RECOMBINANT EXPRESSION OF ORGANOPHOSPHORUS ACID ANHYDROLASE IN BACTERIA: TOWARDS ENVIRONMENTAL BIOSENSORS

M.Sc. Thesis

By

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DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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RECOMBINANT EXPRESSION OF ORGANOPHOSPHORUS ACID ANHYDROLASE IN BACTERIA: TOWARDS ENVIRONMENTAL BIOSENSORS

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Submitted in partial fulfillment of the requirements for the award of the degree

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **RECOMBINANT EXPRESSION OF ORGANOPHOSPHORUS ACID ANHYDROLASE IN BACTERIA: TOWARDS ENVIRONMENTAL BIOSENSORS** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from May 2018 to June 2019 under the supervision of Dr. Abhijeet Joshi , Assistant Professor, Discipline of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore and Dr. Prashant Kodgire, Associate Professor, Discipline of Biosciences and Biomedical Engineering, Indian Institute of Technology 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.

(PRIYANKA YADAV)

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

Signature of the Supervisor

(Dr. Abhijeet Joshi)

PRIYANKA YADAV has successfully given his/her M.Sc. Oral Examination held on **26.06.2019.**

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Date:

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Dedicated to

My Beloved Family

Abstract

The extensive use of organophosphorus compounds (OPs) in pesticides in order to increase the crop production has led to the accumulation of these highly toxic chemicals in soil water, and food chain. Also the terrorist events happening worldwide lead to the proliferation of the hazardous chemical agents like VX, VR (nerve agents) and chemical contamination outbreak in the food supply. OPs are the most commonly used insecticides and pesticides, which cause a wide range of long-lasting and life-threatening conditions. Due to the acute toxicity and long-term side effects of OP compounds, their timely, on-the-spot and rapid detection has gained importance, for the efficient healthcare management. In this respect, several OP degrading enzymes have gained the spotlight in developing the enzyme-based biosensors, owing to their high activity and broad specificity. Among these microbial enzymes Organophosphorus acid anhydrolase (OPAA) is able to hydrolyze the variety of bonds**,** like P-F, P-O, P-S and P-CN. OPAA is able to hydrolyze nerve agents and OPs which prompted our interest of using it as the potent agent which can be used for designing the enzyme based biosensor. There have been very few reported works of OPAA activity on paraoxon and methyl parathion and our work shows the increased activity of variant of OPAA (FL) on paraoxon than the previously reported works. In the present piece of work variant of OPAA is expressed, purified and tested for its activity against paraoxon. OPAA showed its optimum activity at pH 8.5 and 50° C. The purified enzyme is further immobilized into the alginate beads for the detection of paraoxon. Furthermore, to improve the detection limits, and sensitivity, free OPAA as well as beads of OPAA and alginate are employed in conjunction with FITC.

Keywords: Organophosphorus acid anhydrolase (OPAA), Paraoxon, Acetylcholinesterase (AChE), Chemical warfare nerve agents, Biosensor

LIST OF PUBLICATIONS

1. Jain, M., **Yadav, P**., Joshi, A., Kodgire, P. 2019, Advances in detection of hazardous organophosphorus compounds using organophosphorus hydrolase based biosensors. Crit Rev Toxicol. (in press)

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ABBREVIATIONS

AChE	Acetyl cholinesterase
BChE	Butrylcholinesterase
MPH	Methyl parathion hydrolase
OPAA	Organophosphorus acid anhydrases
OPH	Organophosphorus hydrolase
OPs	Organophosphorus compounds
PTE	Phosphotriesterase
BSA	Bovine serum albumin
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
FITC	Fluorescein isothiocyanate
SEM	Scanning electron microscopy
CD	Circular dichroism spectroscopy
OD	Optical density

Chapter 1

Introduction

In recent times, increased in population has led to higher demand for food supplies, thus necessitating increased yield from existing arable land, and expanding the types and quantity of agricultural produce. A direct consequence of the massive agricultural expansion is an increased use of pesticides in order to enhance the productivity of the crops. Over 5.6 billion pounds of pesticides are used globally every year (1). The most well-known commercial pesticides are classified broadly into three categories: organochlorines, carbamates, and organophosphates. Of these, organophosphates - also called phosphotriesters due to the presence of three phosphodiester bonds (2), are of substantial interest due to their low-cost of synthesis and high efficacy. Strikingly, OP-based compounds today constitute anywhere between 33% of available pesticides in developed countries and 50-60% in the developing world (3). Incidentally, these compounds exhibit toxicity when absorbed through the skin, ingested or inhaled. In fact, the World Health Organization reports show that around 200,000 people suffer annually from pesticide related poisoning due to direct or indirect exposure to organophosphorus compounds (OPs) (4). For researchers in medical and environmental science, the use of OPs has been of particular concern due to its lower biodegradability compared to newer pesticides and their tendency to block the active site of important enzymes in neurons such as acetylcholinesterase (AChE) and butrycholinesterase (BChE), which break down and removes acetylcholine from the nerve synapse (5), (6). Thus, the inability to break down acetylcholine results in neurological problems such as convulsions, tremors, and even death (7). Although OP poisoning was found to be treatable using a combination of atropine and pralidoxime

(8), such medication has several side effects (9) and is not as effective in patients with prolonged exposure due to an effect known as the aging effect, in which a non-enzymatic loss of alkyl chain from the phosphate group in the OP compound renders the inhibited/blocked AChE into a non-reactivatable form (10). Strikingly, Poisoning by OPs at about 10-20% has a high rate of mortality that exceeds normal expectations for exposure to toxic chemicals such as mercury, cyanide, and cadmium (11) and hence is a cause of concern for healthcare professionals to this day (12), necessitating measures to facilitate convenient and accurate detection of OPs in humans and environment.

Initially separation method like chromatography was used for the pesticides analysis in the environment. But it prevented adequate monitoring and had other limitations (13). Various detoxification methods for pesticides like landfill and incineration were also employed. However, these were adequate for a very short period of time because after sometime pesticides leak into the surrounding, soil, water bodies causing further concern (14). For the degradation purpose of pesticides incineration was employed by the US Environment Protection Agency (EPA). But because of the requirement of high temperature to destroy pollutant, high energy requirement, cost and toxic emission of pesticides it met the great opposition by the public (15).

An alternative method of pesticides detection was promoted many years back using enzymes. The main enzymes that were utilized at that time were AChE and BChE (16), (17), (18), (19). Both the enzymes play a key role in the functioning of the nervous system (13). The OPs compounds and nerve agents were detected based on the inactivation of the enzymes in proportion to the OPs and nerve agents present. Based on this property of the OPs various inhibition based biosensors have been designed for the accurate detection of the OPs (20,21). But AChE and BChE are also inhibited by various other chemical agents apart from nerve agents and OPs such as hypochlorite, detergents, carbamates, heavy

metals, fluoride, and nicotine so one cannot rely on the detection of the inhibition based biosensors using AChE and BChE (22). Also, it involves prior clean up procedure of instrument, require tedious process of extraction so not so suitable for the detection of the OPs and nerve agents on the daily basis. So there is need for the development of the biosensor that is rapid, selective, and sensitive for the detection of presence of OPs and nerve agents.

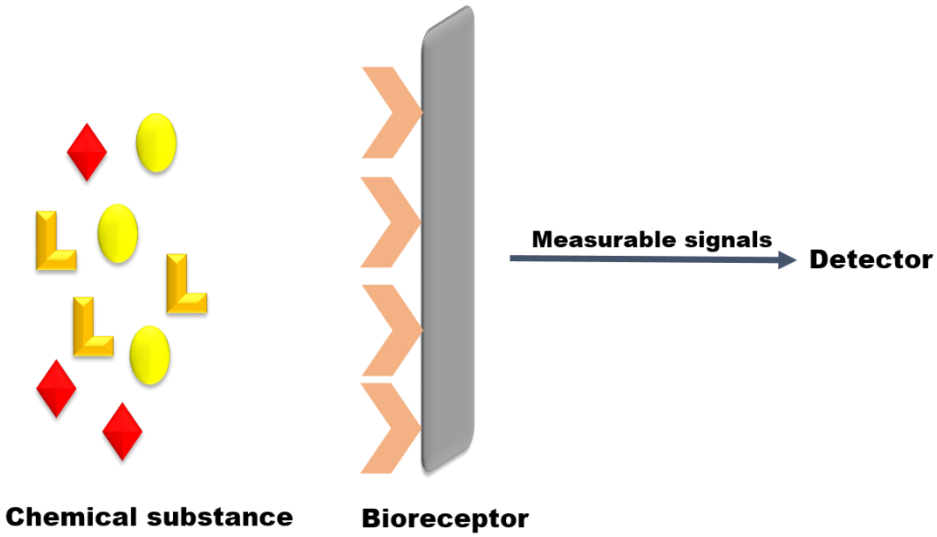


Fig. 1.1: **Diagrammatic representation of biosensor.** Biosensors includes two components, a biological component and a physiochemical detector. The biological component can be protein or the whole cell immobilized (represented as bio receptor) which on reacting with the analyte (represented as chemical substances) produces signal which are amplified and detected.

In 1971, first evidence of biodegradation of pesticides was reported (23). But the enhanced impact of the biodegradation was observed after mid 1980s in the field. And since then number of microbial enzymes (like Phosphotriesterase) have been used for the purpose of detection and detoxification of the OPs and nerve agents. Now in today's

world enzymes are the most commonly utilized biological recognition considering the ease of application and translation (24). **Fig. 1.1** shows the diagrammatic representation of one such method of using enzyme for the detection purpose of OPs and nerve agents.

Chapter 2

Literature review and Problem Formulation

2.1 Organophosphorus compounds (OPs)

OPs are also known as phosphotriesters because of the presence of the three phosphodiester bonds. Structurally OPs have alkyl or aryl groups that are either bonded to phosphorous via oxygen or sulfur link or they are directly bonded to the phosphorus. Leaving group attached to the phosphorus may be an aromatic group, halogen, aliphatic or heterologous cyclic group (25). The central atom phosphorus, if bound to the sulphur then the OPs are called as thions and if the central atom is bound to the oxygen then such OPs are called as oxons as shown in **Fig. 2.1** (26). General structure of OPs includes R_1 and R_2 that are alkyl or aryl groups either bonded to phosphorous via the oxygen or sulfur link or they are directly bonded to the phosphorous. X group attached to the phosphorous may be an aromatic group, halogen, aliphatic or heterologous cyclic group (2,25). It has been reported that continuous or long exposure of the OPs leads to the deposition of these compounds at the depot site, from where they are uninterruptedly released into the blood and from there they reach the nervous system where their target is the AChE enzyme. These compounds inactivate family of serine hydrolyze (lipases and esterases) that are crucial for proper functioning of human body (26).

2.2 Nerve agents

Nerve agents belong to the class of organic chemicals and sometime are also known as nerve gases. These are reported to be used as chemical warfare agents during wars, and even in terrorist attacks. Nerve agents are classified into two categories G-agents and V-agents. The nerve agents like soman, sarin, tabun, and cyclosarine are among the highly toxic nerve agents that altogether compromise the G-series nerve agents

(27). The V-series includes VX, VR where V stands for venomous (28). Nerve agents, and OPs compounds, block an enzyme known as acetylcholinesterase (AChE), which is responsible for the breakdown of the neurotransmitter, acetylcholine (ACh) by disrupting the mechanism of the nerve signal transfer to the target organ. Disruption of this mechanism is also known as the nerve agent poisoning. Nerve agents are generally tasteless, appear colorless to amber colored and liquid in nature that may vaporized to gas. Agents like soman has a camphor like odor, tabun has slightly fruity odor, VX and sarin are odorless (29).

The first symptoms of poisoning appears within seconds of exposure to the nerve agents resulting in the profuse salivation, involuntary urination, constriction of pupils, convulsion and defecation. Death may also occurs because of loss of control over the respiratory muscles and other muscles of the body resulting in the cardiac arrest or asphyxiation (21,30). There are different ways by which nerve agents can enter inside the human body. Primary way of entering into the body is through the respiratory system as nerve agents are easily aerosolized or vaporized. These agents can also be absorbed through skin. People subjected to the nerve agent exposure should wear respirator along with full body suit (27).

The metabolism OPs and nerve agent in the human body follow same fate. When a person accidently or deliberately inhale, ingest or absorb the OPs, they reach either to its depot site like muscles, adipose tissue, liver, kidney, or gut or it may get distributed to the central nervous system through the blood circulation. The amount that remained in the blood is eliminated out via urine, expired air or in faeces. *(31,32)* as shown in **Fig. 2.2.**

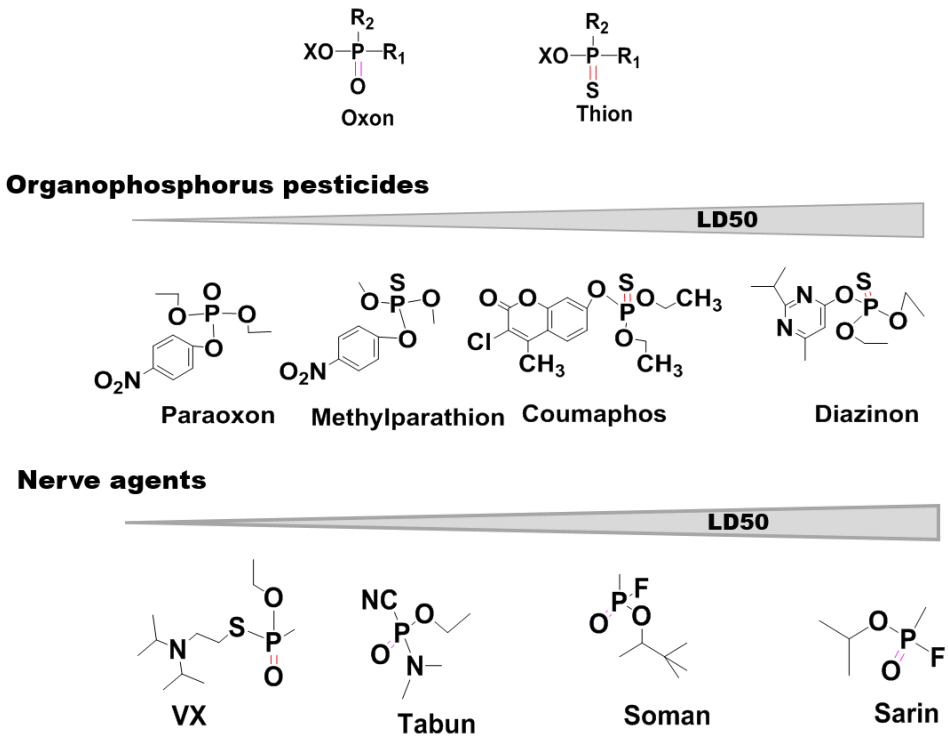


Fig. 2.1: Different organophosphorus compounds and nerve agents. General structure of OPs: R1 and R2 are alkyl or aryl groups that are either bonded to the central atom phosphorous via the oxygen or sulfur link or they are directly bonded to the phosphorous. X group attached to the phosphorous may be an aromatic group, halogen, aliphatic or heterologous cyclic group (2,33).

2.3 Effect of OPs on AChE

AChE is an enzyme that is known for the hydrolysis of acetylcholine, a neurotransmitter that is involved in the transfer of nerve impulses to the effector cells at cholinergic, synaptic as well as neuro-muscular junctions. Several researchers have indicated that OPs mainly interact with the AChE active sites (residues Ser203, His447, and Glu334) through phosphorylation, wherein a covalent bond formed between the central phosphorous atom of the ligand and an oxygen from the side chain of a serine residue as shown in **Fig. 2.3** (34). OPs bind to the hydroxyl

group of the serine at active site to form the phosphorylated enzyme resulting in the accumulation of the Acetylcholine at the synapses because inactivated AChE is unable to hydrolyse acetylcholine (2,35).

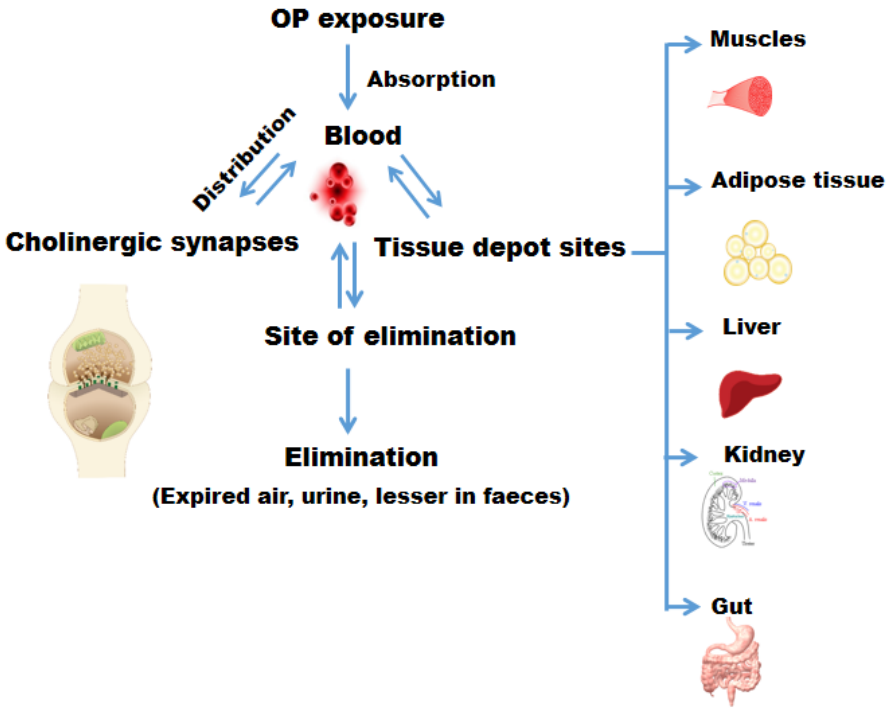


Fig. 2.2: **Fate of OPs and nerve agents after entering into the body.** When a person accidently or deliberately inhale, ingest or absorb the OPs and nerve agents they either get distributed to synapses or tissue depot site, and traces remain of OPs and nerve agents eliminate out

In some cases, the reaction may continue further, causing an alkyl chain loss from the resulting phosphyl alkoxy substituent. This undertaking is known as 'aging' and prevents reactivation of AChE by conventional therapy as shown in **Fig. 2.4**. Once AChE is inactivated, the enzyme loses its ability to hydrolyze acetylcholine, and continuous stimulation is caused by the build-up of acetylcholine in muscle or nerve fibers, causing exhaustion and/or tetany (6). It is speculated that the

overstimulation of glutamate receptors caused by inhibition of both targets AChE and non-AChE causes convulsions, cardiac collapse and respiratory arrest, whereas massive histamine release may be responsible for anaphylactic shocks (36).

Not only AChE OPs also effect certain receptors like thyroid receptor, androgen receptor and estrogen receptor. The long term effect of disrupting endocrine receptor is still unknown (37), (38), (39), (40), (41), (42), (43), (44), (45), (46). Thus, OPs target, not only AChE but also other enzymes, which make these a severe health hazard and thus warrants urgent development of detection as well as remediation techniques (36).

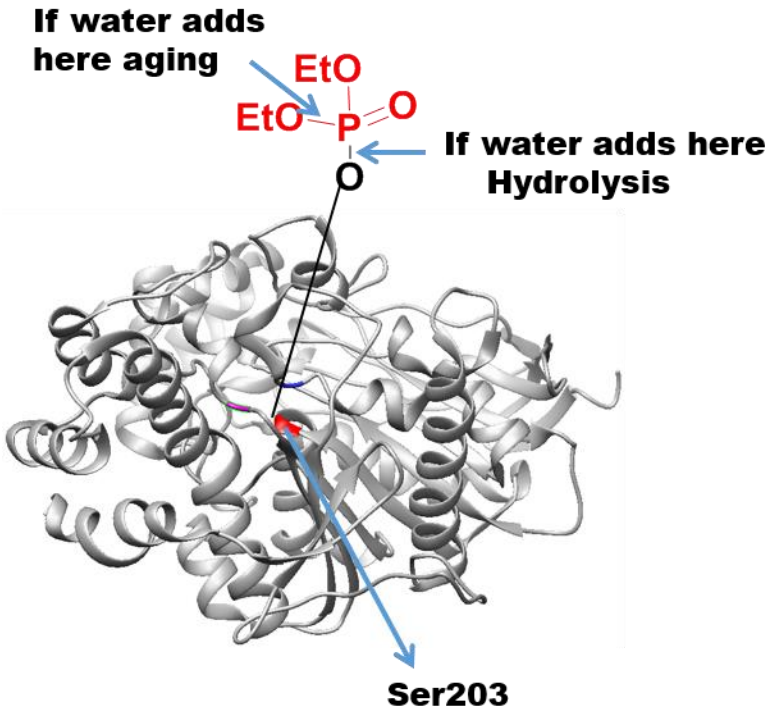


Fig. 2.3: **Structure of human Acetylcholinesterase (PDB ID-4PQE).** OPs binds to the AChE active site with the hydroxyl group of serine at 203 position resulting in phosphorylation of AChE.

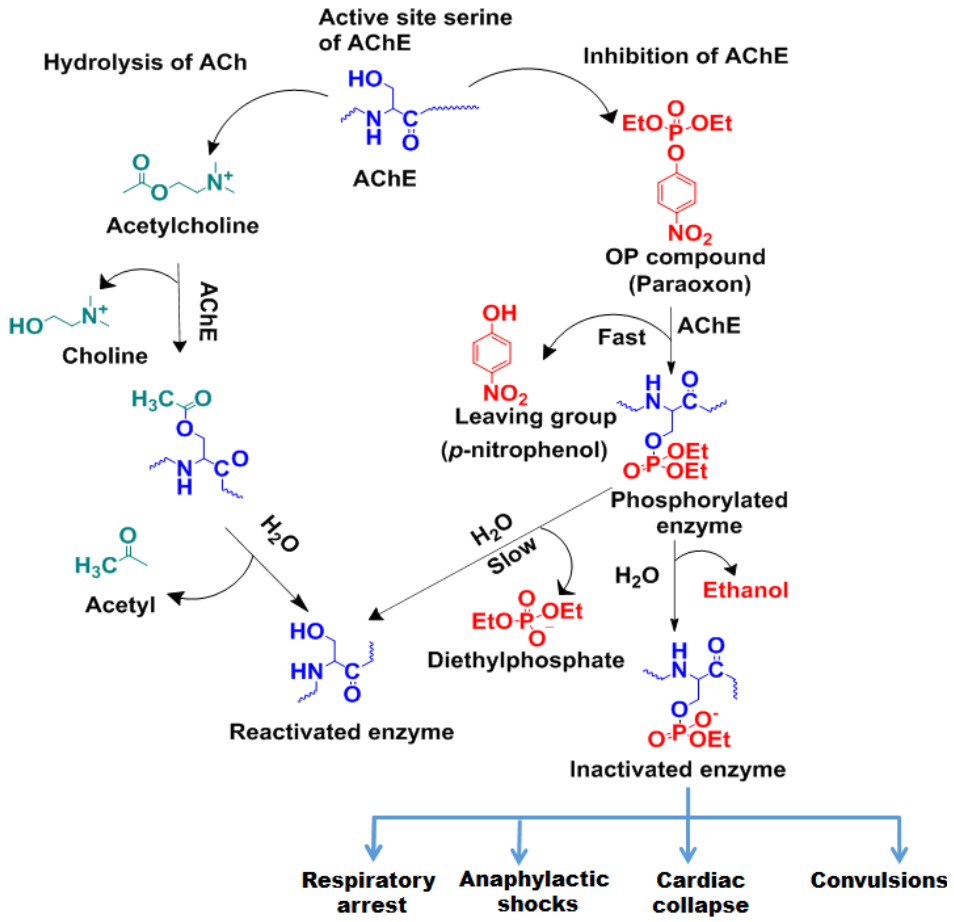


Fig. 2.4: The general mechanism of Acetylcholine hydrolysis by AChE and inhibition of the enzyme on OPs. OPs bind to the hydroxyl group of the serine at active site to form the phosphorylated enzyme resulting in the accumulation of the Acetylcholine at the synapses because inactivated AChE is unable to hydrolyze acetylcholine

2.4 OPs hydrolyzing enzymes

There are numbers of methods known for the degradation of OPs like incineration and chemical neutralization but, among all the advanced technologies known microbial enzyme mode of degradation is the safest. Although the OPs degrading enzymes have been identified in numbers of organisms including mammals, however, the microbial enzymes have

sought more attention and have been found to be more potent in degrading Ops (47). OPs degrading enzymes was first time reported in 1946 by Mazur. These enzymes were later categorized in the category of Phosphotriester hydrolase by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. These enzymes were further classified based on their substrate specificity (35). OPs can be degraded naturally by microbial enzymes such as OPH and methyl parathion hydrolase (MPH) into less toxic or non-toxic compounds like *p*-nitrophenol and diethyl phosphate (48). Although OPH and OPAA are the most studied and well-categorized among all OP degrading enzymes, other enzymes have also been reported which are capable of detoxifying OPs (4).

(i) Paraoxonase (PON)

PON enzyme in human serum has the ability to degrade paraoxon and nerve agents. It is a single chain beta class metalloprotein with central Calcium binding site, 4 helices, 5 sheets and 6 bladed beta-propeller (**Fig. 2.5 B**) (49). Human paraoxanase preferably cleave bonds like P-O, P-C, P-F, P-CN (50). Mammalian paraoxanase is found to be more productive for the degradation of some OPs like cyclosarin and soman than DFPase and OPH.

(ii) Methyl Parathion Hydrolase (MPH)

MPH belongs to a family of metallo beta lactamase and is an aryldialkylphosphatase. The first *mpd* encoding gene for MPH was discovered in *Plesiomonas sp. strain M6* (51). Another closely related gene was identified and sequenced from *Pseudomaonassp*.WBC3. Its structure revealed that it is a Zn (II) containing dimeric enzyme. : It is two chain metalloprotein with 20 helices, 7 sheets and Zinc central atom. It belongs to the class of beta and alpha proteins (52) (**Fig. 2.5** D). It catalyzes the degradation of methyl parathion. Each subunit consists of

hybrid binuclear Zn in which most solvent exposed beta metal cation is replaced with Cd. It is found to be homologous to other metal beta lactamases but does not show any similarity to Phosphotriesterase (51).

(iii) Diisopropyl-fluorophosphatase (DFPase)

DFPase is a 35 kDa protein obtained from the brain of *Loligo Vulgaris squid*. DFP is the main substrate and hydrolyze P-F bond releasing isopropyl phosphate and fluoride. It is a single chain Calcium-dependent Phosphotriesterase with 1 helices and 4 sheets in it structure, that belongs to the class of beta protein having 6-bladed beta-propeller (**Fig. 2.5 E**) (53).Two highly effective calcium sites are required for its stabilization and catalysis function. To identify the residues important for the active site and to interpret the mechanism of enzyme in the reaction, site directed mutagenesis and kinetic studies were performed (53-56). Three histidines were found to play an important role at the active site, in which two histidine H274 and H174 have the role of stabilization and residue H287 was found to behave as a general base catalyst (51).

(iv) *Escherichia coli* (*E. coli*) Aminopeptidase P (Pep P)

It is an exopeptidase of 49.65 kDa that cleaves at the amino terminal only if the following residues are Proline. It is metalloprotein in *E. coli* that consists of 4 Pep monomers and requires two Mn ions for its activity (26). The enzyme adopts “pita bread” fold like creatinase and methionine aminopeptidase that function as a tetramer (57). The two manganese ion at C terminal in the active site of the β sheet is separated by 33Å (**Fig. 2.5 F**). The two ions bridge together via hydroxide ion which acts as the nucleophile in the attack of the peptide bond (58). Its substrate for degradation includes methyl isobutyl and methyl isopropyl groups (26). It shows only 31% sequence similarity with OPAA (4).

(v) Organophosphorus Hydrolase (OPH)

OPH enzyme is isolated from soil microorganisms such as *Pseudomonas diminuta* and *Flavobacterium.* It is a homodimer metalloprotein which is a member of superfamily amidohydrolase. OPH is also known as Phosphotriesterase, organophosphorus degrading enzyme and parathion hydrolase. This enzyme is encoded by the phosphate degrading gene (*opd)* located on the extrachromosomal plasmid (35). It has broad substrate specificity and is known to hydrolyse P-CN, P-O, P-S and P-F bond (48). It utilizes a divalent ion such as $Co^{2+,}$ $Zn^{2+,}$ $Mg^{2+,}$$Ca^{2+,}$ and Fe^{2+} etc. for the nucleophilic attack by activating the hydrolytic water molecules (57). The high resolution X-ray structure showed that the protein folds into αβ barrel motif also known as TIM Barrel (**Fig. 2.5 A**) (35). PTE adopts TIM barrel fold where the active site is present at C terminal of the β barrel (57).

2.5 Organophosphorus Acid Anhydrolase (OPAA)

OPAA is an enzyme isolated from the extract of halophilic *Alteromonas* species and is found to be able to hydrolyze a large number of OPs. It is a monomeric 48 kDa polypeptide metalloprotein having Mn in its natural form. It is a three chain metalloprotein having 54 helices and 6 sheets (**Fig. 2.5 C)**. Protein is capable of performing biological process like depshosphorylation, proteolysis and respond to the toxic substances (59). It has three pockets closer to the binuclear active site. The small pocket is composed of Tyr212, Val342, His343 and Asp45. And the large pocket is made up of residues Leu225, His226. His332, and Arg418. Residues Tyr292 and Leu366 formed the leaving group. It is able to hydrolyze organophosphate triesters and the protein appears to be dipeptidase (35). It displays stereo specificity similar to phosphotriesterase (PTE). It also shows high activity in hydrolyzing the P-F bond in sarin,

soman, and DFP among others. However, it is less effective in hydrolyzing P-CN, P-O bond (35).

Thousands of fatalities in the developing world due to pesticides poisoning along with the prospective threat of intentional release of nerve gases has made the treatment of such poisoning a matter of great concern and there is urgent need to focus for the therapeutics intercession (11,60). Combinations of few drugs are used currently to treat nerve agents poisoning such as Benzodiazepines to treat seizures, pralidoxime (also known as 2-PAM) to reactivate the inhibited acetylcholinesterase, atropine a muscarinic antagonist (30).

Recently, another method has been emerged for treating organophosphate poisoning via using potential catalytic enzyme that can detoxify these poisons in the blood. These catalytic enzyme particularly hydrolyze the organophosphates into nontoxic compounds before they act on the acetylcholinesterase at the neuromuscular junction. Along with the current treatment of using combination of drugs this approach of using hydrolyzing enzyme work in a complimentary manner to improve the survival rate.

2.6 OPAA based biosensor

Biosensors are the analytical devices used for the detection of chemical substances that combines biological components with the physiochemical detectors are called biosensors. Biosensors have emerged as leading analytical techniques owing to miniaturization of transduction, development of microelectronic circuitry and interfacing using bio-recognition units. Cost effective and miniaturized analytical tools have led to the transformation of sophisticated instruments into the point of care devices. Enzymes are the most commonly utilized biological recognition considering the ease of application and translation.

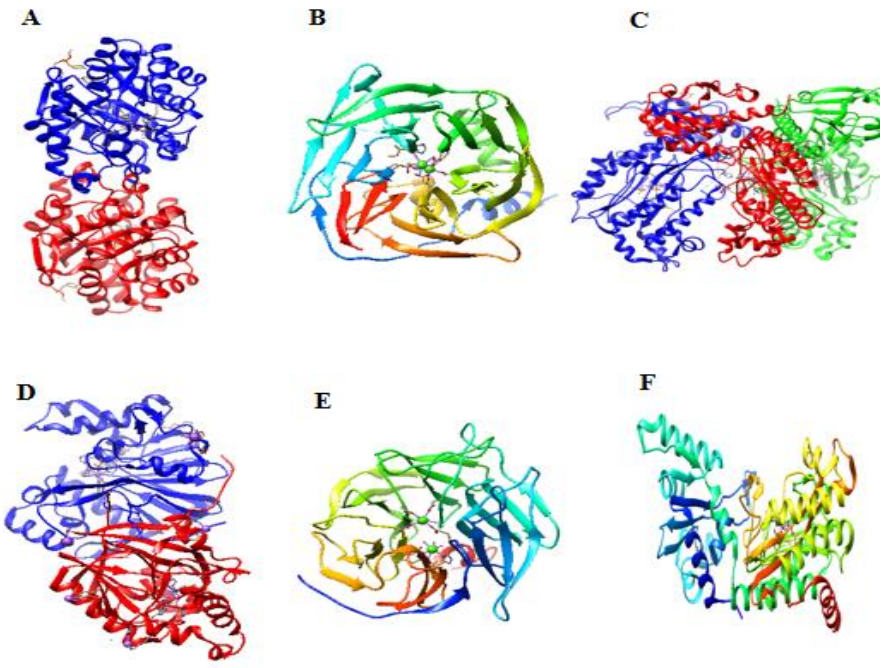


Fig. 2.5: Structure of degrading enzymes. (A) PTE from Pseudomonas diminuta (OPH) "1HZY": Two chain metalloprotein with 46 helices and 6 sheets and the Zinc as its central atom. It belongs to the alpha and beta protein class having a TIM beta/alpha barrel-fold (61) (B) Serum Paraoxonase "1V04": Single chain beta class metalloprotein with central calcium binding site, 4 helices, 5 sheets, and 6 bladed beta-propeller fold (49). (C) OPAA "3L7G": It is a three chain metalloprotein having 54 helices and 6 sheets with central atom Manganese in its structure. Protein is capable of performing biological process like depshosphorylation, proteolysis and respond to the toxic substances (59). (D) MPH "1P9E": It is two chain metalloprotein with 20 helices, 7 sheets, and Zinc central atom. It belongs to the class of beta and alpha proteins (52). (E) DFPase "1E1A": It is a single chain Calcium-dependent PTE with 1 helices and 4 sheets in its structure, that belongs to the class of beta protein having 6-bladed beta-propeller (53). (F) Aminopeptidase P "1WL9" from E. coli, a protease with manganese at its center comprising of two domains.. It belongs to the class of alpha and beta proteins (57).

Typically, the detection systems used in conjunction with biological components can be classified into electrochemical (potentiometric, amperometric, and conductometric) and optical (UV–visible and fluorescence) systems of measurement. Detection systems employing potentiometric measurement utilize changes in potential with corresponding changes in current which is the result of reduction and oxidation potential of the electrochemical reaction. On the other hand, amperometric detection systems work on the basis of changes in current according to changes in chemical concentrations of interest. They typically utilize two/three electrodes systems constituted as a reference, a working electrode and an auxiliary electrode. Amperometric detection systems are believed to be superior due to the linear increase which solves the perils, such as salt screening and exponential or logarithmic increase with the concentration in a potentiometric detection system. The conductometric detection systems measure resistivity or electrical conductivity of analyte samples based on the ions and electron numbers. The effect produced is also influenced by temperature and pH. Electrical impedance is another phenomenon utilized by detection systems wherein the resistance of biological component and ionic conductance across it is utilized for the measurement. The optical detection systems typically utilize photons as mediating components in electrical transduction in comparison to direct use of electrons in electrochemical detectors. Intensity, quenching efficiency, decaying time, anisotropy, radiant energy transfer, etc. are the important parameters measured while using absorption, reflectance or fluorescence using visible, ultraviolet (UV), near-infrared (NIR) radiations in photometric measurement. Some optical detection tools also work on the principle of evanescent waves and the phenomena known as total internal reflection, occurring due to the changes in the refractive index. Some hybrid mechanisms like electro chemiluminescence, amperometric-

potentiometric, piezoelectric, and magneto-elastic detection systems are also utilized in several reports of biosensors.

The OPAA enzyme isolated from an aerobic, short rod, gram negative bacterium *Alteromonas sp.* has drawn a keen focus due to its efficiently high expression in *E.coli* as well as its high activity against nerve agent GD (62).

A group of scientist in the year 2000, proved that by encapsulating OPAA in conjugation with 2-PAM and atropine in lysosome to provide 25 LD_{50}s of protection against DFP (63).

A broad spectrum antidote and an effective enzymatic antidote to nerve agents should have a cocktails of enzymes that not only catalysis the racemic material efficiently, but should also be specifically directed towards enantiomers that are more toxic, of all the relevant substrate. In the year 2012, proper stereochemistry was coupled with the good catalytic efficiency with the H257Y/L303T mutant of phosphotriersterase microbial enzyme for G agent substrates (64). The variant so created had approximately 10 times greater catalytic efficiency than that of wild type PTE on cyclosarin and sarin and approximately 100 times greater catalytic efficiency on soman. It also possessed greater activity on P(-) or S_p enantiomer than the less toxic P(+) or R_p enantiomer of cyclosarine because of reversal stereospecificity preference than that of the wild type PTE.

In the year 1993, Luqi Pie and group determined OPAA in blood. To develop a pioneered approach OPAA was encapsulated in erythrocytes. OPAA activity was determined with paraoxon as substrate. Activity was reported based on the amount of liberation of p-nitrophenol and diethylphosphate formed during the reaction. Detection limit was reported 0.01 mM of p-nitrophenol (65).

In 2004, Simonian and his group invented a new approach of using a multienzyme strategy for the detection of various classes of neurotoxins based on the substrate specificity of the enzymes. OPAA was used as a

modified pH sensitive field effect transistor. Sensor response of DFP was reported and linear order kinetics was found between 12.5 to 500 μM concentration of DFP whereas barely any measurable signals was reported in case of paraoxon (66).

Before 2015, there was no report of OPAA action against V-type nerve agents and there were no previous report of its engineering (67). Out of 517 amino acids of OPAA from *Alteromonas sp.* only 440 found to affect the activity of the enzyme. Truncation of 77 amino acids from the C-terminal had shown not to affect the activity of the enzyme (67). Thus OPAA structure was engineered by Steven P. Harvey's group to improve the catalytic efficiency of the enzyme. Using site directed mutagenesis created few mutations in the small pocket in order to increase the specific activity of the enzyme. Two mutants FL (Y212F and V342L) and FLYD (Y212F, V342L I215Y, and H343D) were produced (67). The mutant of OPAA so produced possessed more than 30 fold enhancement in the catalytic efficiency on VR racemic.

In the 2017, Xiao Yunzhu and his group showed that OPAA obtained from the coastal sediment of south China form *Pseudoalteromonas sp.* SCSIO 04301 (GIMCC 1.828) activity on methylparaoxon and paraoxon. They reported specific activity OPAA against methylparaoxon approximately 0.0314 U/mg and specific activity against paraoxon was found approximately 1.604 U/mg (68).

Earlier in 2003, Sarita V. Mello and her group build a biosensor in which they developed a Langmuir and Langmuir-Blodgett (LB) monolayer film of OPAA. One layer of OPAA was labeled fluorescein isothiocyanate (FITC) a fluorescent probe. It was deposited on to the quartz slide and tested against DFP as sensor. The stability of the LB film as sensor showed the potential of the system as a biosensor and a clear pronounced response was reported (69). On hydrolysis of the bond, it generates 2 protons and alcohol, which are often electroactive or

chromophoric (4,70). The two proton produced reduces the pH of the environment and thus drop the intensity of the FITC.

So we hypothesized that FL, mutant of OPAA if used against paraoxon may show increased catalytic efficiency against paraoxon and can be used for the development of the potential biosensor against OPs and the nerve agents.

2.7 Objective

The microbial enzyme attracted widespread attention not only because it is economic but also for environment benefits. Biodegradation of the OPs reduced mammalian toxicity by several magnitudes. OPAA, can be combined with pH electrode or a field-effect transistor based pH indicator to measure the protons produced or optical transducer to quantify the amount of *p*-nitrophenol produced during hydrolysis based on the chromophore produced on hydrolysis of paraoxon (70). Free OPAA or immobilized OPAA can also be employed in conjugation with Fluorescein isothiocyanate (FITC) which expected to shows decrease in its fluorescence intensity with drop in pH as shown in **Fig. 2.6**. OPAA has been proved to be an effective biocatalyst for hydrolysis of OPs and nerve agents, owing to which it has been considered for the purpose of detection and/or remediation of OPs (51). So there is need of development of an enzyme based biosensor which is safe, non-corrosive, cheaper, more user friendly, rapid, stable, sensitive, selective and healthy technology.

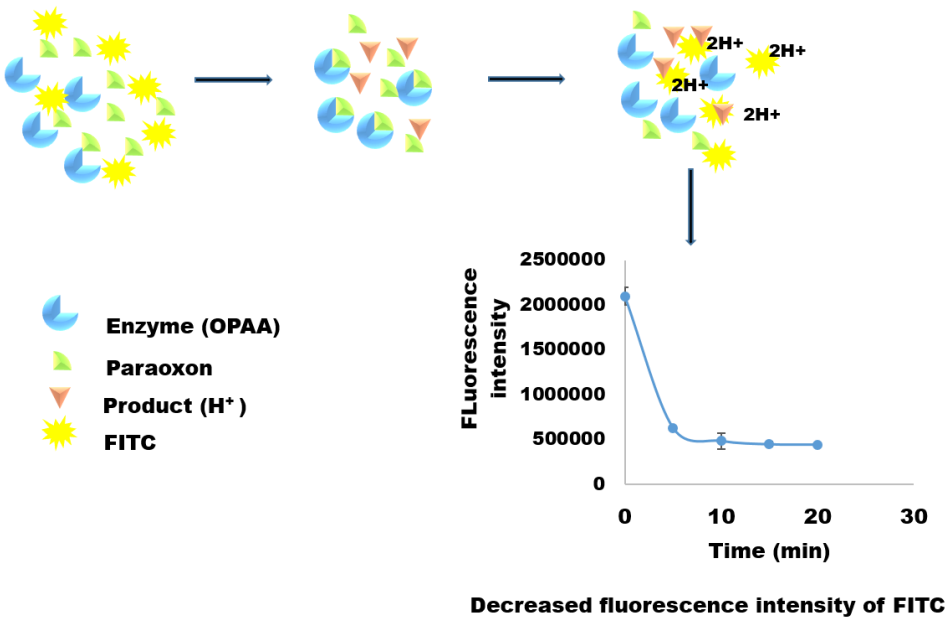


Fig. 2.6: **Diagrammatic representation of detection of OPs in FITC conjugated OPAA.** Reaction mixture containing paraoxon on hydrolysis by recombinant OPAA in presence of FITC drop the pH of the reaction mixture resulting in the intensity drop of the FITC.

Chapter 3

Materials and Methods

3.1 Materials

(i) Strains: LB/ Amp plate streaked with FL was obtained from Prof. Steven Harvey, U.S. Army Edgewood Chemical Biological Center, MD, 21010-5424, USA. The *E. coli* cell lines, DH5α and Rosetta were used as host cells for cloning and expressing OPAA. pET28a vector (Novagen) used for cloning and expressing the protein.

(ii) Chemicals: Favor Prep plasmid extraction mini kit, GeneJET gel extraction kit (Thermo Scientific), GeneJET purification kit (Thermo Scientific), Agarose special low EED (HiMedia), Luria Bertani Broth Miller (HiMedia), Kanamycin (HiMedia), *Nde*I and *Bam*HI. (NEB). dNTPs, Ligase, *Pfu* DNA Polymerase, EtBr, Lysozyme ,Tris-Cl, NaCl, Imidazol, Bis Tris Propane buffer, methylparathion, paraoxon (Sigma Aldrich), $MnCl_2$, $CaCl_2$, Alginate, Bradford (HiMedia), FITC dextran, FITC isomer, DMSO were used for cloning, expressing and purification and activity of recombinant OPAA gene.

3.2 Methods

3.2.1 Plasmid isolation

E. coli cell having the required plasmid was grown in Luria Bertani broth (from HiMedia) containing antibiotic Kanamycin (from HiMedia). The plasmid was isolated from *E. coli* cell using favor prep plasmid extraction kit. The well grown bacterial culture was centrifuged at 11000 x g for 1 min to pellet down the cells. The pellet was then washed with STE buffer (5M NaCl, 1M Tris, 0.5M EDTA). FAPD1 buffer containing RNase A was added to the washed pellet and the cells were resuspended by pipetting. To the resuspended cells, FAPD2 buffer was added, mixed

with the cells by gently inverting the tubes 5-10 times and to lyse the cells tubes were incubated at room temperature for 2-5 minutes. FAPD3 buffer was added to the lysed cells and the cell lysate was neutralized by gently inverting 5-10 times immediately.to clear the lysate the tubes were centrifuged at full speed. The supernatant so obtained was then carefully transferred into the FAPD column and centrifuged again at 11000 xg for 30 seconds. The flow-through was discarded followed by addition of W1 to the FAPD column and the column was again centrifuged at 11000 xg for 30 seconds. The flow-through so obtained is discarded and wash buffer is added followed by centrifugation for 30 seconds at 11000 xg. The flow-through was discarded and the column was centrifuged at high speed for an additional 3 min to dry the column. The FAPD column was then placed into a fresh microcentrifuge tube and the plasmid was eluted in 50 µl of elution buffer by adding the elution buffer to the center of the membrane.

3.2.2 Polymerase chain reaction (PCR)

The template DNA was amplified using primer PK702F and PK706R (Sigma) by *Pfu* DNA Polymerase. The master mix was prepared using primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, template DNA, and *Pfu* DNA polymerase. The master mix was aliquot into PCR tubes. The negative control was prepared similarly but without template DNA. Program of thermo cycler was set for PCR reaction with initial denaturation at 95° C followed by 30 cycles of denaturation at 95° C for 2 minutes and 30 seconds respectively, annealing at 59° C for 30 seconds and extension at 72° C for 2 minutes. Finally, the final extension was set at 72° C for 10 minutes. The PCR products were analyzed by agarose gel electrophoresis.

3.2.3 Colony PCR

Colony PCR was performed to confirm the ligation of the insert into the vector. A colony of bacteria was initially replica plated on a fresh

plate and then added as the template instead of the DNA to the master mix of the PCR following the same procedure as described for the PCR.

3.2.4 Restriction Digestion of the DNA

Sequential digestion of pET28a vector and double digestion of OPAA was done using enzyme *Nde*I and *Bam*HI as per manufactures instructions at 37° C for 60 minutes, followed by heat inactivation of the enzyme at 65° C for 10 minutes.

3.2.5 Gel elution of digested DNA

The digested DNA fragments were extracted from the gel using GeneJET purification column. The agarose gel having the DNA fragments was cut into pieces using clean scalpel and the pieces were placed into the fresh weighed microcentrifuge tubes and weight of the gel slice was recorded. Equal volume of the binding buffer is added to the gel. To completely dissolve the gel, gels were incubated at 50° C -60° C and the tubes were invert mixed until the gel was completely dissolved. The gel solution was transferred to the column and centrifuged for 1 min. The flow-through so obtained was discarded and the column was washed with the wash buffer by centrifuging them for 1 minute. The column was centrifuged for additional 1minute to completely remove the residual wash buffer. The DNA was eluted in 50 µl of elution buffer and stored at -20° C.

3.2.6 Competent cell preparation

Competent cells were prepared by using $CaCl_2$. Cells were grown overnight in Luria Bertani broth without any antibiotic. Overnight grown bacterial culture is again inoculated for secondary culture into the LB broth in 1:100 ratio and incubated at 37° C till the culture's OD reached 0.4-0.5 at 600nm. All the subsequent steps were then done on the ice. Log phase cell culture was first incubated on ice for 10 minutes and then

centrifuged at 4° C for 10 minutes to pellet down the cells at 5000 rpm. Cells were then suspended in chilled $CaCl_2$ (10 ml of $CaCl_2$ for 25 ml of cell culture) and were incubated on ice for 45 minutes. Cells were again pellet down and cells were gently suspended into 2 ml chilled $CaCl_2$. After 4 hours incubation on ice cells was aliquot into prechilled microcentrifuge tubes with 15 % prechilled glycerol. Cells were then stored at -80° C.

3.2.7 Ligation

Predigested DNA vector and insert with *Nde*I and *Bam*HI were ligated using T4 DNA Ligase (New England Biology) and 1x ligation buffer. The reaction mixture was incubated at 22° C for 1 hour followed by heat inactivation at 65° C for 10 minutes.

3.2.8 Transformation

The transformation was done by the heat shock method. Competent cells were though on ice for some time. The required amount of DNA to be ligated were taken into the microcentrifuge tube and given heat shock by incubating tubes at 42° C for 90 seconds followed by 120 seconds incubation on ice. 4 times of competent cell, LB broth was added to each tube and incubated for 90 minutes at 37° C. Transformed cells were then spread on to the LB Kanamycin plate.

3.2.9 Agarose gel electrophoresis

DNA, RNA can be separated in agarose matrix by applying electric fields. The shorter molecule travels the larger distance in the agarose gel and the larger molecule travels the shortest distance. For preparing wells into the gel, molten agarose with a fluorescent dye Ethidium bromide (EtBr) for staining the sample were poured into the casting tray of electrophoresis unit and allowed to solidify. EtBr intercalates into the rings of DNA which can then be seen under UV. The electrophoresis unit is filled with the 1X TAE buffer and the samples were

mixed into the loading dye then loaded into the wells. The loading dye contains glycerol to give density to the sample and bromophenol blue to track the movement of the sample into the gel. Electric fields were then applied to separate the sample of different size from the mixture. The run gel was then analyzed under transilluminator.

3.2.10 Expression of recombinant *E. coli*

A single colony of transformed cells were inoculated into LB broth containing 0.01 mg/ml Kanamycin and were grown overnight at 37° C. The secondary culture of the cells was done in fresh LB broth and cells incubated on incubator shaker at 37° C until the OD reaches 0.8. The culture was then induced with 1 mM IPTG and 1.9 mM $MnCl_2$ and incubated at 20° C for 20 hours on the incubator shaker at 220 rpm. The cells were then harvested by centrifuging the cell culture at 8000 rpm for 10 minutes. The pellet obtained was then washed and suspended into the 50 mM Tris buffer (pH 7.4) with 10 % glycerol. To lyse the cells 400 µg/ml lysozymes were then added to the resuspended cells and incubated at 30° C for 20 minutes followed by sonication of 1 minute cycle at 50 % Amplitude and centrifugation of cell lysate at 14000 rpm for 10 minutes. To confirm the induction 12 % SDS PAGE gel was then run.

3.2.11 Purification of the recombinant OPAA

Protein was purified using affinity chromatography (Ni NTA sephrose beads) at 4° C. The input sample was prepared such that it had the composition as the equilibration buffer (500 mM NaCl, 50 mM Tris of pH 8, and 10 mM Imidazole). Input sample was then loaded into the pre equilibrated column containing Ni NTA tagged sephrose beads and incubated for 2 Hrs. on shaker at 100 rpm. The column was then centrifuged in swinging bucket centrifuge at 100 xg and the flow through was collected. The column was then washed with W30 (50 mM Tris of pH 8.0, 500 mM NaCl, and 30 mM Imidazole) and W50 (50 mM Tris of pH

8.0, 500 mM NaCl and 50 mM Imidazole). The purified protein was eluted using elution buffer (50 mM Tris of pH 8.0, 500 mM NaCl and 150 mM Imidazole). Affinity chromatography derive the benefits from the difference in the interaction between biomolecules in the mobile phase and the stationary phase. Here, the stationary phase was Ni-NTA tagged sepharose beads and the mobile phase is the crude extract of protein loaded. The protein of our interest has His tag at its N-terminal so it binds to the stationary phase and the other protein elute out first by removing the weaker interaction in the washes. Target protein binds with higher affinity to the stationary phase and does not come out with the washes. The elution buffer overcome the strong interaction and the protein is eluted

3.2.12 Desaltation and concentration of the purified recombinant OPAA

The purified protein was in 500 mM NaCl, 50 mM Tris of pH 8.0, and 150 mM Imidazole. It was then desalted and concentrated to 50 mM NaCl, 50mM Tris of pH 8.0 and 15 mM Imidazol. The purified protein was loaded into the amicon and centrifuged at 4000 xg until the volume of the protein in the amicon reaches 10 times lesser than the loaded protein. The volume was again made up to the initial volume using 50 mM Tris of pH 8.0. Amicons were again centrifuged at 4000 xg until the volume was reduced to 10 times lesser than the intial volume. The temperature was maintained 4° C throughout the procedure of desaltation and concentration. The desalted and concentrated protein was then collected in the fresh autoclaved micro centrifuge tube and tested for its activity against OPs.

3.2.13 SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) is run to separate the protein on to the gel depending on their size by applying the electric field and using discontinuous polyacrylamide gel as the support medium. SDS in the gel act as a surfactant and impart

negative charge thus covering the charge of the protein depending on charge-to-mass ratio. The basic pH reduces the positive charge of the protein and intrinsic charge is also negligible in comparison to the SDS loaded. In the discontinuous SDS PAGE first the protein migrate into the stacking gel of pH 6.8 and then through the resolving gel of pH 8.8. Difference in pH leads to the stacking effect at the junction between the stacking gel and the separating gel. The running buffer mainly contain Tris-glycine with SDS. The glycine in pH 6.8 act as a zwitter ion and at alkaline pH it remains in deprotonated state, slightly negatively charged. In the stacking gel the slightly negative charge Cl^- ion move in front thus the protein is sandwiched between the Cl^- ion and the positively glycinate ion. In resolving gel the glycinate exist as negatively charged thus losing the slowing positive charge and become the leading ion which causes the appearance of the bands after staining. When the external electric field is applied protein migrate towards positive electrode anode with different migrating speed. The gel act as sieve resulting into the separation of the protein. The smaller protein travel larger distance compare to the larger protein. At the end of the SDS PAGE electrophoresis the gel is stained in the coomassie dye. The amount of protein can also be estimated using the gel by intensity of the band.

12 % gel was casted using glass plates, spacer, comb, casting tray, and casting stand. Samples were prepared using 1X loading dye and heating at 95° C for 5 minutes. Samples were loaded and the gel was run in the 1X running buffer. The gel was stained and destained for the required period of time and the gel was analyzed.

3.2.14 Activity assay of the recombinant OPAA

1 µl of purified protein was mixed with 50 mM CHES [2-(N-cyclohexylamino) ethane-sulfonic acid] buffer of pH 9.0, 1 mM methyl parathion (Sigma), 0.024 mM $MnCl_2$ and the final volume was made up to 1 ml with SDW and the reaction was incubated at 25° C for 20 minutes.

Similarly to check the activity of OPAA on paraoxon, Bis Tris propane buffer of pH 8.5, 1 mM $MnCl_2$ was used at 50 °C for 15 minutes. OPAA activity was calculated by measuring the increase in absorbance of product (*p*-nitrophenol) formed at 400 nm using extinction coefficient 17,000 M/cm for *p*-nitrophenol in case of methyl parathion and 10101 M/cm in case of paraoxon. Specific activity was calculated in U/mg.

3.2.15 Encapsulation of the BSA and recombinant OPAA in alginate microsphere using an ultrasonic atomizer

BSA was encapsulated as a model protein compound in 0.7 % sodium alginate by using an ultrasonic atomizer with a 4 % $CaCl_2$ as collection bath. The 0.35 % BSA and 0.7 % of sodium alginate was sprayed (0.3 ml/min) and using ultrasonic atomizer at frequency 130 KHz and 3.5 Watt of power and collected in $CaCl_2$ collection bath kept on the magnetic stirrer at 1400 rpm. The $CaCl_2$ with BSA encapsulated alginate was then centrifuged and the beads so obtained were washed with water. Encapsulated beads were obtained by centrifuging at 7000 rpm for 15 minutes. Similarly, desalted OPAA was encapsulated in alginate beads using the same protocol. After three washing the beads were suspended in 1ml of sterile distilled water and used further for the detection of the OPs.

3.2.16 Characterization the morphology of the beads using scanning electron microscopy

To check the morphology of the beads so formed scanning electron microscopy (SEM) was done. Sample preparation is the most important step while taking the image of the biological samples. Small concentration of beads were sprayed into thin layer evenly on to the glass slide. The slide was kept on the desiccator for 3 hours in which silica adsorbs all the moisture present in the sample. The sample was the coated with copper metal and used for the imaging purpose.

Two different ways were used for the estimation of the encapsulation efficiency of BSA and OPAA. First, the supernatant so obtained after the first centrifugation was collected and Bradford was performed for the estimation of the protein in the supernatant. Second, beads were boiled at 95° C for 15 minutes and the beads are then loaded on the SDS PAGE along with the input and the first wash.

3.2.17 Biosensing studies of paraoxon in solution phase using fluorescence spectroscopy

The protein and FITC-dextran were physically mixed. The protein and the FITC dextran (2 mg/ml) were mixed in equal ratio 1:1 (w/w). The mixture was kept on shaking condition for three hours at the room temperature. After 3 hours of incubation 1 mM $MnCl_2$, paraoxon (5 µM-1.5 mM) was added to the mixture of protein and FITC-dextran and the volume was made upto 1 ml. Decrease in the intensity was observed for 10 minutes and the reaction was kept at 50°C throughout the experiment. Decrease in the intensity of FITC because of the H^+ obtained on hydrolysis of the paraoxon, was observed using spectrofluorometer. During measurement of fluorescence excitation of FITC was 490 nm and emission was 500 nm-550 nm with silt width of 2 nm was recorded.

3.2.18 Biosensing studies of paraoxon using encapsulated OPAA

Encapsulated beads are mixed with FITC isomer (prepared in DMSO) were kept on shaking condition at room temperature for three hours. The beads were then washed with 1ml water thrice and then suspended in the water and used for the detection of OPs similar to the soluble fraction.

Chapter 4

Results and discussion

4.1 Cloning of OPAA with N-terminal His-tag

In order to enhance the expression and simplify the purification of the protein, the variant of OPAA gene (FL) was cloned downstream of the strong promoter (T7) into the expression vector pET28a. The pSE420 expression carrying recombinant OPAA gene (FL) was double digested with *Nde*I and BamH1 simultaneously pET28 vector was also double digested. The cloning was performed such that N-terminal Met codon, 6X His tag and the stop codon of 3' was retained. **Fig. 4.1** shows the cloning strategy. Cloning of OPAA in pT28a was confirmed by colony PCR, restriction analysis of the recombinant plasmid construct, followed by Sanger sequencing. On restriction analysis, a release of 1323 bp band from the recombinant plasmid was observed on to the agarose gel (**Fig. 4.2).** The recombinant plasmid was then transformed into Rosetta cell.

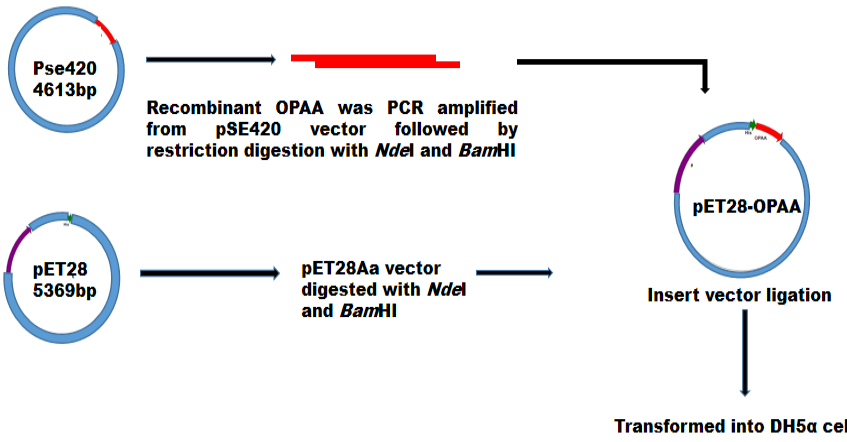


Fig. 4.1: **Construction of recombinant plasmid pET28a with recombinant OPAA gene.** Recombinant plasmid was constructed by ligating the fragments by Ligase.

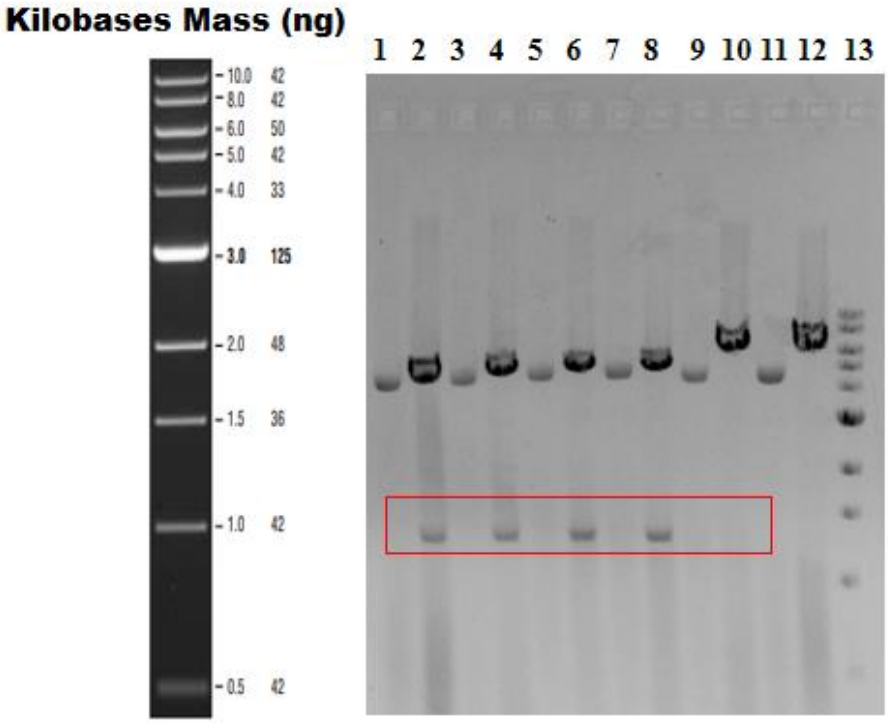


Fig. 4.2: **Conformation of clone was done by restriction analysis.** On restriction analysis release of 1323 bp was observed.

4.2 Expression of recombinant OPAA

OPAA protein is a cytoplasmic protein so to enhance the expression of the protein, the transformed *E. coli* cells were induced with IPTG and grown to mid-log phase. The bacterial cells grow exponentially hence express the protein in the large amount at the mid-log phase. As OPAA is a cytoplasmic protein the expressed protein was obtained in soluble form by lysing the cell wall of the bacteria by adding Lysozyme. Lysozyme is glycoside hydrolase, which hydrolyses the β 1, 4 glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in peptidoglycan. The cells were further agitated by applying high sound energy (>20 KHz) using ultrasonicator. The protein so obtained in the soluble fraction was then confirmed by running the SDS PAGE. OPAA protein is of 51 KDa but the His tag at the N-terminal added 1 KDa to the

molecular weight of the protein, as a result, a 52 KDa band was observed confirming the presence of approximately 70 % protein in the soluble fraction (**Fig. 4.3, lane no. 2)**.

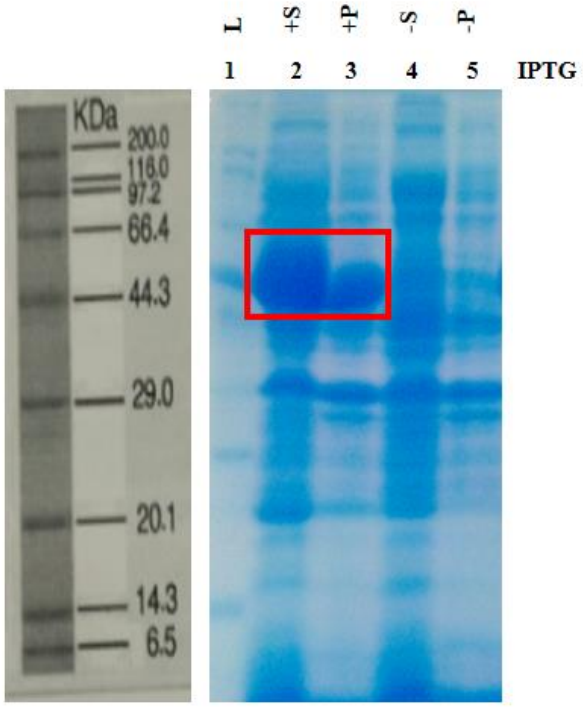


Fig. 4.3: **SDS PAGE showing the recombinant OPAA of 52 KDa**. Lane1 carries the protein ladder, lane 2 carries supernatant of induced sample, lane 3 carries pellet of induced sample and lane 4 and 5 supernatant and pellet of induced samples respectively.

4.3 Purification of recombinant OPAA

The recombinant OPAA with N-terminal His-tag was purified from the crude extract by affinity chromatography using Ni-NTA tagged sepharose beads. The N-terminal His-tag binds to the nickel and the protein lacking His-tag elute out in the washes. The homogeneity of the purified protein was tested by running the SDS PAGE. A single band was observed in Lane 11, 12, 13 indicating the apparent homogeneity of the purified protein (**Fig. 4.4).**

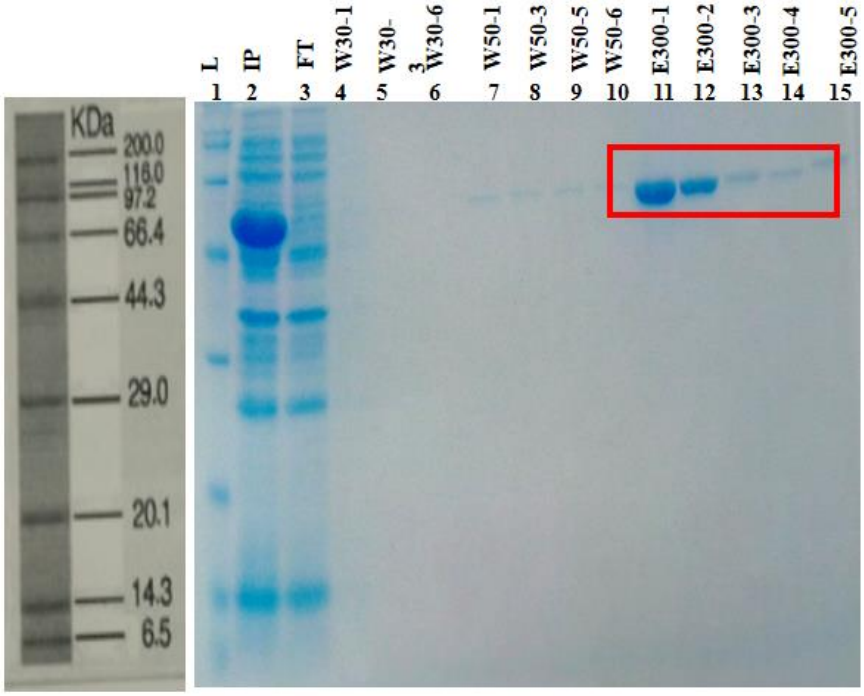


Fig. 4.4: **SDS PAGE showing the purified recombinant OPAA protein.** Lane 1 carries the protein ladder, lane 2 and 3 have input and flow throw respectively, lane 3 -10 carry washes and lane 11-15 carry elution of the purified protein.

4.4 Activity assay

4.4.1 Activity of OPAA using micro titer plate reader in soluble fraction

The activity of the protein was measured UV-spectrophotometrically by determining the amount of *p*- nitrophenol formed on hydrolysis of paraoxon at 50° C and methyl parathion at 50° C. Activity and specific activity of the crude extract and the purified protein is shown in table 1. The protein was found to be active at pH 8.5, 1 mM $MnCl_2$ and Bis-Tris Propane buffer in case of paraoxon. And the activity of recombinant OPAA was tested on methyl parathion with CHES buffer of pH 9.0, 1 mM $MnCl_2$ and also with Bis-Tris Propane buffer. Activity and specific activity of the protein was found to be comparable in case of the protein produced when the cells were grown in the presence or absence

of IPTG in case of methyl parathion **(Fig. 4.5)** whereas in case of paraoxon specific activity of enzyme in presence of IPTG was found to be 2.4 folds higher than the specific activity of the protein produced in absence of IPTG. The specific activity of the purified OPAA 4.132 $\mu molmin^{-1}$ mg^{-1} was found to be higher than the reported 1.6 $\mu molmin^{-1}$ mg^{-1} (68) . The activity of OPAA is defined as the amount of enzyme required to hydrolyse 1 µmole of methyl parathion and is expressed in U/ml (µmol min^{-1} ml^{-1}) and specific activity is expressed in U/mg. The activity and specific activity was calculated by using the following formula:

$$\text{Activity} = \frac{OD \times \text{Total volume of the reaction} \times 10^{-3} \times 10^{6}}{\text{Extinction coefficient} \times \text{Time} \times \text{Volume of protein}} \quad \ldots 1)$$

$$\text{Specific activity} = \frac{\text{Activity}}{\text{Concentration of protein}} \quad \ldots 2)$$

Table 1: Comparison of the activity of lysate and purified recombinant OPAA with different OPs.

S.No	Sample (OPAA)	Substrate (1mM)	Activity (U/ml)	Specific activity (U/mg)
1	Un-induced	Methyl parathion	0.20	0.60 ±0.02
2	Induced	Methyl parathion	0.20	0.56 ± 0.01
3	Un-induced	Paraoxon	0.26	0.08 ± 0.00
4	Induced	Paraoxon	5.77	1.74 ± 0.08
5	Purified	Paraoxon	1.80	4.13 ± 0.03

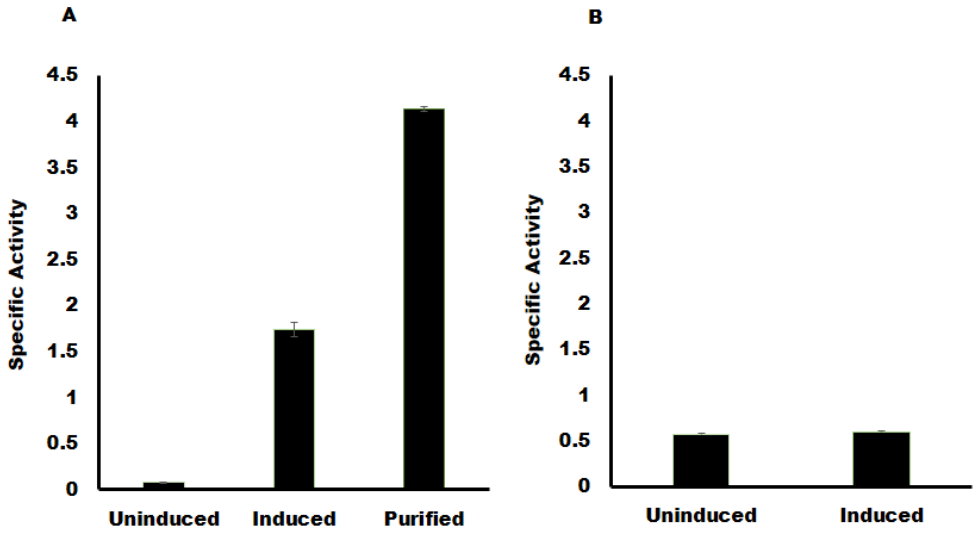


Fig. 4.5: **Histogram showing the comparison of the activity of recombinant OPAA on different substrates** (a) paraoxon (b) methyl parathion when the cells induced in the presence and absence of IPTG.

4.4.2 Solution phase biosensing studies using fluorescence spectrophotometer

OPAA on hydrolysis of OPs release p-nitro phenol and two protons. The protons released lowers the pH of the reaction mixture. FITC is a fluorescent dye, intensity of which is sensitive to pH. Intensity of FITC decreases as H^+ concentration of the reaction mixture increases, lowering of pH. It is one of the most commonly used fluorophores (71). Seven phototropic forms are known of fluorescein (72). This property of FITC was exploited to detect the presence of OPs. Different concentration of buffer was used and different ratio of protein and FITC (w/w) was used for the detection of OPs. The reaction mixture was prepared with 1mM $MnCl_2$, paraoxon, Bis Tris Propane buffer, Protein, FITC and sterile distilled water. It was observed that the reaction mixture with no activity

buffer showed larger decrease in the intensity in comparison with the reaction mixture with the activity buffer in it as shows in the **Fig. 4.6A**. This could be because the presence of buffer neutralizes the H^+ so released during the reaction owing to the buffering capacity of the buffer. The activity was further checked by varying the amount of protein and FITC and it was observed that 1:1 (Protein: FITC) showed greater extent of decrease in the intensity of FITC because of the release more H^+ in the reaction mixture as shown in **Fig. 4.6B**. With increasing the concentration of paraoxon, intensity of FITC decreased faster showing the more number of H^+ released.

To calculate the linear range and limit of detection (LOD), different concentration of paraoxon from 5 µM to 1.5 mM were tested with OPAA. The linear range of the protein was to be between 100 µM to 1.5 mM with the linear regression $R^2 = 0.99$ (**Fig. 4.7)**. Using the slope of the linear regression and standard deviation of the samples LOD was calculated theoretically as shown below and was found to 1.5 µM.

$$\text{LOD} = \frac{3 \text{ X Standard deviation}}{\text{Slope of regression of linear range}} \qquad \ldots 3)$$

Slope of regression of linear range = 10.20 % / µM

$$\text{Therefore, LOD} = \frac{3 \text{ X } 5.08\%}{10.20 \text{ \% / µM}}$$

= 1.49µM

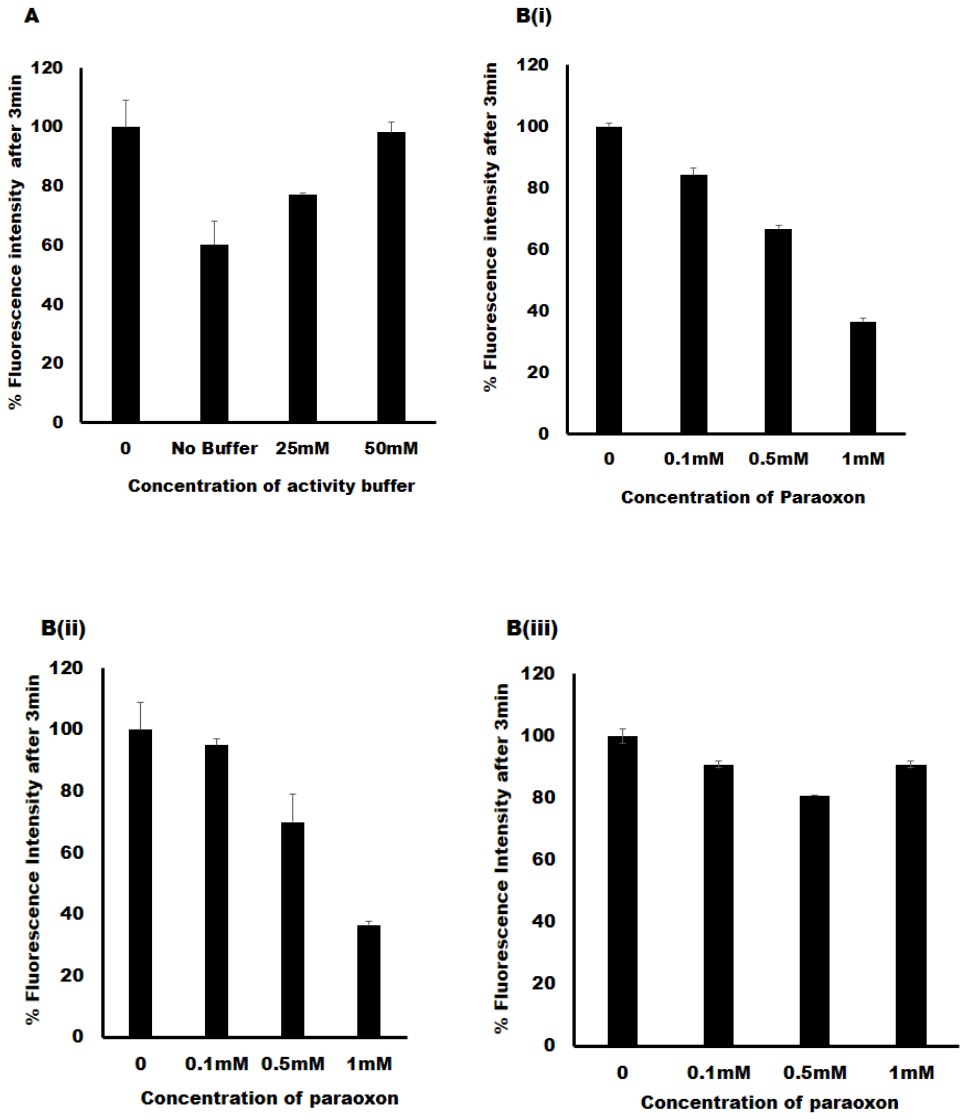


Fig. 4.6: **Optimization of activity assay (A)** Optimization of concentration of Bis-Tris Propane buffer. Drop in the intensity of FITC was more in case of the reaction mixture in which no activity buffer was used. **(B)** Optimization of protein: FITC (w/w). **(i)** 1:1 **(ii)** 3:1 **(iii)** 5:1. Highest activity was observed in case of Protein: FITC (1:1) W/W.

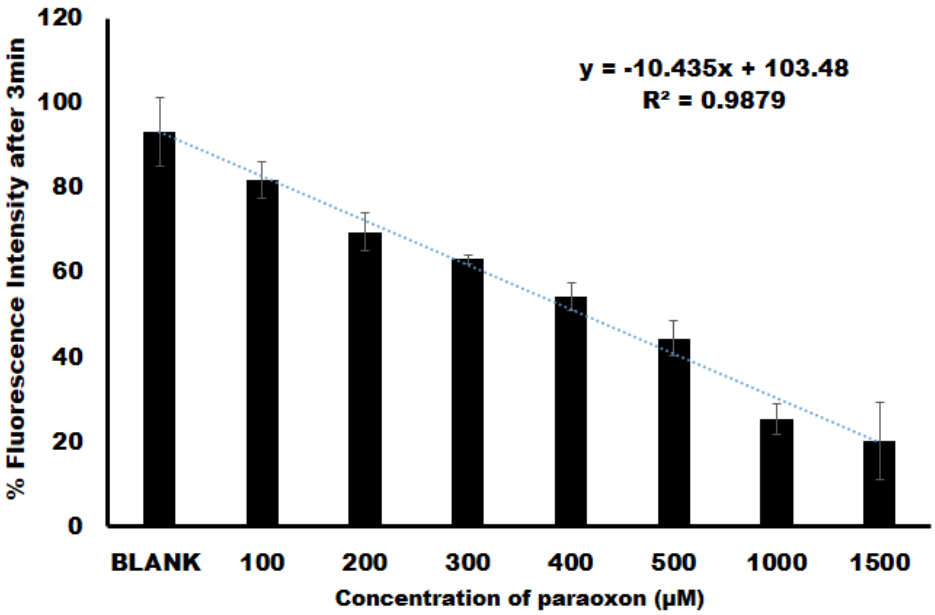


Fig. 4.7: Histogram showing the change in the fluorescence intensity. Change in the fluorescence intensity of FITC as compared to the blank after 3 minutes of incubation at 50° C. The linear range of paraoxon detection was observed between 100 µM to 1.5 mM.

4.5 Encapsulation of the protein BSA and OPAA in alginate microspheres

Encapsulation of protein in polymer aim at maintaining the activity and the function of the protein over a prolonged time, reduce the chances of interference, and to increase the sensitivity biosensor response. To start with, we tried encapsulating BSA into the sodium alginate by using $CaCl_2$ as crosslinker as shown in the **Fig. 4.8**. Alginate is a natural polymer with numbers of favorable properties like biocompatibity, ease of gelation, it enables the protein to retain its activity for longer period of time and also maintain its function. BSA was found to be encapsulated into the alginate microsphere on protein estimation by Bradford or SDS PAGE gel**.** Encapsulation step using the ultrasonic atomizer may lead to alteration in

secondary structure of protiens leading to further denaturation. In order to test the alterations in secondary structure of protiens circular dichroism was performed for 0.1 mg/ml BSA and 0.1 mg/ml BSA sprayed using ultrasonic atomizer. No change in the secondary structure of the protein was confirmed by circular dichroism (CD) (**Fig. 4.9).**

Similary recombinant OPAA protein was encapsulated in 0.7% agninate using ultrasonic atomizer. And on running SDS gel it was determined that the protein was encapsulated inside the beads (shown in **Fig. 4.10**) and the beads further were used for the detection of OPs. On the SDS gel no protein band was observed in the lane of wash and a visible band observed in lane 3 shows the presence of protein inside the beads.

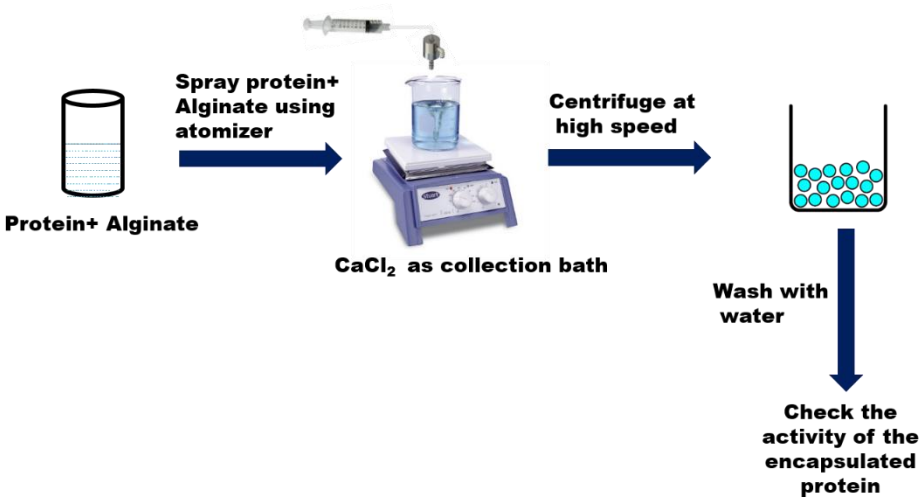


Fig. 4.8: **Diagrammatic representation of the encapsulation procedure.** Protein and alginate mixture were sprayed in $CaCl_2$ which act as a cross linker and aid in beads formation. Beads formed were then centrifuged at high speed and washed with water.

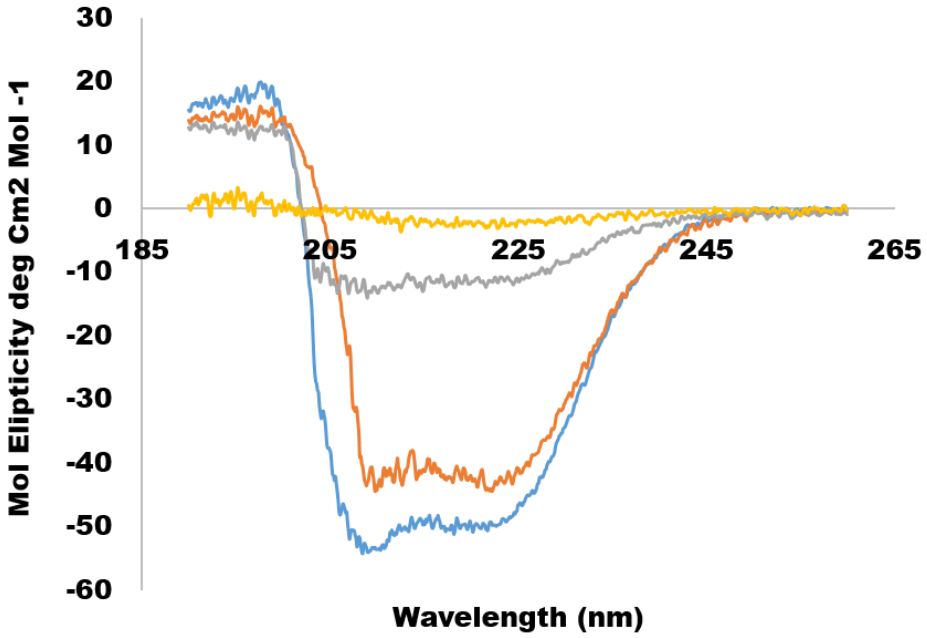


Fig. 4.9: **To check the conformation of the secondary structure of protein**. No secondary structure alteration was observed because of vibration when BSA was sprayed through an atomizer.

4.6 Characterization

The surface characterization of alginate beads before and after encapsulation of BSA was investigated by Scanning electron microscopy (SEM). Spherical shaped beads ranging from 1 µm to 10 µm in diameter were observed in SEM, in case of only alginate microspheres and BSA encapsulated alginate microspheres proving that encapsulation of protein does not changed the morphology of the beads. Once OPAA was encapsulated in 0.7 % alginate spherical beads of 15 µm in size were formed, shown in **Fig. 4.11.**

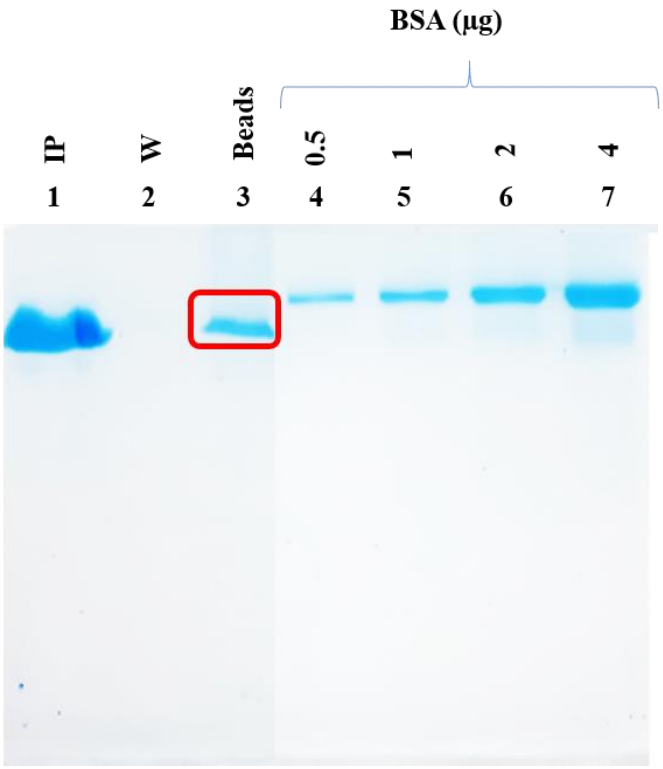


Fig. 4.10: SDS PAGE gel confirmed the encapsulation of OPAA into alginate microsphere. Presence of band in lane number 3 shows that the protein was being encapsulated inside the alginate

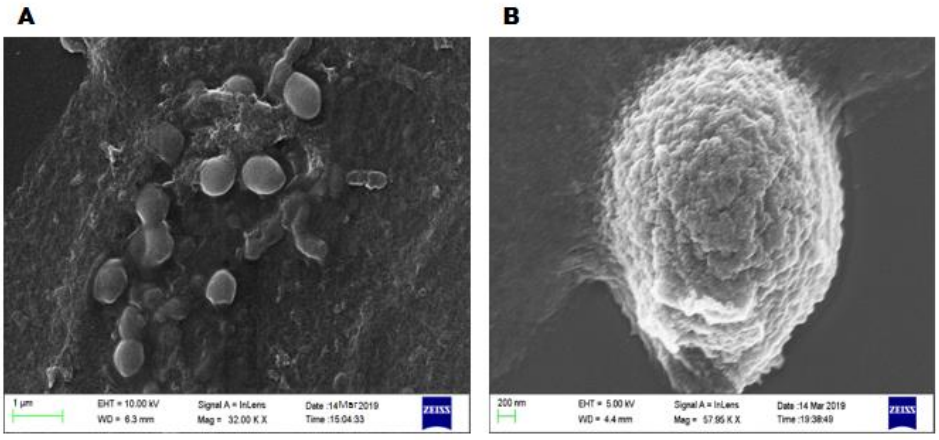


Fig. 4.11: **Characterization of encapsulated recombinant OPAA inside the alginate microsphere** A: Group of encapsulated OPAA inside 0.7 % alginate. B: Magnified image of the bead.

4.7 Biosensing studies using Fluorometric assay

4.7.1 Activity assay of the encapsulated recombinant OPAA on paraoxon

As described in the 3.4 section, activity was performed with 1mM $MnCl_2$, Bis-Tris Propane buffer, paraoxon at 50° C. But now instead of soluble protein, beads were used in the activity reaction. The reaction continued for 15 min and then the reading was taken using micro titer plate reader at 400 nm. For the formation of p-nitophenol, the substrate (paraoxon) has to permeat inside the beads. The following **Table 2** shows the activity of encapsulated beads using microtiter plate reader.

Table 2: Biosensing studies of Paraoxon using encapsulated OPAA

Sample	Substrate	Activity (U/ml)	Standard deviation
Encapsulated recombinant OPAA	Paraoxon	0.73 ± 0.03	0.03

4.7.2 Activity assay using Fluoroscence spectrophotometer

Protein encapsulated beads were incubated with FITC isomer prepared in DMSO (dimethyl sulfoxide) for 3 hours. DMSO is an aprotic solvent which dissolves both polar and non-polar substances. After 3 Hours of incubation beads were washed with 1ml of water, centrifuged for 15 min at 7000 rpm thrice to wash off the additional unconjugated FITC. FITC conjugated alginate microspheres were then used to detect the presence of OPs. The reaction mixture of activity contain 1 mM $MnCl_2$, paraoxon and sterile distilled water and decrease in the intensity of FITC was observed for 10 minutes shown in **Fig. 4.12**. The drop in the intensity was observed because of formation of H^+ on hydrolysis of OPs.

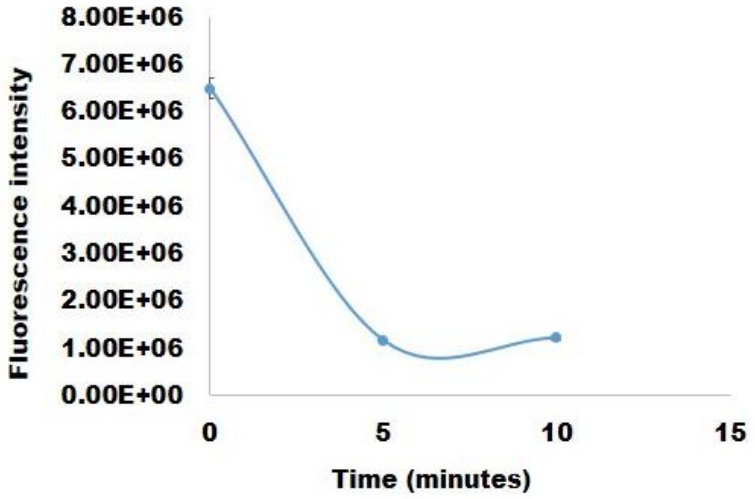


Fig. 4.12: **Significant drop in the FITC intensity**. When the rection mixture containing encapsulated OPAA, FITC and paraoxon significant drop in the intensity of FITC was observed from intial point to 5min when the reaction mixture was incubated at 50°C.

Chapter 5

Conclusion and future aspects

OPs degrading enzymes have attracted a widespread attention because of the toxicity of pesticides and use of nerve agents as the chemical warfare agents. Overall we present an OPAA enzyme based biosensor which is safe, simple for the user and benign to the environment for the detection of the paraoxon. This work provide information about the cloning, expression, purification, encapsulation of OPAA into alginate and developing a pH based biosensor.

The variant of OPAA (FL) gene was isolated from *p*SE420 expression vector, received from Prof. Steven Harvey, US Army Edgewood Chemical Biological Center, USA was cloned into pET28 expression vector with N-terminal His-tag. The recombinant OPAA was expressed in *E.coli* cells at 20° C, purified and was tested for its activity against methyl parathion and paraoxon. The specific activity of OPAA using colorimetric assay was observed 4.13 ± 0.027 U/mg against paraoxon which is 2.6 fold higher with a response time of 15 minutes in comparison of reported wild type OPAA (1.6 ± 0.06 U/mg, 30 minutes) (68). The activity was further validated using Fluorometric assay using pH sensitive fluorophore, FITC. To the best of my knowledge, linear range of paraoxon detection with the OPAA has not been reported so far. And we observed the intensity of FITC dropped within a response time of 3 minutes giving linear range of detection between 100 µM -1.5 mM. The protein was encapsulated into alginate using ultrasonic atomizer and encapsulation was confirmed by running SDS gel and by Bradford assay. No change in the secondary structure of protein was observed when encapsulated into alginate and it was confirmed by CD. Morphology of the beads were observed by SEM. Average size of the beads so formed was found to around 10 µM. Beads were further used for the detection of the

OPs using pH sensitive FITC. Drop in the intensity of FITC was observed on hydrolysis of OPs.

For the pesticides detection most of the technologies known so far are costly and cannot be used directly in the field. There is a high demand of more cost effective and efficient method development.

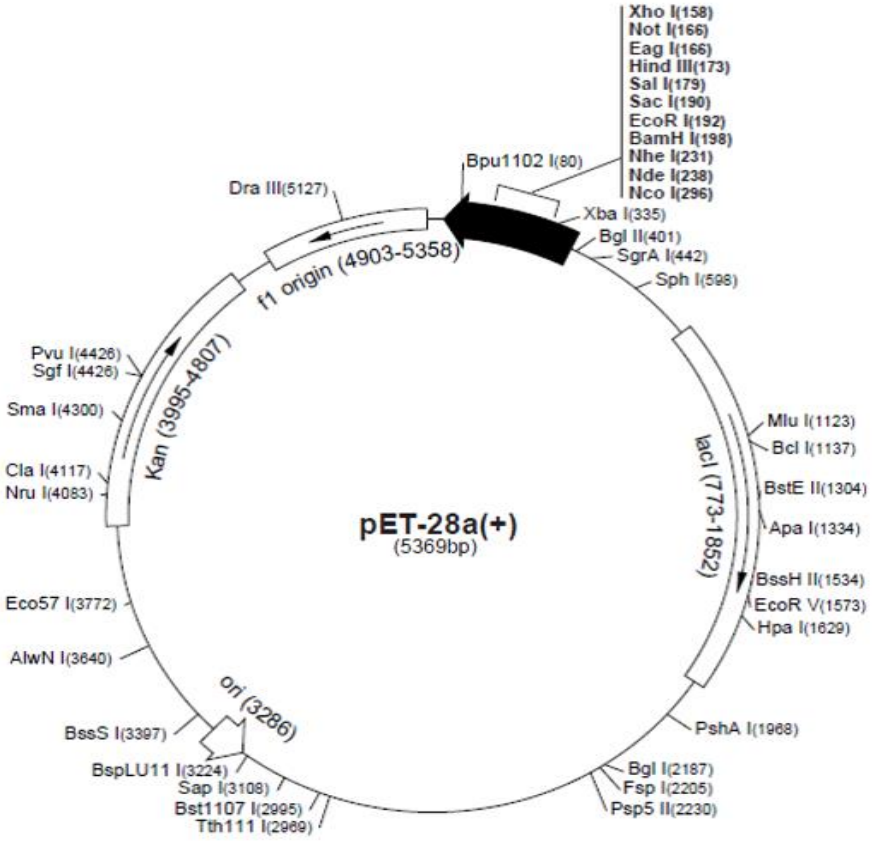
Application of genetic engineering can be used to develop a biosensor that can combines OPAA specific to certain pesticides and nerve agents with other OPs degrading enzyme in order to enhance the capability of the biosensor to degrade several pesticides more cost effectively and rapidly. Since OPAA is a cytoplasmic protein, if OPAA is expressed on the surface of the where it can come in contact with environment. Within a short time of exposure, sensitivity, and since protein need not to be taken out but whole cell can be immobilized which increases the stability of the biosensor.

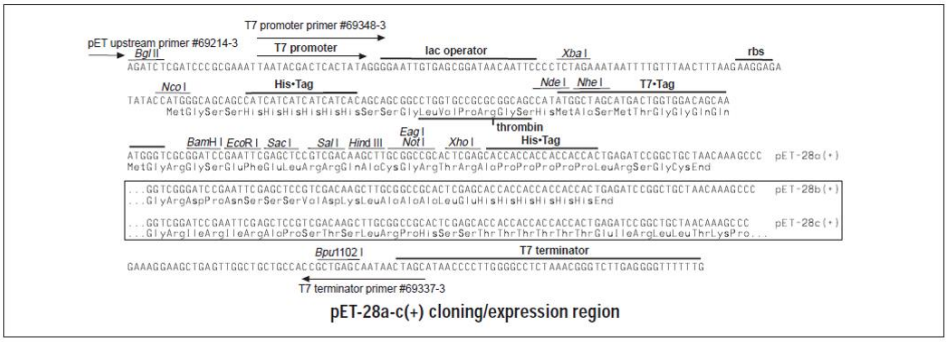
Although OPAA is of non-human origin and is associated with pharmacokinetic and immunogenicity challenges. Production of OPAA in diverse production system that includes surface modification can result into detoxification of pesticides and nerve agents within the blood circulation. Thus fulfilling the eligibility of ideal bioscavangers with no cytotoxicity and prolonged circulation.

Chapter 6

Annexure

1. Plasmid map of pET28a





2. List of primers

Primer	Sequence (5'-3')[a]	Restriction site[b]
PK702F	GCGCG **CATatg** aat aaa tta gcg gtg tta tac gct gaa c	*Nde*I
PK706R	ATAT **GGATCC** tta atc gag ctc tag ctc gcg	*Bam*HI

a. Nucleotides in small case are complimentary to the genome sequence.

b. Underlined (bold case) in the corresponding sequence.

Chapter7

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