is a promising approach for the bioremediation of contaminated soils and water. Enzymes can break down the chemical bonds of pesticides, converting them into less toxic and more biodegradable compounds. Organophosphate-degrading enzymes, such as organophosphate hydrolase and paraoxonase, degrade organophosphate pesticides are one of the examples. These enzymes can hydrolyze the ester bonds of organophosphate compounds, converting them into non-toxic products. Enzymatic degradation of pesticides offers several advantages over traditional remediation methods, such as being cost-effective, eco-friendly, and less disruptive to the environment. However, there are still challenges that need to be addressed, such as the optimization of enzyme activity and stability under different environmental conditions and the need for efficient delivery methods to ensure the proper distribution of enzymes in contaminated sites. This study is the first report on the degradation of OP-Pesticide Coumaphos using the enzyme OPAA-FL, validated using different experiments. The study further shows the optimum temperature and pH conditions for the enzyme activity for Coumaphos degradation.

Chapter 5

Development of Bioremediation Strategies of Organophosphorus Pesticides using Enzyme Immobilization

5.1 Introduction

Pesticides are chemical compounds used to kill, repel, or control pests. With increasing urbanization and a growing population, the use of pesticides is rising immensely. Reports suggest about 3 million tons of global usage of pesticides annually [30]. Of various classes of pesticides, organophosphate (OP) pesticides are commonly used and pose a risk to human life. Organophosphorus (OP) pesticide poisoning is a significant public health issue, with over 200,000 fatalities and several million nonfatal instances happening annually. Reports show that pesticides are the leading cause of poisoning in India, accounting for one-third of pesticide poisoning cases worldwide [13]. OP-Pesticides like Ethyl Paraoxon, Coumaphos, Malathion, Methyl Parathion, Chlorpyrifos, and Dimethoate are commonly used as herbicides. Chemical warfare agents (CWFAs) such as sarin, soman, tabun, and VX are also OP compounds. Literature has revealed that many nations and terrorist organizations have stored about 2,00,000 tons of these CWFAs.

OP pesticide poisoning occurs by inhibiting an enzyme called Acetylcholine Esterase (AChE) by phosphorylating it. AChE is a crucial enzyme in our nervous system and is responsible for the breakdown of acetylcholine. The irreversible inhibition of AChE by OP Pesticides leads to acetylcholine accumulation and neurological complications.

Not only are pesticides harmful to human life, but they also have dire environmental consequences. The pesticide movement in the environment can occur by air, aerosols, natural water bodies, soil or plants, and the food we eat. Pesticide pollution is a significant problem in India. Pesticide pollution is causing the contamination of soil and water. Moreover, organophosphate pesticides are hard to degrade, resulting in their accumulation in the environment [36]. Pesticides in

water resources occur due to run-offs from agricultural lands or industrial wastes [37]. Waste from the pesticide industry pollutes water resources as hazardous waste is released without treatment. Some reports suggest that the incidences of neurodegenerative diseases like Parkinson's disease increase with the consumption of water contaminated with common OP pesticides like chlorpyrifos, diazinon, and dimethoate. The conventional physical and chemical degradation methods are inappropriate to apply to sizeable, contaminated water bodies as they incur high operational costs and lead to secondary contamination. Enzymatic Bioremediation is flourishing as it is specific, and the by-products are harmless.

But bioremediation using soluble enzymes has many disadvantages example, instability, one-time usage, and sensitivity to non-optimal conditions. To overcome the stated problems, the enzymes are generally immobilized onto solid support [38]. Immobilization of enzymes provides many benefits, like reusability, stability in organic solvents and at higher temperatures, reduced cost of downstream processing, and easy separation of biocatalysts. Immobilized enzymes also have an increased tolerance to toxic compounds [39].

Immobilization of enzymes is done on various matrices or support, which could be Natural polymer, synthetic polymer, or inorganic molecules. One of the most common methods of immobilization is Calcium-Alginate cross-linking. Alginate is a natural polymer isolated from brown algae; it consists of linear copolymers of β-(1–4) linked d-mannuronic acid and β-(1–4)-linked l-guluronic acid units. Alginate is a preferred matrix for enzyme immobilization because it is elementary, economical, mild, and does not have toxic effects on the cells. The possible disadvantage of alginate is its low mechanical strength and susceptibility to biodegradation.

The objective of the current study is to immobilize OPAA-FL in alginate beads to maintain its activity for more extended periods and use it for performing enzymatic Bioremediation for the detoxification of pesticide-contaminated water.

5.2 Results and Discussions

5.2.1 Immobilization of OPAA-FL-variant proteins in Alginate Beads via Encapsulation

Proteins are encapsulated to retain activity for an extended period and assure stability. OPAA-FL variant was encapsulated using $CaCl_2$ as the cross-linker **Fig. 5.1(A)**. Alginate was chosen as the matrix for encapsulation because of its biocompatibility, ease of use, and simple chemistry. Enzyme Loaded beads were formed from 2% Alginate. After 3-4 cycles of washes, the beads were used for characterization experiments. The immobilization efficiency of protein-loaded beads was found to be 80-85% higher than the earlier reports of OPAA-FL encapsulation in alginate. The efficiency was calculated via Gel estimation **Fig. 5.1(B)** and Bradford analysis. All the concentrations were validated via ImageJ software.

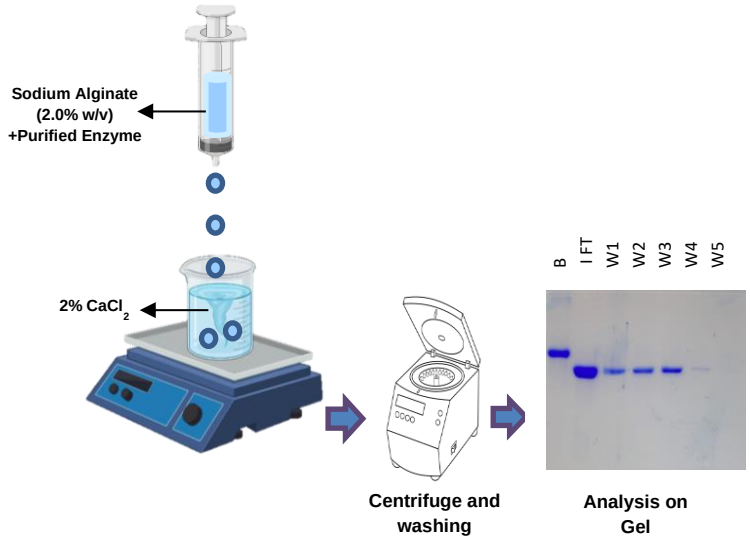


Figure 5.1. Immobilization of OPAA-FL Variant into alginate Beads via Encapsulation. (A) schematic representation of the procedure of formation of alginate beads for encapsulation of OPAA-FL. (B) Gel Estimation of the amount of protein loaded to find the immobilization efficiency, B- BSA, I- Input, FT- Flow through, W1,2,3,4,5- Wash 1,2,3,4,5.

5.2.2 Impact of pH and Temperature on the activity of Immobilized OPAA-FL as compared to free OPAA-FL against Ethyl Paraoxon

To determine whether the matrix affects the optimum pH and temperature of the protein, a determination of the impact of pH and temperature on the activity of OPAA-FL was made. To compare the optimum temperature for free and immobilized OPAA-FL with Ethyl Paraoxon, the activity was determined at a temperature ranging from 20 °C-70 °C in the presence of 1 mM $MnCl_2$, 1 µLof purified OPAA-FL, 50 mM Bis-Tris Propane buffer pH 8.5, and 5 Enzyme Immobilized Beads respectively and the reaction volume was made up to 250 µL. The activity increased gradually until 50 °C and then decreased for free and immobilized enzymes. The result of the experiment, as shown in **Fig. 5.2(A),** intimated that 50 °C is the optimum temperature for both free and encapsulated OPAA-FL with Ethyl Paraoxon.

To know the optimum pH for free and immobilized OPAA-FL with Ethyl Paraoxon, the activity was determined at 50 °C using a pH range from 6-10 in the presence of either 50 mM Sodium Phosphate Buffer (pH- 6, 6.5, 7, 7.5, 8) or 50 mM Bis-Tris Propane buffer (pH 8.5, 9, 10), 1 mM $MnCl_2$, 1 µLof purified OPAA-FL and 5 Enzyme Immobilized Beads respectively and the reaction volume was made up to 250 µL. The optimum activity was observed at Ph 8.5 for both free and encapsulated OPAA-FL, as shown in **Fig. 5.2. (B).**

5.2.3. Kinetic studies of Immobilized OPAA-FL as compared to free OPAA-FL against Ethyl Paraoxon

After checking the optimum pH and temperature for the encapsulated protein, it was essential to check whether the encapsulated Protein follows Michaelis-Menten kinetics or not. So, the kinetic parameters were calculated using Ethyl Paraoxon as substrate at a concentration of 0.1-9 mM and are reported in **Fig. 5.3** and **Table 5.1**. The kinetic parameters were calculated according to the published literature [34].

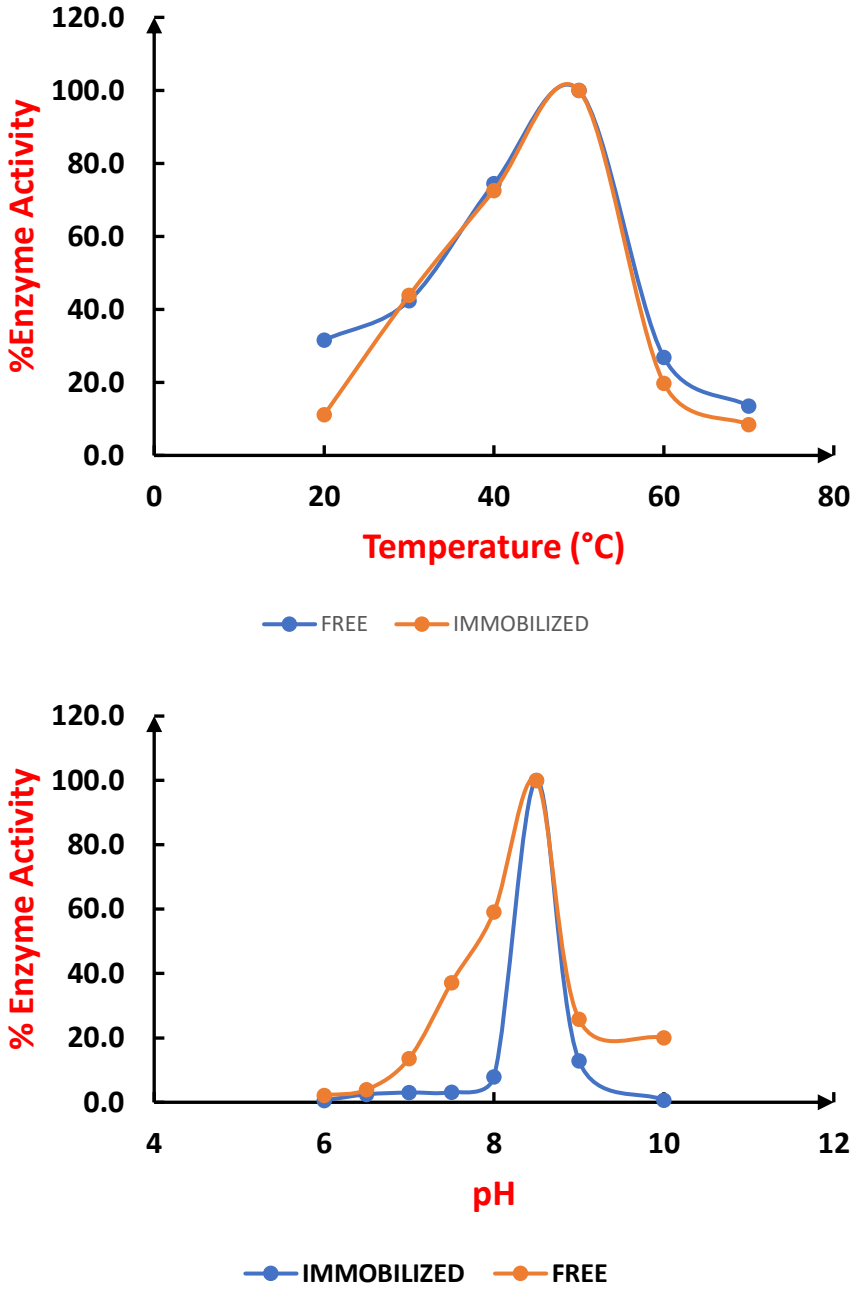


Figure 5.2. Impact of temperature and pH on the free and Encapsulated OPAA-FL activity against Ethyl Paraoxon. (A) Effect of Temperature on Activity of Free and Encapsulated OPAA-FL with Ethyl Paraoxon. (B) Effect of pH on the activity of free and Encapsulated OPAA-FL with Ethyl Paraoxon. The error bars show the standard deviation, and the results are the average of two identical samples.

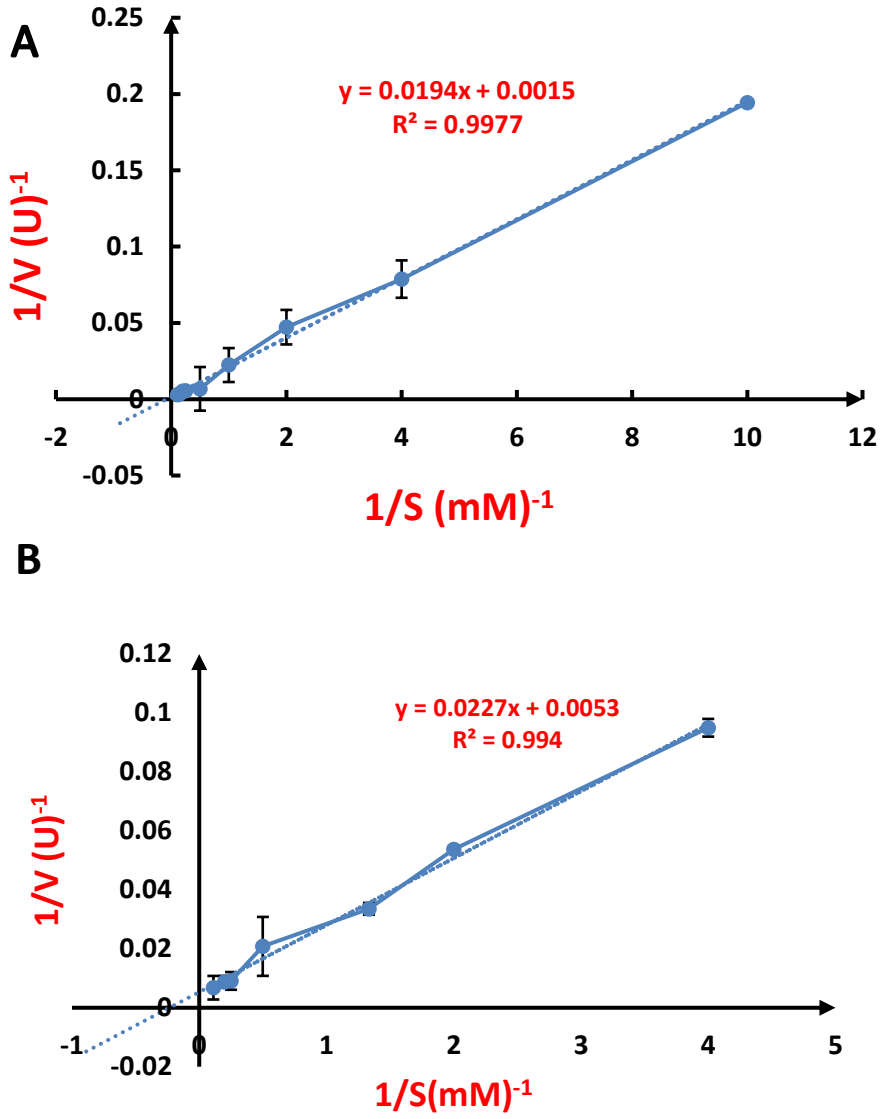


Figure 5.3. Lineweaver Burk plot for OPAA-FL Variant against Ethyl Paraoxon. (A) For Encapsulated Enzyme (B) For Purified Enzyme. The error bars show the standard deviation, and the results are the average of two identical samples.

Table 5.1. Kinetic Parameters for Free and Encapsulated OPAA-FL with Ethyl Paraoxon

Sample	Ethyl Paraoxon (mM)	V_{max} (U)	K_m (mM)	K_{cat} $(min)^{-1}$	K_{cat}/K_m $(mM^{-1} min^{-1})$
Purified OPAA-FL	0.1 to 9	188.68	4.28	$2 * 10^6$	$4 * 10^5$
Encapsulated OPAA-FL	0.1 to 9	666.67	12.93	$7 * 10^6$	$5 * 10^5$

5.2.4 Colorimetric Sensing of Immobilized Enzyme as Compared to Free OPAA-FL against Ethyl Paraoxon

The colorimetric biosensing activity of Encapsulated and purified OPAA-FL was determined using Ethyl Paraoxon as the substrate in concentrations ranging from 50-2000 µM (**Fig. 5.4)**. A linear range of 100-2000 µM for the detection of ethyl paraoxon was observed with a LOD of 1 µM for free enzyme and 9 µM for the encapsulated enzyme. The sensitivity of such estimation was found to be 0.0063 U/µM for free enzyme and 0.0072 U/µM for encapsulated OPAA-FL. **Table 5.2** summarizes the sensing parameters for Colorimetric Sensing of Immobilized Enzyme as Compared to Free OPAA-FL against Ethyl Paraoxon.

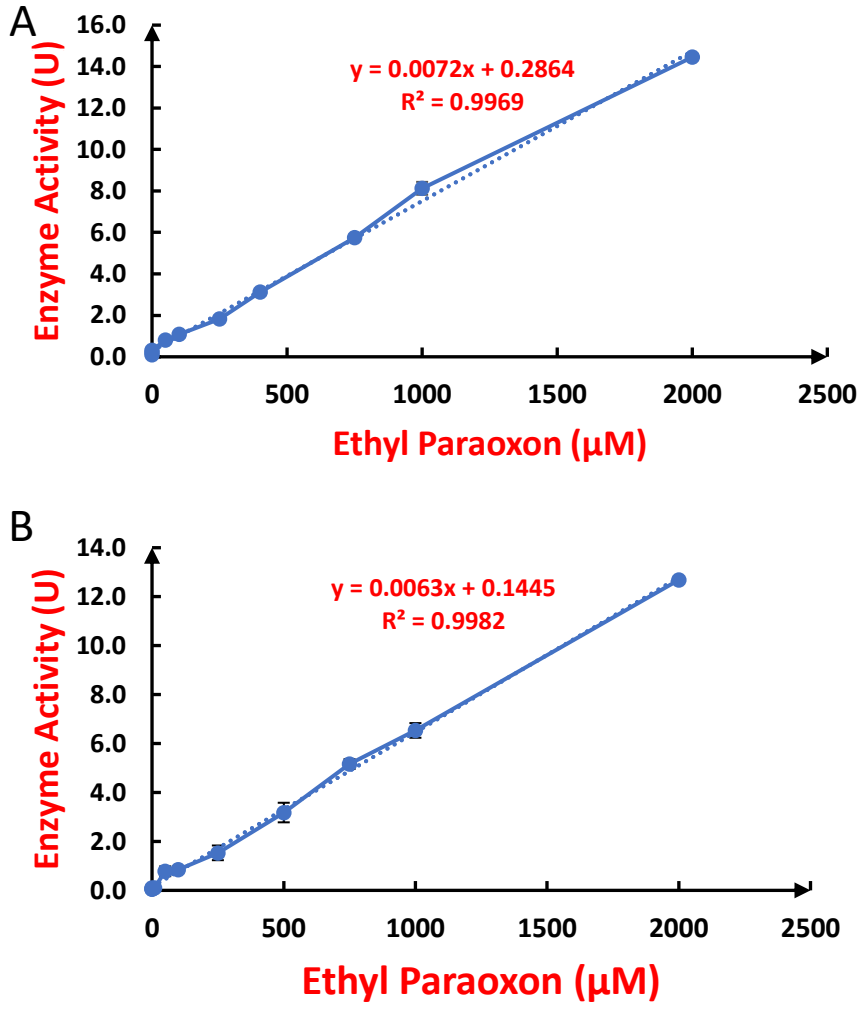


Fig. 5.4: Colorimetric Sensing of Ethyl Paraoxon using OPAA-FL enzyme (A) For Encapsulated Enzyme (B) For Purified Enzyme. The error bars show the standard deviation, and the results are the average of two identical samples.

Table 5.2 Sensing Parameters for Colorimetric Sensing of Immobilized Enzyme as Compared to Free OPAA-FL against Ethyl Paraoxon

SAMPLE	LINEARITY	LOD	LOQ	SENSITIVITY	RESOLUTION	Response Time
Encapsulated Enzyme	0.997	9.4 µM	28.20 µM	0.0072 U/µM	2.85 µM	15 minutes
Free Enzyme	0.9982	1.04 µM	3.14 µM	0.0063 U/µm	0.317 µM	15minutes

5.2.5 Reusability Studies of Immobilized OPAA-FL against Ethyl Paraoxon

One of the significant advantages of enzyme immobilization is the reusability of the enzyme, which is not the case with free enzymes. So, the efficiency of reusability was determined by checking the % activity with the same beads for consecutive cycles. To prepare the beads for the next cycle, they were rinsed extensively to remove residual products. The efficiency of reusability **(Fig. 5.5)** was calculated as the ratio of immobilized enzyme activity in each cycle to its initial activity [38].

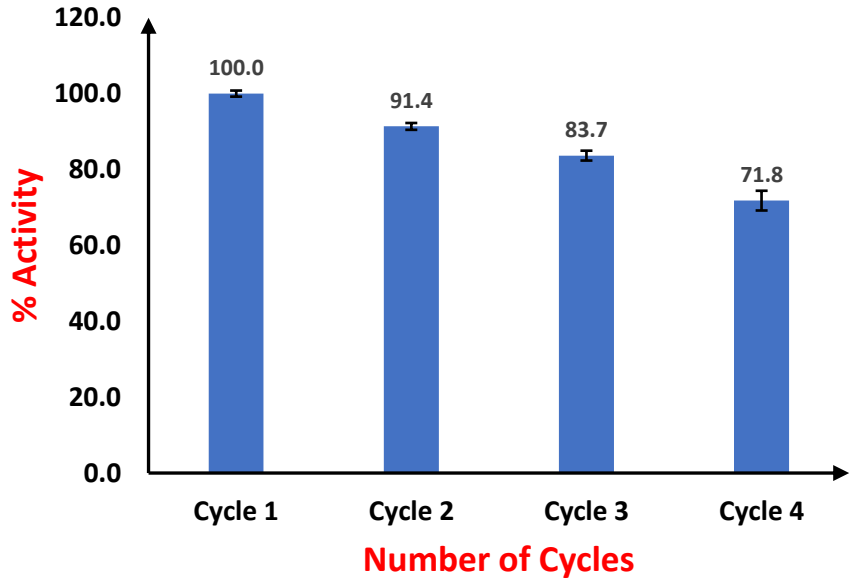


Figure 5.5. Reusability Studies of Immobilized OPAA--FL against Ethyl Paraoxon. The efficiency of reusability decreased by around 10% with each cycle. The error bars show the standard deviation, and the results are the average of two identical samples.

5.2.6 Storage Stability Studies of Immobilized OPAA-FL against Ethyl Paraoxon

The enzyme's storage stability was examined to establish its long-term viability. The encapsulated protein was stored at 4 °C, and activity was measured for 30 days. The results for storage stability are shown in **Fig. 5.6**. The storage effectiveness is determined by the ratio of immobilized enzyme activity to their initial activity following a set

amount of time in storage. It was observed that after 7 days, too, there was hardly any loss in protein activity. But after 30 days of incubation, the activity reduced to about 47%.

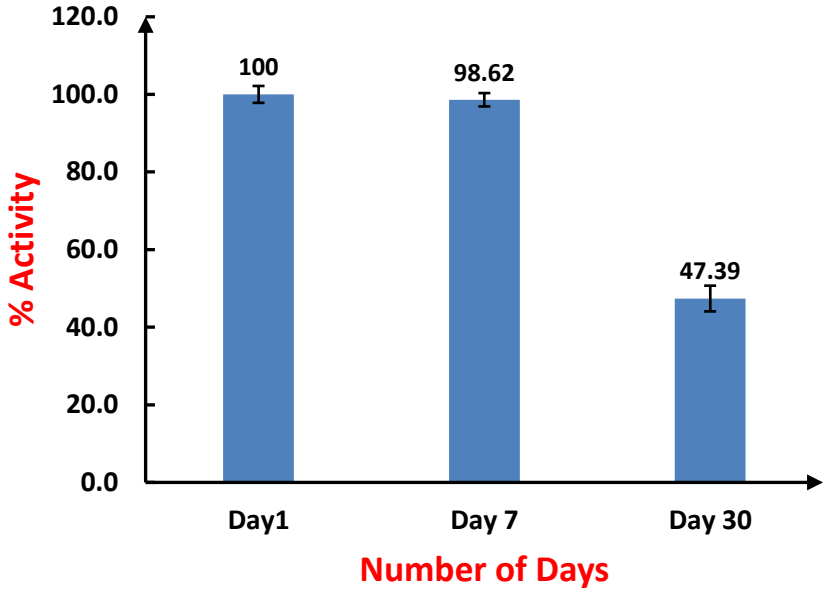


Figure 5.6. Storage Stability Studies of Immobilized OPAA-FL against Ethyl Paraoxon. The error bars show the standard deviation, and the results are the average of two identical samples.

5.2.7 Impact of different solvents on the activity of Free vs. Immobilized OPAA-FL

To check the activity of the enzyme in different organic solvents, 50mM Bis-Tris Propane buffer (pH 8.5), 1 mM $MnCl_2$, 1 µL of purified OPAA-FL, and 5 Enzyme Immobilized Beads were taken, and the reaction volume was made up to 250 µL. The reaction mixture was incubated for 15 minutes at 50 °C. Using the PNP's extinction value of 17,000 M/cm, the rise in absorbance of PNP produced at 410 nm was measured to assess enzyme activity. **Fig. 5.7(A)** shows that the immobilized enzyme is active in Organic Solvents like Methanol, Ethanol, and NaCl; the % activity in SDS is found to be least while it was maximum in the case of NaCl. In **Fig. 5.7 (B)** comparison of the % enzyme activity of Immobilized vs. free enzyme is shown where the

activity of the immobilized enzyme is taken as 100% in all cases. It is obvious that the decrease in the activity of the free enzyme in the presence of solvents like Methanol, Ethanol, Or SDS is because of the enzyme not being able to withstand these conditions, whereas immobilized enzyme is protected in the matrix and can withstand the above conditions.

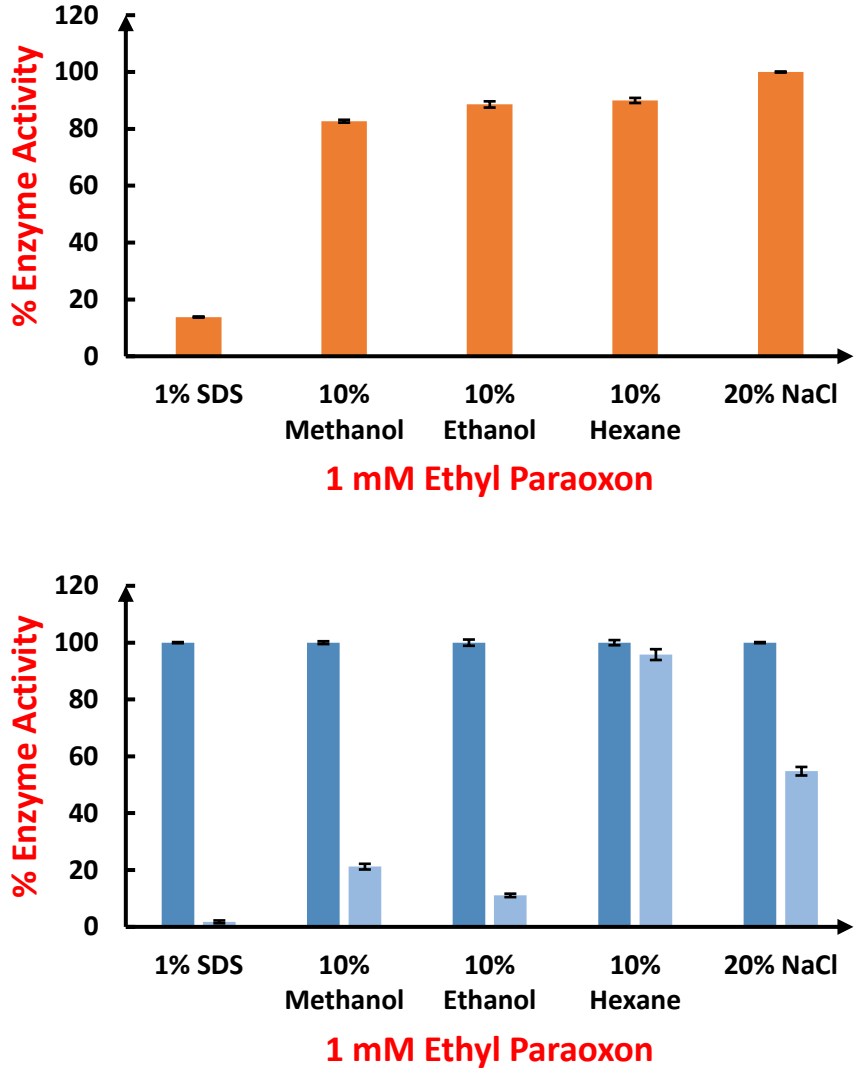


Figure 5.7. Effect of Different Solvents on Activity of Free vs. Immobilized Enzyme. (A) Effect of different solvents on the activity of immobilized enzyme showing its stability in various solvents, (B) Comparison of activity of free vs. immobilized enzyme in different solvents. The error bars show the standard deviation, and the results are the average of two identical samples.

5.2.8. Degradation studies of OP pesticide Ethyl Paraoxon contaminated water using Immobilized OPAA-FL and Free OPAA-FL

Degradation of ethyl paraoxon in pesticide-contaminated water using encapsulated water was done. The effluents released from the pesticide industry are not treated and contaminate the water bodies. Generally, 150-200 ppm of organophosphates are present in industrial effluents, and the permissible limit for the same is only 2-3 ppm. So, to achieve the degradation to acceptable limits lab scale study was performed using a 150-ppm substrate. Preliminarily, the degradation was performed with 0.003U and 0.01U of encapsulated protein, and the %hydrolysis of ethyl paraoxon was measured at designated periods using a Synergy H1 BioTek microplate reader. **Fig. 5.8 (A, B)** shows hydrolysis of around 20% hydrolysis in 120 minutes and 30% hydrolysis in 60 minutes, respectively, using the free enzyme. **Fig. 5.8 (C, D)** shows around 50% hydrolysis in 120 and 60 minutes using 0.003 U and 0.01 U immobilized enzymes, respectively. The results intimated that encapsulated enzyme has better tolerance for high temperature for longer duration as compared to free enzymes. Hence further experiments to check the hydrolysis of Ethyl Paraoxon were performed using Encapsulated Enzyme.

Further, for enzyme concentration optimization, the hydrolysis studies were performed using HPLC (Prominence-i LC-2030 C (Shimadzu, Japan)) instrument. A C18 (5 μm) (4.6 x 250 mm) HPLC column (Shimadzu, Japan) was used with mobile phase in isocratic mode comprised of 70: 30 of Methanol: Milli Q water. The UV detector was set at 254 nm with a flow rate of 1 ml/min. Briefly, the degradation was performed with 0.15 U of encapsulated protein, and the % hydrolysis of ethyl paraoxon was measured at designated periods.

The calibration curve for Ethyl Paraoxon **(Fig. 5.9)** was plotted for substrate concentrations ranging from 0-150 ppm. The curve was found to be linear, with a LOD value as low as 0.12 ppm and a resolution

of around 0.03 ppm. **Table 5.3** shows the sensing parameters of the calibration curve of Ethyl Paraoxon using HPLC.

Fig. 5.10 shows the results of degradation studies using 0.15 U enzyme analyzed on HPLC, showing that 93% of pesticide was hydrolyzed in only 5 minutes, and almost 99% of hydrolysis was obtained in just 60 minutes. This implies that OPAA-FL encapsulated into alginate beads is suitable for decontaminating Pesticide contaminated water to achieve permissible limits. **Fig. 5.11. (A, B, C, D)** shows HPLC graphs for Standard Substrate EP (Rt-6.0) and Degradation rates at 0, 15, and 60 minutes respectively.

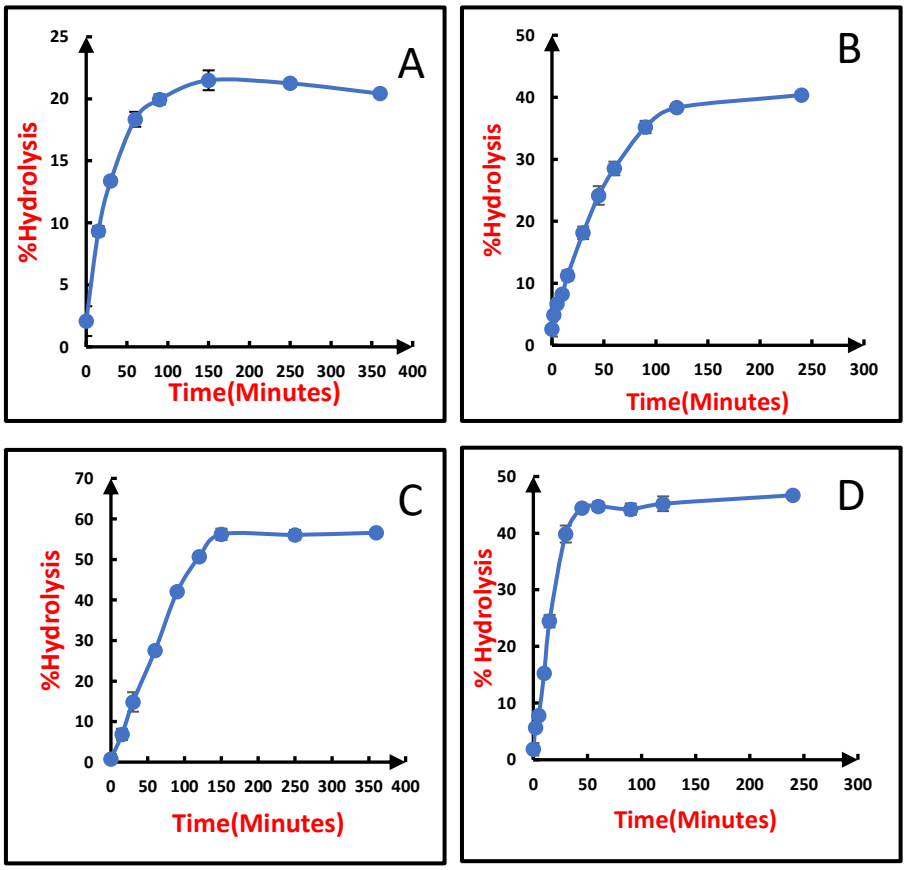


Figure 5.8. % Hydrolysis of Ethyl Paraoxon using Free and Immobilized Enzyme (A) Only 20% of hydrolysis was achieved in 120 minutes using 0.003 U of Free Enzyme (B) About 30% hydrolysis was achieved using 0.01 U free enzyme in 60 minutes. (C) 50% of hydrolysis was achieved in 120 minutes using 0.003 U of Immobilized Enzyme (D) 50% hydrolysis was achieved using 0.01 U immobilized enzyme in 60 minutes. The error bars show the standard deviation, and the results are the average of two identical samples.

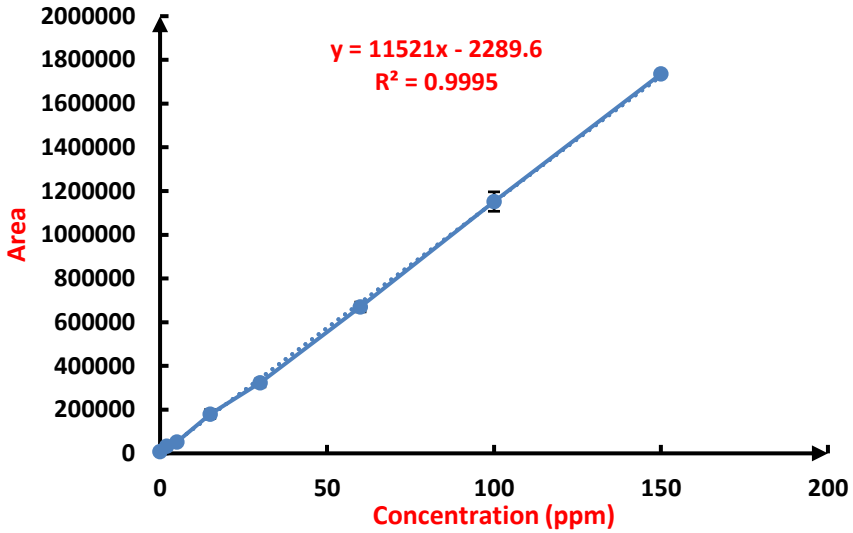


Figure 5.9. Calibration curve of Ethyl Paraoxon using HPLC. The error bars show the standard deviation, and the results are the average of two identical samples.

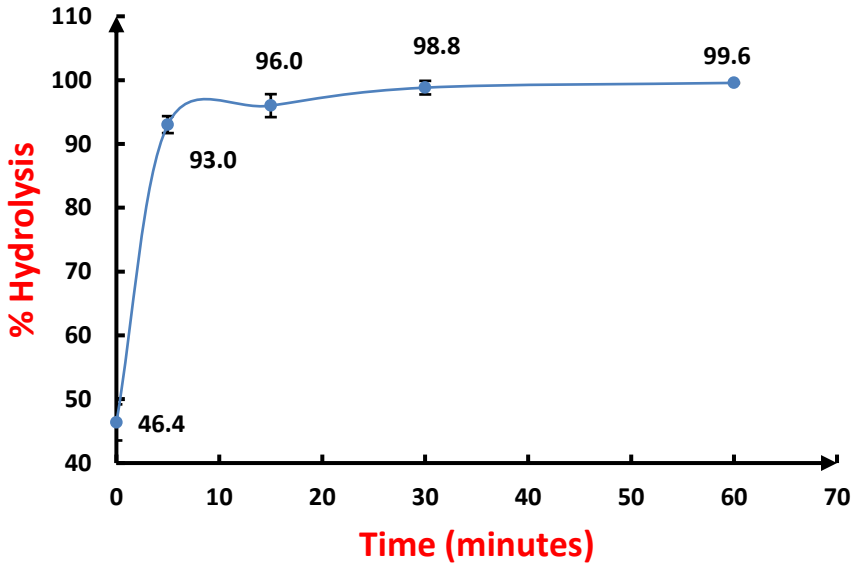


Figure 5.10. % Hydrolysis of Ethyl Paraoxon using Immobilized Enzyme analyzed on HPLC. The results indicate hydrolysis of about 93% EP in around 5 minutes and about 99% hydrolysis within 60 minutes.

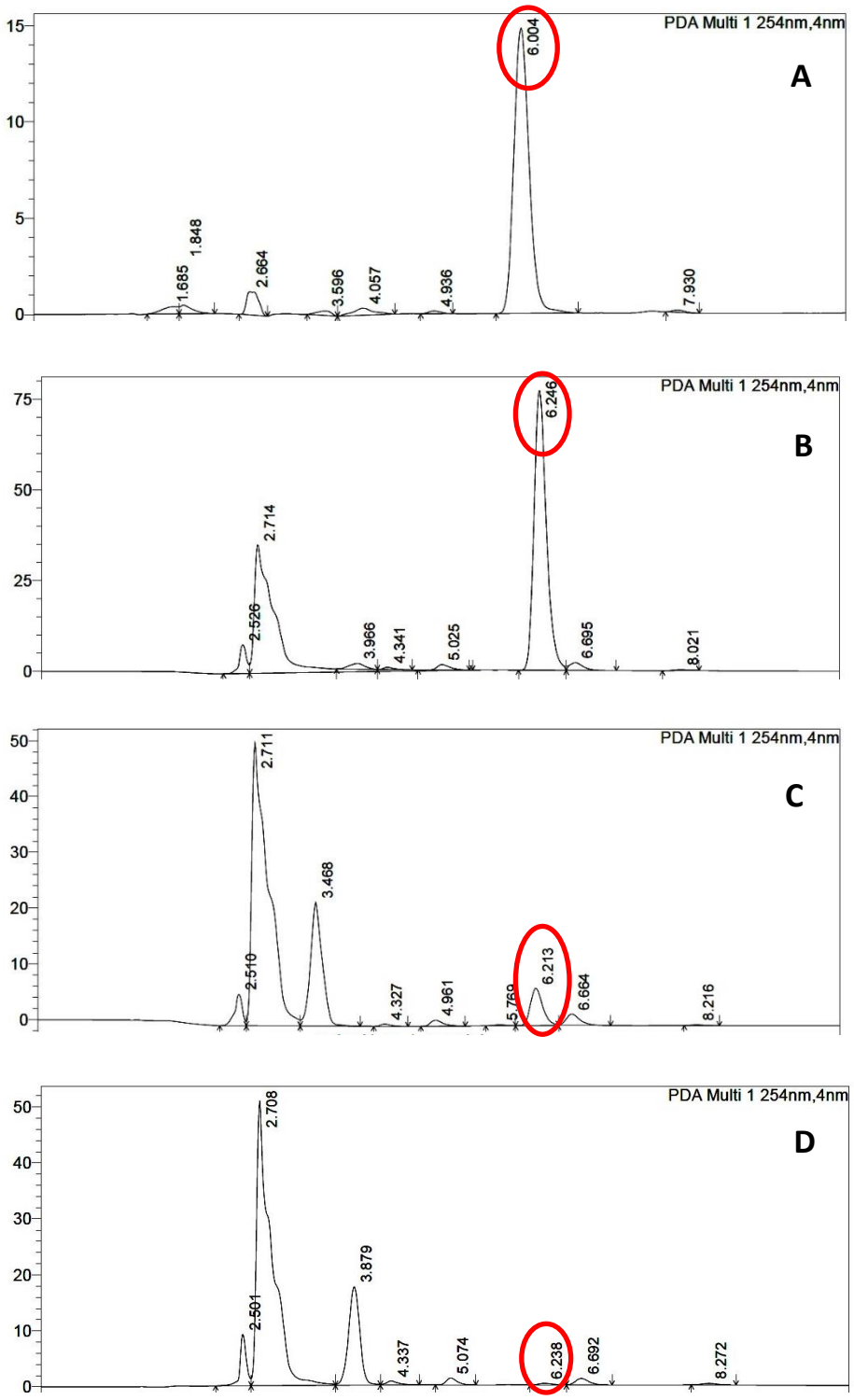


Figure 5.11. **HPLC graphs for the degradation of Ethyl Paraoxon** (A) Standard Substrate Rt 6.0 minutes. (B) Enzymatic degradation using immobilized enzyme at 0 minutes, Substrate peak at Rt 6.2 minutes. (C) Hydrolysis after 15 minutes showed a reduced substrate peak at Rt 6.2 minutes and a product peak at 3.4 minutes. (D) Hydrolysis of Ethyl Paraoxon after 60 minutes showed a diminished substrate peak.

Table 5.3 Sensing Parameters for Calibration curve of standard substrate Ethyl paraoxon using HPLC.

LINEARITY	LOD	LOQ	SENSITIVITY	RESOLUTION
0.9995	0.12 ppm	0.36 ppm	11521/ppm	0.039 ppm

5.3 Summary

Enzymatic bioremediation is a process that utilizes enzymes to break down or convert pollutants into less harmful substances. Enzymatic bioremediation can be used to treat a wide range of pollutants, including petroleum hydrocarbons, pesticides, and heavy metals. This technology offers several advantages over traditional remediation methods, such as being cost-effective, eco-friendly, and less disruptive to the environment. This study involves the use of immobilized OP hydrolyzing enzyme OPAA-FL into alginate beads and has shown around 99% hydrolysis of Ethyl Paraoxon within 60 minutes. This study also shows the stability of immobilized enzymes in different solvent conditions, their storage stability, and reusability. Enzymatic bioremediation is a promising approach for cleaning up contaminated sites and restoring the health of ecosystems.

Chapter 6

Real-Life Application of Organophosphorus Pesticide Degrading Enzymes

6.1 Introduction

With increasing urbanization, contact with pesticides is inevitable, and it can occur via clothes, the food we eat, or surfaces we encounter. Pesticides are used to eliminate pests in agriculture. They are indispensable in producing controlled quantities and qualities of food. But despite their merits, they are toxic to the environment[40]. Excessive use of pesticides in agricultural fields exposes soil, air, and crops to harmful chemical pesticides. Rain and wind are generally the carriers of pesticides from their point of application to the crops nearby. Prolonged usage of pesticides leads to the accumulation on fruits and vegetable surfaces and renders them highly toxic to human beings[40].

In this study, we will focus on the real-life application of Organophosphate hydrolyzing enzymes for the degradation of pesticide-contaminated fruits, vegetables, water, textiles, etc.

Water use is significant for life, and its quality is non-negotiable. Water pollution is a primary concern for humanity, and one of the pollutants is a pesticide. There are incidences of OP pesticides in drinking water, leading to neurodegenerative diseases. Hence there is a need to develop a safe, economical, reliable, and environmentally friendly method for pesticide removal [41]. The OP pesticides can be removed by physical disinfection, chemical decontamination, and Biodegradation. The highest degradation efficiency, gentle settings, and regulated progress make biodegradation the most promising option for pesticide decontamination. In biodegradation techniques, enzymatic degradation has proven to be more favorable than microbial degradation owing to better efficiency and comparatively better degradation of OP pesticides.

Food is a basic need of life, but the safety of the food that we are eating is questionable. Fruits and vegetables are a significant part of the human diet. They are rich sources of nutrients, macro as well as micronutrients. It is generally advised to eat fruits and vegetables in raw form. Cooked and processed food is said to have lost the required nutrients that are otherwise retained in their raw form. Thousands of tons of pesticides are sprayed on crops each year, leading to their accumulation. So there is an urgent need to develop strategies that can detect and degrade pesticides in food products like fruits, vegetables, grains, etc. the currently available methods for pesticide removal are washing, peeling, blanching, treatment with chlorine, NaCl, hydrogen peroxide, organic acids, essential oils, electrolyzed water, ozone, ultrasound UV light, etc

One of the modes of entry for Pesticides is via absorption into the skin. Hence it is always advised to wear protective clothing while handling pesticides. For agricultural workers, pesticide exposure via skin contact is also widespread. Direct contact with these contaminants on the skin has dire consequences; it can cause reproductive disorders, neurological diseases, respiratory disorders, diabetes, and even cancer.

In the present study, real-life applications like decontamination of textiles and spiking recovery experiments were performed. This was done to check the interference effects in the detection and degradation of different matrices by OPAA-FL

6.2 Results and Discussion

6.2.1 Preparation of calibration curves for sensing of Ethyl paraoxon for Spike and Recovery Experiments

A calibration curve for purified OPAA-FL was determined using Ethyl Paraoxon as the substrate in concentrations ranging from 50-2000 µM (**Fig. 6.1)**. A linear range of 100-2000 µM for the detection of ethyl paraoxon was observed with a LOD of 1 µM for the free enzyme. The sensitivity of such estimation was 0.0063 U/µM for OPAA-FL. **Table 6.1** summarizes the sensing parameters for the Colorimetric Sensing of OPAA-FL against Ethyl Paraoxon.

For spiking and recovery experiments, Household tap water was taken from the water supply in Simrol, Indore, Madhya Pradesh, India. The tap water was spiked with different concentrations of Ethyl Paraoxon to check the stability and interferences imposed on OPAA-FL in a real-life scenario. The experiment was used to calculate the percent recovery, which was indicative of the accuracy of our enzyme OPAA-FL. The % recoveries in household tap water were 100% and an error of ± 2% **(Fig. 6.2)**. This indicates that household water has negligible interference in estimating spiked EP.

6.2.2. Decontamination of Paraoxon on Textiles

The decontamination experiment of paraoxon on textiles: cotton was done to check the application of the enzyme in the pesticide decontamination field. Cotton clothes were cut into squares (2 X 2 cm^2) and were contaminated with 250 µmol ethyl paraoxon. 50 µg of the enzyme was dissolvedin 10 ml of different solutions: Buffer, 1%SDS, 20%NaCl, Tap Water, and 0.5% Soap Solution. **Fig. 6.3** shows the %Hydrolysis of Ethyl Paraoxon after 20 minutes of incubation in each of the solutions mentioned above at 50°C by measuring the production of PNP at 410 nm.

Contact with different solutions, like detergents, salts, etc., is inevitable in real-life, so decontamination was studied in the presence of other solutions. **Fig. 6.3(D)** shows about 80-100% hydrolysis of the pesticide in around 60 minutes. No hydrolysis was seen in the presence of SDS.

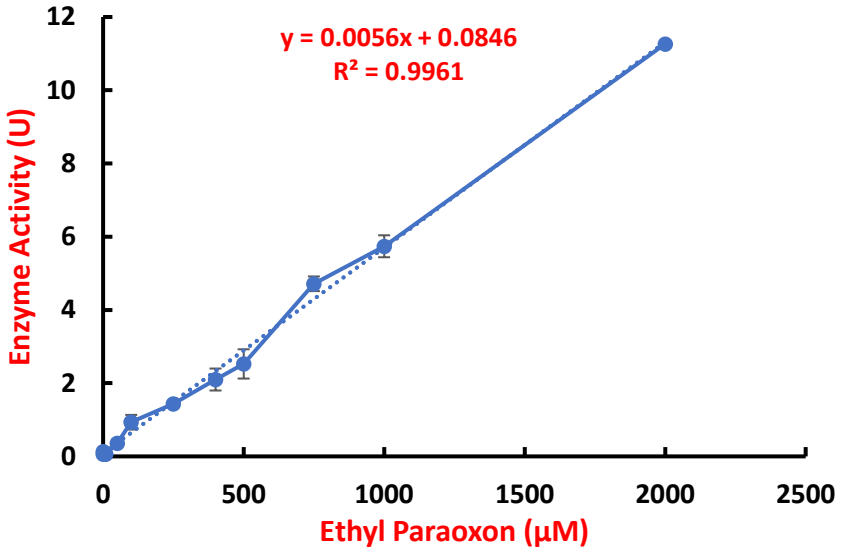


Figure 6.1 Calibration Curve for Colorimetric Biosensing of Ethyl paraoxon using OPAA-FL Variant. The error bars show the standard deviation, and the results are the average of two identical samples.

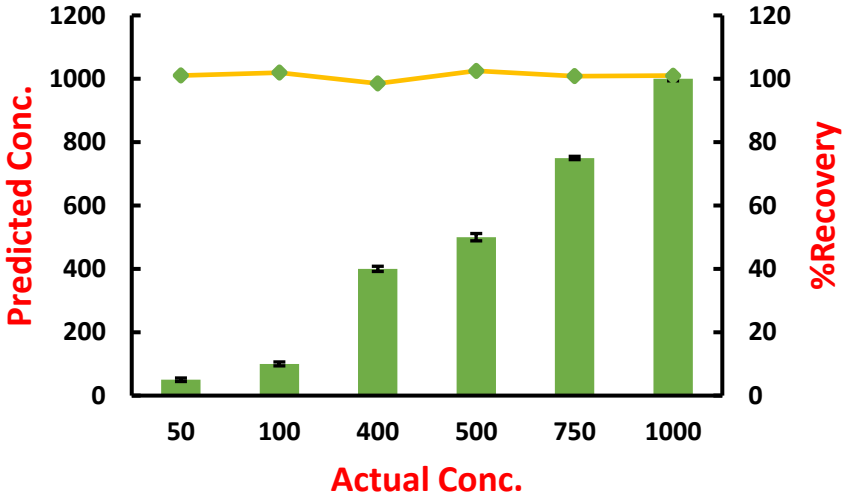
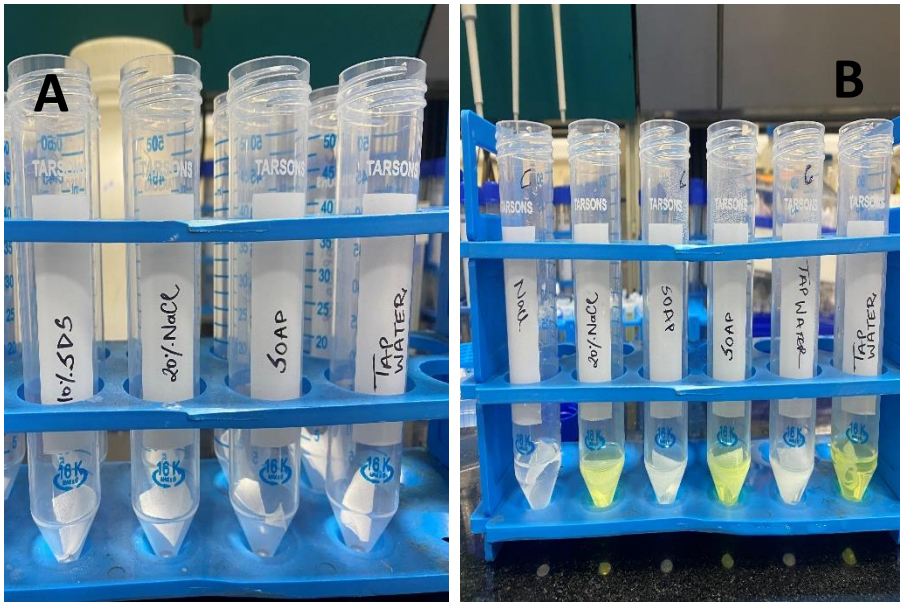
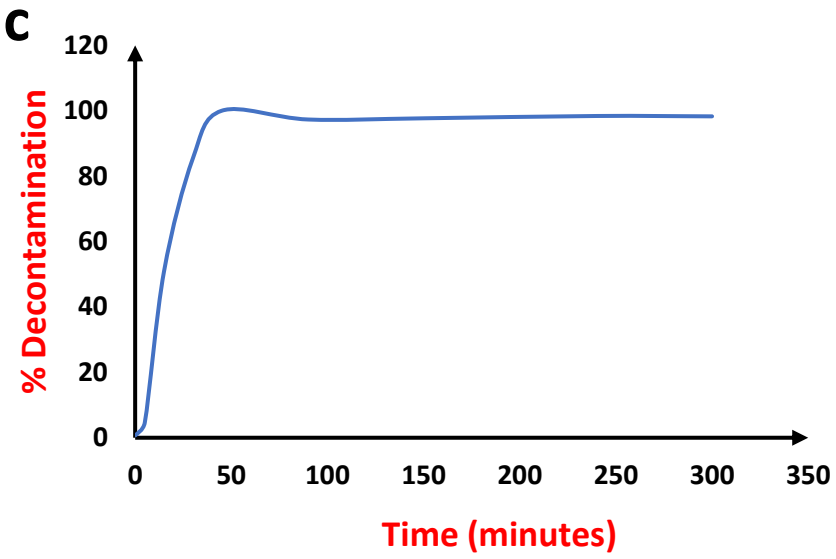


Figure 6.2. Spike Recovery Experiments of OPAA-FL with Tap Water. The error bars show the standard deviation, and the results are the average of two identical samples.

Table 6.2 Sensing Parameters of calibration curve of OPAA-FL against Ethyl Paraoxon

LINEARITY	LOD	LOQ	SENSITIVITY	RESOLUTION	Response Time
0.9961	1.17 µM	3.54 µM	0.0056 U/µm	0.357 µM	15 minutes


A
B
10% SDS
20% NaCl
SOAP
TAP WATER


C
% Decontamination
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Time (minutes)

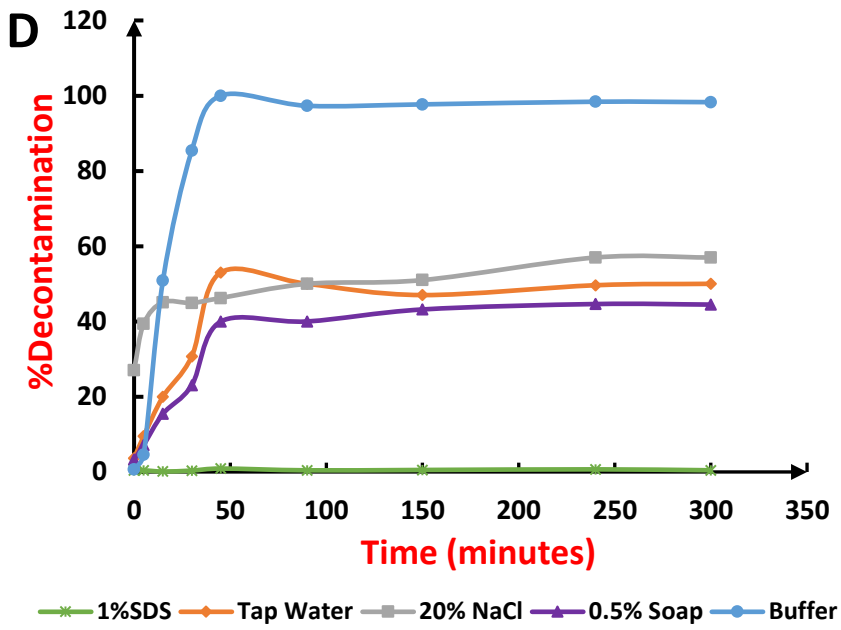


Figure 6.3. Decontamination studies on cotton Fabric: A) Experimental tubes before the start of the experiment. B) Yellow color in experimental tubes (with enzyme) and no color in control tubes (without enzyme) shows Pesticide Degradation in different solutions. C) Graph: The decontamination experiment of cotton fabric shows around 80% of paraoxon is removed after 60 minutes. D) Decontamination of cotton in the presence of different Fabric solutions showing hydrolysis of Ethyl Paraoxon in the presence of NaCl, Tap Water, and Soap Solution, but not in the presence of SDS.

6.3 Summary

This study finds the real-life application of OP- pesticide degrading enzymes. To study the same, it is important to check interference of real matrices or environment in degradation of pesticides by the enzymes. This study involves spike and recovery experiments using tap water showing a 100% recovery indicating no interference from tap water in degradation. Further decontamination studies on textiles shows the ability of enzymes to hydrolyze Pesticide adsorbed on cotton textiles, an application that could be escalated to practical situations.

Chapter 7

Conclusions and Future Perspectives

In conclusion, using pesticides has become widespread in modern agriculture to protect crops and increase yields. However, the indiscriminate use of pesticides, particularly organophosphate (OP) pesticides, has been associated with adverse environmental and health effects. OP pesticides are known to pose a significant risk to human health, as well as to non-target species and the environment. The toxic effects of OP pesticides are primarily due to their ability to inhibit acetylcholinesterase (AChE), an essential enzyme that regulates the transmission of nerve impulses. When OP pesticides bind to AChE, they prevent its normal function, leading to the accumulation of the neurotransmitter acetylcholine and resulting in a range of symptoms, including nausea, vomiting, abdominal cramps, diarrhea, headache, dizziness, weakness, fatigue, sweating, difficulty breathing, blurred vision, muscle twitching, tremors, convulsions, coma, and even death.

To address the negative impacts of OP pesticides, there is a growing need for effective strategies for their detection and degradation. Analytical techniques, such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), have been developed to detect and quantify OP pesticides in environmental samples. These techniques can provide valuable information on the presence and extent of pesticide contamination, which is essential for practical remediation efforts, but these are sophisticated techniques. There is a need to develop an easy, user-friendly, and economical method for pesticide detection and degradation.

We studied the degradation of pesticides using recombinant enzymes OPAA-FL and Nus-OPH. After expressing and purifying the enzymes, we checked their activity against a common pesticide Ethyl Paraoxon. Surprisingly we got twice more activity for OPAA-FL than

what was earlier reported. We further studied the activity of our enzymes against other OP Pesticides and found some positive results against Coumaphos. Coumaphos is an organophosphate pesticide widely used in agriculture for pest control. Coumaphos on exposure to environmental conditions such as sunlight, air, and water, it can degrade into several by-products, including chlorfenvinphos, 3,5,6-trichloro-2-pyridinol (TCP), and diethyl-thiophosphate (DETP). The degradation products of coumaphos can persist in the environment and potentially pose a risk to human health and the environment. TCP has been found to have toxic effects on aquatic life and can accumulate in human tissues. Chlorfenvinphos has been classified as a possible human carcinogen, and DETP has been associated with neurological effects.

We checked and confirmed coumaphos degradation by OPAA-FL using many techniques such as TLC, HPLC, and LC-MS. Our study is the first report on the degradation of coumaphos by OPAA-FL. The study further included determining kinetic parameters for Coumaphos degradation by OPAA-FL and its optimum pH and temperature.

In addition to detection, the degradation of OP pesticides is also essential for the remediation of contaminated environments. Several methods have been developed for the degradation of OP pesticides, including chemical, physical, and biological approaches. Bioremediation is a promising approach that uses microorganisms to degrade OP pesticides into non-toxic by-products. Bioremediation can be further enhanced through immobilized enzymes, which offer several advantages over free enzymes, including increased stability and reusability.

We studied the degradation of a common OP pesticide Ethyl Paraoxon using encapsulated OPAA-FL into alginate beads. The immobilized enzyme showed similar pH and temperature as the free enzyme suggesting the matrix does not affect its optimum activity. Moreover, the encapsulated enzyme followed the Michaelis-Menten Kinetics. We further checked our encapsulated enzyme's reusability and

storage stability and found that it retained over 98% for Day 7 and 48% for Day 30. The encapsulated enzyme could also be reused for about 7-8 cycles with less than 10% loss in activity. The immobilized enzyme was also found to be active in various solvents. Then we studied the bioremediation of pesticide-contaminated water at a lab scale using the encapsulated enzyme and found out that the immobilized enzyme is capable of hydrolyzing 99% of ethyl paraoxon within 60 minutes using as low as 0.15 U of enzyme.

We also tried to study the interference of real-life matrices in pesticide degradation by OPAA-FL. For this, we performed spike and recovery experiments in household tap water and got a percent recovery of about 100-102%. We also studied the decontamination of cotton textiles using OPAA-FL and achieved around 80% degradation.

In conclusion, the use of pesticides has become a ubiquitous practice in modern agriculture, but it has come at a cost to human health and the environment. The use of OP pesticides has been associated with significant adverse impacts, including the risk of poisoning and the contamination of water and soil. Practical strategies for detecting and degrading OP pesticides are necessary to address these issues. Bioremediation using immobilized enzymes offers a promising approach for effectively and sustainably removing OP pesticides from contaminated environments. However, it is essential to continue research to optimize and develop these strategies for effective and sustainable pesticide management. Adopting integrated pest management practices can also play an indispensable role in reducing the reliance on chemical pesticides and mitigating the negative impacts of pesticide use.

Future work for this research could be to explore the possibilities of using different matrices for Enzymatic Bioremediation of OP-pesticides. Developing a Lab scale cartridge system for the degradation of pesticide-contaminated water. Practical studies that apply to the research findings of this study can be explored.

APPENDIX-A

Different OP Pesticide Permissible Limits "ppm- Parts Per Million, µg/L stands for microgram per liter."		
Pesticide	**permissible Limits (ppm or µg/L)**	**Guideline Issued by**
Chlorpyrifos	0.03µg/L	EU Drinking Water Directive
	0.1µg/L	US EPA Drinking Water Maximum Contaminant Level
	0.5µg/L	WHO Guideline Value
	0.7µg/L	Canada Drinking Water Quality Guidelines
Diazinon	0.02µg/L	EU Drinking Water Directive
	0.3µg/L	US EPA Drinking Water Maximum Contaminant Level
	0.03 ppm(soil)	US EPA
Malathion	0.02µg/L	EU Drinking Water Directive
	0.2µg/L	US EPA Drinking Water Maximum Contaminant Level
	2 ppm (soil)	US EPA
Parathion	0.0002µg/L	EU Drinking Water Directive
	0.006µg/L	US EPA Drinking Water Maximum Contaminant Level
	0.09 ppm (soi)	US EPA

OPAA-FL Variant Gene Sequence: OPAA-FL variant contains mutations at Y212F and V342L.

MNKLAVLYAEHIATLQKRTREIIERENLDGVVFHSGQAKR

QFLDDMYYPFKVNPQFKAWLPVIDNPHCWIVANGTDKPKL

IFYRPVDFWHKVPDEPNEYWADYFDIELLVKPDQVEKLLP

YDKARFAYIGEYLEVAQALGFELMNPEPVMNFYHYHRAYK

TQYELACMREANKIAVQGHKAARDAFFQGKSEFEIQQAYL

LATQHSENDTPFGNIVALNENCAILHYTHFDRVAPATHRS

FLIDAGANFNGYAADITRTYDFTGEGEFAELVATMKQHQI

ALCNQLAPGKLYGELHLDCHQRVAQTLSDFNIVNLSADEI

VAKGITSTFFPHGLGHHIGLQLHDVGGFMADEQGAHQEPP

EGHPFLRCTRKIEANQVFTIEPGLYFIDSLLGDLAATDNN

QHINWDKVAELKPFGGIRIEDNIIVHEDSLENMTRELELD

Different genotypes of OPAA and their Kinetic Parameters [42]				
Enzyme	**Genotype**	**K_m (µM)**	**K_{cat} (min^{-1})**	**K_{cat}/ K_m ($min^{-1} M^{-1}$)**
WT	**Wild-type**	**1.20E + 04 ± 4.31E + 02**	**9.02E + 03 ± 6.32E + 02**	**1.33E + 06 ± 1.41E + 05**
FL	**Y212F/V342L**	**4.52E + 04 ± 4.62E + 03**	**5.31E + 03 ± 1.01E + 03**	**8.50E + 06 ± 2.49E + 06**
FY	**Y212F/V342Y**	**1.10E + 04 ± 1.80E + 03**	**5.62E + 03 ± 1.57E + 03**	**1.96E + 06 ± 8.65E + 05**
FI	**Y212F/V342I**	**1.63E + 04 ± 1.99E + 03**	**6.00E + 03 ± 1425.09**	**2.72E + 06 ± 9.80E + 05**

REFERENCES

[1] V. De Luca, L. Mandrich, and G. Manco, "Development of a Qualitative Test to Detect the Presence of Organophosphate Pesticides on Fruits and Vegetables," *Life*, vol. 13, no. 2, Art. no. 2, Feb. 2023, doi: 10.3390/life13020490.

[2] A. Chowdhury, S. Pradhan, M. Saha, and N. Sanyal, "Impact of pesticides on soil microbiological parameters and possible bioremediation strategies," *Indian J. Microbiol.*, vol. 48, no. 1, pp. 114–127, Mar. 2008, doi: 10.1007/s12088-008-0011-8.

[3] S. Handa, N. Agnihotri, and G. Kulshreshtha, "Effect of pesticide on soil fertility," *Pestic. Residues Significance Manag. Anal.*, pp. 184–198, 1999.

[4] H. Hafez, W. H.-P. Thiemann, A. A. M. D. Re, E. Capri, L. M. Padovani, and M. Trevisan, "Persistence and biodegradation of diazinon and imidacloprid in soil," 2003.

[5] L. Rani *et al.*, "An extensive review on the consequences of chemical pesticides on human health and environment," *J. Clean. Prod.*, vol. 283, p. 124657, Feb. 2021, doi: 10.1016/j.jclepro.2020.124657.

[6] S. Kumar, G. Kaushik, M. A. Dar, S. Nimesh, U. J. López-chuken, and J. F. Villarreal-chiu, "Microbial Degradation of Organophosphate Pesticides: A Review," *Pedosphere*, vol. 28, no. 2, pp. 190–208, Apr. 2018, doi: 10.1016/S1002-0160(18)60017-7.

[7] M. Jokanović, P. Oleksak, and K. Kuca, "Multiple neurological effects associated with exposure to organophosphorus pesticides in man," *Toxicology*, vol. 484, p. 153407, Jan. 2023, doi: 10.1016/j.tox.2022.153407.

[8] O. A. Lenina and K. A. Petrov, "Balanced modulation of neuromuscular synaptic transmission via M1 and M2 muscarinic receptors during inhibition of cholinesterases," *Sci. Rep.*, vol. 12, no. 1, Art. no. 1, Feb. 2022, doi: 10.1038/s41598-022-05730-w.

[9] kumar naveen, "harmful effects of pesticide on human health".

[10] S. Y. Ganie, D. Javaid, Y. A. Hajam, and Mohd. S. Reshi, "Mechanisms and treatment strategies of organophosphate pesticide induced neurotoxicity in humans: A critical appraisal," *Toxicology*, vol. 472, p. 153181, Apr. 2022, doi: 10.1016/j.tox.2022.153181.

[11] T. Čadež, D. Kolić, G. Šinko, and Z. Kovarik, "Assessment of four organophosphorus pesticides as inhibitors of human acetylcholinesterase and butyrylcholinesterase," *Sci. Rep.*, vol. 11, no. 1, Art. no. 1, Nov. 2021, doi: 10.1038/s41598-021-00953-9.

[12] M. Thakur, I. L. Medintz, and S. A. Walper, "Enzymatic Bioremediation of Organophosphate Compounds—Progress and Remaining Challenges," *Front. Bioeng. Biotechnol.*, vol. 7, 2019, Accessed: Apr. 26, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fbioe.2019.00289

[13] H. Mali *et al.*, "Organophosphate pesticides an emerging environmental contaminant: Pollution, toxicity, bioremediation progress, and remaining challenges," *J. Environ. Sci.*, vol. 127, pp. 234–250, May 2023, doi: 10.1016/j.jes.2022.04.023.

[14] A. Basso and S. Serban, "Industrial applications of immobilized enzymes—A review," *Mol. Catal.*, vol. 479, p. 110607, Dec. 2019, doi: 10.1016/j.mcat.2019.110607.

[15] E. Vilanova and M. A. Sogorb, “The Role of Phosphotriesterases in the Detoxication of Organophosphorus Compounds,” *Crit. Rev. Toxicol.*, vol. 29, no. 1, pp. 21–57, Jan. 1999, doi: 10.1080/10408449991349177.
[16] “Microbial Degradation of Organophosphorus Xenobiotics: Metabolic Pathways and Molecular Basis - ScienceDirect.” https://www.sciencedirect.com/science/article/abs/pii/S0065291106510033 (accessed Apr. 28, 2023).
[17] “Engineering the Organophosphorus Acid Anhydrolase Enzyme for Increased Catalytic Efficiency and Broadened Stereospecificity on Russian VX | Biochemistry.” https://pubs.acs.org/doi/full/10.1021/acs.biochem.5b00624 (accessed Apr. 28, 2023).
[18] G. Mechrez, M. A. Krepker, Y. Harel, J.-P. Lellouche, and E. Segal, “Biocatalytic carbon nanotube paper: a ‘one-pot’ route for fabrication of enzyme-immobilized membranes for organophosphate bioremediation,” *J. Mater. Chem. B*, vol. 2, no. 7, pp. 915–922, 2014, doi: 10.1039/C3TB21439G.
[19] S. Mortaza Robatjazi, M. Reihani, S. Mahboudi, S. Mohammad Hasanpour, and M. Ali Nasiri Khalili, “IMMOBILIZTION OF ORGANOPHOSPHORUS HYDROLASE ENZYME ON FERRIC MAGNETIC NANOPARTICLES AND INVESTIGATION OF IMMOBILIZED ENZYME STABILITY,” *J. Microbiol. Biotechnol. Food Sci.*, vol. 6, no. 6, pp. 1295–1299, Jun. 2017, doi: 10.15414/jmbfs.2017.6.6.1295-1299.
[20] S. R. Caldwell and F. M. Raushel, “Detoxification of organophosphate pesticides using a nylon based immobilized phosphotriesterase fromPseudomonas diminuta,” *Appl. Biochem. Biotechnol.*, vol. 31, no. 1, pp. 59–73, Oct. 1991, doi: 10.1007/BF02922126.
[21] M. Sirotkina, I. Lyagin, and E. Efremenko, “Hydrolysis of organophosphorus pesticides in soil: New opportunities with ecocompatible immobilized His6-OPH,” *Int. Biodeterior. Biodegrad.*, vol. 68, pp. 18–23, Mar. 2012, doi: 10.1016/j.ibiod.2011.12.004.
[22] M. Sharifi, S.-M. Robatjazi, M. Sadri, and J. M. Mosaabadi, “Immobilization of organophosphorus hydrolase enzyme by covalent attachment on modified cellulose microfibers using different chemical activation strategies: Characterization and stability studies,” *Chin. J. Chem. Eng.*, vol. 27, no. 1, pp. 191–199, Jan. 2019, doi: 10.1016/j.cjche.2018.03.023.
[23] S. K. Falahati-Pour, A. S. Lotfi, G. Ahmadian, and A. Baghizadeh, “Covalent immobilization of recombinant organophosphorus hydrolase on spores of *Bacillus subtilis*,” *J. Appl. Microbiol.*, vol. 118, no. 4, pp. 976–988, Apr. 2015, doi: 10.1111/jam.12744.
[24] J. Orbulescu, C. A. Constantine, V. K. Rastogi, S. S. Shah, J. J. DeFrank, and R. M. Leblanc, “Detection of Organophosphorus Compounds by Covalently Immobilized Organophosphorus Hydrolase,” *Anal. Chem.*, vol. 78, no. 19, pp. 7016–7021, Oct. 2006, doi: 10.1021/ac061118m.
[25] D. M. Munnecke, “Hydrolysis of organophosphate insecticides by an immobilized-enzyme system,” *Biotechnol. Bioeng.*, vol. 21, no. 12, pp. 2247–2261, 1979, doi: 10.1002/bit.260211207.
[26] J. Bao *et al.*, “ELP-OPH/BSA/TiO2 nanofibers/c-MWCNTs based biosensor for sensitive and selective determination of p-nitrophenyl substituted organophosphate pesticides in aqueous system,” *Biosens. Bioelectron.*, vol. 85, pp. 935–942, Nov. 2016, doi: 10.1016/j.bios.2016.05.094.

[27] A. H. Mansee, W. Chen, and A. Mulchandani, “Detoxification of the organophosphate nerve agent coumaphos using organophosphorus hydrolase immobilized on cellulose materials,” *J. Ind. Microbiol. Biotechnol.*, vol. 32, no. 11–12, pp. 554–560, Dec. 2005, doi: 10.1007/s10295-005-0059-y.
[28] M. Shimazu, A. Mulchandani, and W. Chen, “Thermally triggered purification and immobilization of elastin-OPH fusions,” *Biotechnol. Bioeng.*, vol. 81, no. 1, pp. 74–79, Jan. 2003, doi: 10.1002/bit.10446.
[29] N. Suthiwangcharoen and R. Nagarajan, “Enhancing Enzyme Stability by Construction of Polymer–Enzyme Conjugate Micelles for Decontamination of Organophosphate Agents,” *Biomacromolecules*, vol. 15, no. 4, pp. 1142–1152, Apr. 2014, doi: 10.1021/bm401531d.
[30] M. Jain, P. Yadav, B. Joshi, A. Joshi, and P. Kodgire, “Recombinant organophosphorus hydrolase (OPH) expression in E. coli for the effective detection of organophosphate pesticides,” *Protein Expr. Purif.*, vol. 186, p. 105929, Oct. 2021, doi: 10.1016/j.pep.2021.105929.
[31] “A novel biosensor for the detection of organophosphorus (OP)-based pesticides using organophosphorus acid anhydrolase (OPAA)-FL variant | SpringerLink.” https://link.springer.com/article/10.1007/s00253-020-11008-w (accessed Dec. 03, 2022).
[32] T. Jindal, D. K. Singh, and H. C. Agarwal, “Persistence and degradation of coumaphos in model cattle dipping vats,” *J. Environ. Sci. Health B*, vol. 37, no. 1, pp. 33–42, Jan. 2002, doi: 10.1081/PFC-120002895.
[33] “A novel organophosphate hydrolase from Arthrobacter sp. HM01: Characterization and applications - ScienceDirect.” https://www.sciencedirect.com/science/article/abs/pii/S0960852422001997 (accessed Dec. 03, 2022).
[34] P. K. Robinson, “Enzymes: principles and biotechnological applications,” *Essays Biochem.*, vol. 59, pp. 1–41, 2015, doi: 10.1042/bse0590001.
[35] X. Zheng, L. Wang, L. Qi, and Z. Dong, “A Novel Organophosphorus Acid Anhydrolase from Deep Sea Sediment with High Degradation Efficiency for Organophosphorus Pesticides and Nerve Agent,” *Microorganisms*, vol. 10, no. 6, Art. no. 6, Jun. 2022, doi: 10.3390/microorganisms10061112.
[36] S. Zhao, W. Xu, W. Zhang, H. Wu, C. Guang, and W. Mu, “Overview of a bioremediation tool: organophosphorus hydrolase and its significant application in the food, environmental, and therapy fields,” *Appl. Microbiol. Biotechnol.*, vol. 105, no. 21, pp. 8241–8253, Nov. 2021, doi: 10.1007/s00253-021-11633-z.
[37] M. Syafrudin *et al.*, “Pesticides in Drinking Water—A Review,” *Int. J. Environ. Res. Public. Health*, vol. 18, no. 2, Art. no. 2, Jan. 2021, doi: 10.3390/ijerph18020468.
[38] U. Colak and N. Gençer, “Immobilization of paraoxonase onto chitosan and its characterization,” *Artif. Cells Blood Substit. Biotechnol.*, vol. 40, no. 4, pp. 290–295, Aug. 2012, doi: 10.3109/10731199.2011.652258.
[39] J. Ha, C. R. Engler, and J. R. Wild, “Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads,” *Bioresour. Technol.*, vol. 100, no. 3, pp. 1138–1142, Feb. 2009, doi: 10.1016/j.biortech.2008.08.022.
[40] J. Fenik, M. Tankiewicz, and M. Biziuk, “Properties and determination of pesticides in fruits and vegetables,” *TrAC Trends Anal. Chem.*, vol. 30, no. 6, pp. 814–826, Jun. 2011, doi: 10.1016/j.trac.2011.02.008.
[41] M. Singh Sankhla, M. Kumari, K. Sharma, R. Kushwah, and R. Kumar,

"Water Contamination through Pesticide & Their Toxic Effect on Human Health," *Int. J. Res. Appl. Sci. Eng. Technol.*, vol. 6, Jan. 2018, doi: 10.22214/ijraset.2018.1146.

[42] S. Y. Bae *et al.*, "An OPAA enzyme mutant with increased catalytic efficiency on the nerve agents sarin, soman, and GP," *Enzyme Microb. Technol.*, vol. 112, pp. 65–71, May 2018, doi: 10.1016/j.enzmictec.2017.11.001.