Recombinant expression, purification, and development of enzyme-based biosensor for OP pesticides detection

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree
of
Master of Science

by

JUNAID AHMED

BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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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 BIOSENSOR FOR OP PESTICIDES DETECTION** 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 own work carried out during the time period from June 2022 to May 2023 under the supervision of Dr. Prashant Kodgire Professor, Department of Biosciences and Biomedical Engineering and Dr. Abhijeet Joshi, Associate professor Department of Biosciences and Biomedical engineering , 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.

Junaid Ahmed (26-05-23)
Signature of the student with date
(JUNAID AHMED)

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

P.V. Kodgire
Signature of the Supervisor of M.Sc. thesis

(Prof. Prashant Kodgire)

AB Joshi
Signature of the Supervisor of M.Sc. thesis

(Dr. Abhijeet Joshi)

Mr. JUNAID AHMED has successfully given his/her M.Sc. Oral Examination held on **9th May 2023.**

AB Joshi
Signature(s) of Supervisor of MSc thesis
Date: 26-05-2023

Sharad Gupta
Signature of PSPC Member
Date: 26-05-2023

P.V. Kodgire
Convener, DPGC
Date: 26-05-2023

Signature of PSPC Member
Date: 26.05.2023

ACKNOWLEDGEMENTS

Achieving one's goals is not an effort of a single person, but rather the advice, assistance, suggestions, and inspiration of many people. As a result, it gives me the deepest pleasure to express my sincere gratitude, regard, and acknowledgment to them.

Firstly, I would like to express my heartfelt gratitude to my thesis supervisor **Prof. Prashant Kodgire** and **Dr. Abhijeet Joshi** for their invaluable guidance, support, and encouragement throughout my research journey and for providing a wonderful opportunity to work in their lab. Their continuous guidance timely advice, scientific approach, and support always helped in moving forward. I am indebted to them for always finding time between their busy schedules to discuss my results and clear my doubts. This research could not have been carried out without their consistent leadership and motivation.

I would also like to thank the **Director, Dr. Suhas Joshi** for allowing me to join this prestigious institute. Also, I would like to extend my deepest thanks to my PSPC member **Dr. Amit Kumar (Head, Discipline of Biosciences and Biomedical Engineering)** and **Dr. Sharad Gupta, Dr. Parimal Kar (Course coordinator)** for their continuous support in various aspects during the M.Sc. journey. I would humbly like to express my gratitude to all the faculty members of BSBE who taught me various courses during M.Sc course work.

I would like to extend my sincere gratitude to all lab members **Mr. Rahul Chaudhari**, **Mr. Brijeshwar Singh**, **Ms. Kanika, Ms. Sayali**, **Ms. Kritika Malik**, **Ms. Isha Dhingra**, **Ms. Sushma**, **Mr. Mohanchaitnya** and my profound gratitude goes to **Mr. Ankit Jaiswal** and **Ms. Surbhi Jaiswal** for their invaluable support, scientific discussion and always keeping me motivated, energetic and always there for their kind support and valuable time.

Additionally, I would like to thank all my batchmates for keeping me motivated, energetic, and always having fun and providing emotional support during a challenging time my friends **Mr. Rahul Sharma, and Mr. Vinod Yadav** Who are like my family away from home.

I would like to express my sincere and deep gratitude to my parents the source of my life, my father **Atta Mohd,** and my mother **Akbar Noor** for their unconditional love, care, and support and, I am also very grateful to my elder brother **Mohd Shabir (IAS)** who guides me at every single stage of my life and made me capable to achieve my goals. Last but not least, I would like to thank Almighty **God** for granting me strength and health and all others who knowingly or unknowingly helped me in this journey.

JUNAID AHMED

Abstract

This thesis aims to explore detection and bio-remediation strategies for (OP) pesticide contamination. OP pesticides are widely used in agricultural and public health practices, and their toxicity to humans, non-targeted animals, and the environment has raised a concern. The research will focus on identifying reliable and sensitive detection methods for OP pesticides in soil and water systems including the use of enzyme-based bio sensing and other reliable analytical techniques. The study will also investigate the potential of bioremediation techniques to degrade OP pesticides, including microbial degradation and phytoremediation. The finding of this research will contribute to developing effective and sustainable strategies for the detection of OP pesticide contamination will have potential implications for environmental and public health concerns. Several OP pesticides degrading enzymes have been identified by creating several sites directed mutagenesis to aid in the catalytic efficiency several folds. In our research, a pH-sensitive monomeric teal fluorescent protein(mTFP) is used for fluorometric biosensing along with organophosphorus hydrolase (OPH) a pesticide-degrading enzyme. The recombinant Nus-OPH and mTFP proteins were expressed and purified *in E. coli.* The fluorometric and colorimetric biosensing method with Nus-OPH and mTFP in a 1:1 equimolar ratio was performed with ethyl paraoxon in the linear range 0.01mM to 1mM both for colorimetric and fluorometric. LOD values 0.009mM and 0.0007mM for colorimetric and fluorometric respectively. A fluorometric sensing assay with tap water instead of using distilled water didn't show any effect in the biosensing of ethyl paraoxon even in the contamination of tap water. The future of monomeric teal fluorescent protein in OP pesticide biosensing looks promising. mTFP-based sensors offer a sensitive, selective, and versatile approach for detecting OP pesticides in the environment. With continued research and development mTFP sensors could become an important tool for environmental monitoring and public health protection.

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Abbreviations

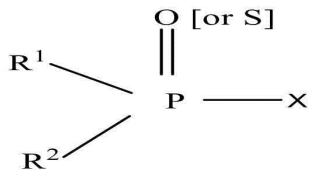
OPH	Organophosphorus hydrolase
OPs	Organophosphorus compounds
NA	Nerve agents
PTE	Phosphotriesterase
BSA	Bovine serum albumin
AChE	Acetyl cholinesterase
BChE	Butrylcholinesterase
MPH	Methyl parathion hydrolase
SDS PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
OD	Optical density
mTFP1	monomeric teal fluorescent protein
GFP	Green fluorescent protein

Chapter 1

1.1. Introduction

Organophosphorus (OP) pesticides have been extensively used for a few years all over the world in agriculture for pest control and crop protection. More than a hundred of these have found Commercial use [1]. It is estimated that 56 billion $ was spent on pesticide production worldwide in 2012. The indiscriminate use of pesticides impacts the environment [2]. The OP compound is one of 29 active ingredients that are currently registered in Japan [1]. Over one-fifth of all pesticides used in developed countries and more than half used in developing countries are accounted for by organophosphorus compounds [1]. Due to their environmental durability, many of these substances will continue to exist in our environment for many years to come. Everyone will eventually come into contact with pesticides due to environmental pollution or occupational use. pesticides residue including physical and biological breakdown products are present in food water and air. exposure to all these complicated mixtures of chemical, active components, and by-products included in technical formulations such as impurities, solvents, and other components created for storage purposes, occur during all stages of pesticides formulations products and applications [3]. According to their chemical composition, pesticides are categorized into four main groups: organochlorines, organophosphorus, carbamates, pyrethrin, and pyrethroids. OP insecticides are primarily utilized for residential agriculture and vector control purposes [4]. OPs is also used as a nerve agent in chemical warfare (e.g., Sarin gas, Soman, Tabun) Based on their toxicity and clinical use the OP pesticide is classified as highly toxic organophosphate (tetraethyl pyrophosphate, parathion); these are mainly used in agricultural insecticides, intermediate toxic organophosphates (e.g., Caumaphos, chlorpyrifos) mainly used as animal insecticides and there are many low toxic OP compounds e.g., Malathion,

diazinon these are mainly used in household applications and field sprays [5]. OP poisoning causes 3 million poisonings and kills nearly 1,70,000 annually, the majority in developing countries. The main cause of this poisoning is the result of unintentional spills various terrorist attacks, farmers' suicide, and occupational danger [6]. OP compounds are highly lipid soluble, easily absorbed by the intact skin, oral mucous membrane, and from the gastrointestinal tract and respiratory tracts, and very rapidly distributed all over the body tissues, the highest concentration is found in the liver and kidneys. OP compounds can easily cross blood-brain barriers and mainly affect the central nervous system [7]. The main mechanism of action is that these OP compounds inhibit the enzyme acetylcholinesterase by establishing a covalent bond with AChE that degrade acetylcholinesterase into choline and acetic acid, acts both on CNS and PNS once it inactivates the AChE [8]. ACh accumulates throughout nerve systems and muscarinic and nicotinic receptor over stimulation and causes many life-threatening risks like hypotension, diarrhea, abdominal cramps, paralysis, etc. The old approach to detecting and analyzing pesticides had several drawbacks that prevented appropriate monitoring. The use of the enzymatic approach is a substitute for traditional methods of pesticide detection. Structurally OP pesticides can be represented as,



In OP compounds the R1, R2, and X groups attached to phosphorous atom determines the chemical, physical and biological properties. The R1 and R2 groups can be alkyl or aryl groups and X can be aromatic, aliphatic, or heterocyclic. Other groups oxygen or Sulphur double bonded to phosphorous atoms [9].

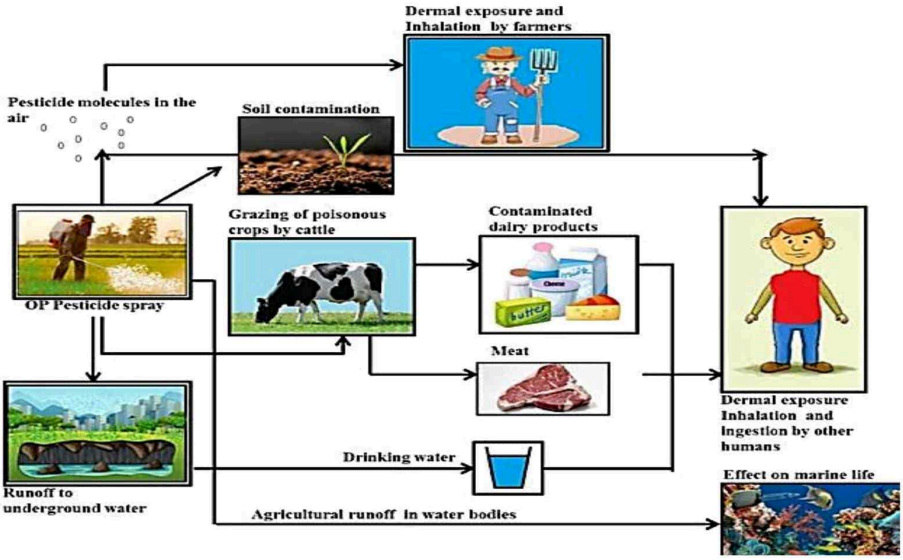


Fig. 1.1. Schematic representation of pesticide poisoning and contamination on different life forms. (*Adapted from Kaushal* et.al.,)

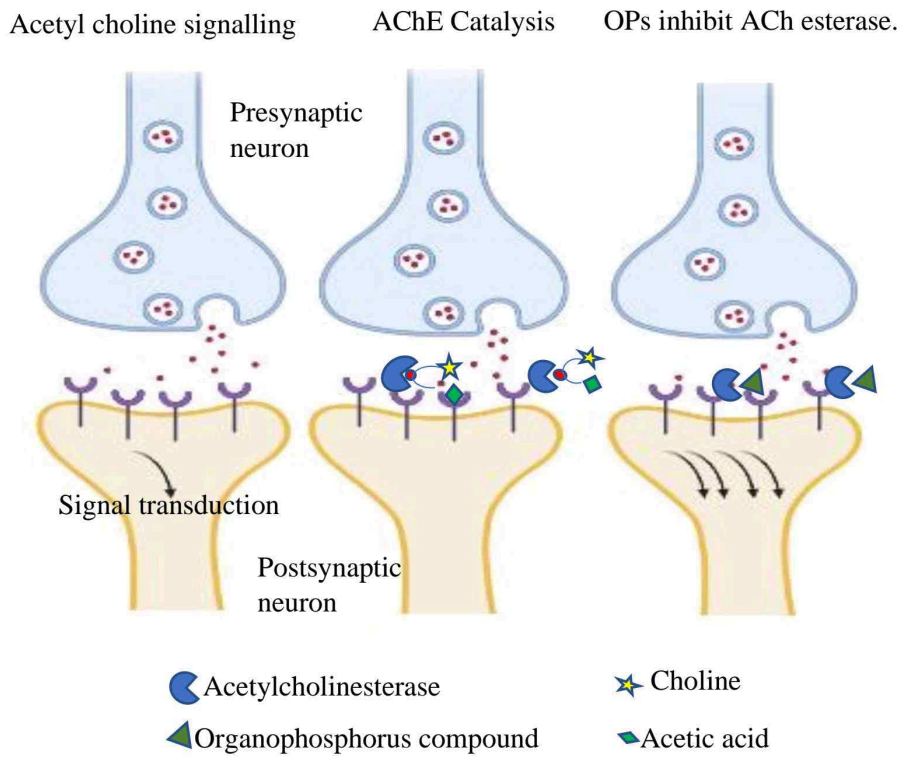


Fig. 1.2. Mechanism of toxicity due to OP poisoning and its effect [made with *Biorender*].

1.1.1 Nerve agents

Nerve agents, commonly referred to as nerve gases, are a subclass of organic compounds. it affects different parts of the body and affects the transmission of nerve signals to muscles and other nerves. Nerve agents work by blocking an active site of the essential enzymes acetylcholinesterase (AChE), which is in charge of the breakdown and release of acetylcholine coming from the nerve synapse. The difficulty of breaking up acetylcholine is associated with neurological problems, such as tremors, convulsions, and even death. According to reports, they are utilized as chemical warfare agents in conflicts and even in terrorist strikes. three categories can be used to categories these nerve agents: (1) the nerve agents tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF), which were all created by the Germans. (2) Agents belonging to the "V-series," where "V" refers to "venomous," such as VE, VG, VM, and VX. (3) GV Series has both G-Series and V-Series characteristics. [10]. These are comprised of highly toxic compounds, and they are 10,00 folds more toxic than OP-based pesticides. 11 people died and more than 5000 were injured when Japanese terrorists detonated nerve gas into the Tokyo subway in march 1995 [11].

1.1.2 Organophosphorus hydrolase

A member of the amidohydrolase superfamily is phosphotriesterase, organophosphorus hydrolase (PTE)/OPH, a homodimer metalloprotein. [12]. It uses a divalent ion like Co^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, or Fe^{2+} through the water molecule hydrolytically for a nucleophilic attack. [13]. According to an x-ray crystallography study, the protein OPH folds into the TIM barrel, a -barrel motif. *Pseudomonas diminuta* and *flavobacterium*, two soil bacteria, are sources of the OPH enzyme. OPH, also referred to as phosphotriesterase (PTE), is an enzyme that breaks down parathion and organophosphates. The extrachromosomal plasmid's phosphate gene (*opd*), encodes this enzyme [14]. It has many different substrate specificities. and can hydrolyse P-F, P-O, P-CN, P-S[15]. Upon hydrolysis by this enzyme, it generates one alcohol

i.e., para nitrophenol and two protons that are chromophores or electroactive [16] The increase in P-Nitrophenol concentration in the presence of increased paraoxon overtime is used to determine the enzyme activity of organophosphorus hydrolase. OPH is an effective catalyst for the degradation of OPs for this it has been considered as a potential candidate for the detection and bioremediation of OPs. pH-sensitive fluorophore is among the transduction platforms that have coupled with organophosphorus hydrolase to detect the low ppm concentration ranges [17]. GFP (Green fluorescent protein) modified *Aequorea* jellyfish provide biochemical and cell biology researchers with a powerful tool. A dimeric variant of protein. cFP484 with significantly greater fluorescent brightness than wildtype protein. In this study mTFP1 (monomeric teal fluorescent protein) was optimized through the addition of dimer breaking mutation an intensive directed evolution using selection for blue-shift emission. The novel mutant protein mTFP1 has very high fluorescent brightness, is light-sensitive, and sensitive to physiologically relevant pH variations [18].

1.1.3 Enzyme-based pesticide detection in the environment.

A common method for detecting organophosphates and carbamates is by inhibiting the acetylcholinesterase enzyme. The term "biosensor" refers to a measurement device combining biochemical elements with electronic elements in close contact and integrated into one unit. Biosensors generate responses by converting substrates into products by the enzymatic reaction on their sensing surfaces. Enzymatic stability is its commercial availability, during storage, and it's all applications these are the factor while selecting an enzyme for an enzymatic biosensor. Many microbes utilize OP compounds for Nitrogen, carbon, and phosphorous. Different bacterial enzymes that degrade OP compounds are Organophosphorus hydrolase (OPH), organophosphorus acid anhydrase (OPAA), and methyl parathion hydrolase (MPH) [19]. The increase in p-Nitrophenol a yellow-coloured product upon enzymatic hydrolysis of paraoxon and malathion is used for the enzymatic activity of OPH and OPAA. Organic dyes and, pH electrodes

are among the transduction materials that have been coupled with OPH to detect the concentration ranges [17]. The detection of OP pesticides using OPH has been accomplished using a variety of techniques, including electrochemical and optical approaches [20]. However, an optical sensing method gives better enzymatic catalytic activity and didn't achieve good detection limits to detect OP compounds. In this study, we used colorimetric and fluorometric sensing methods using a monomeric teal fluorescent protein (mTFP) for sensing ethyl paraoxon.

Table 1.1. Acute cholinergic effects of OPs[21]**.**

Receptors	Site	Sign and symptoms
Mixed	Central nervous system	nausea, nervousness, agitation, headache, disorientation, inability to focus, and respiratory depression.
Muscarinic	Glands Smooth muscles	excessive secretion, including tears (parasympathetic nervous system), sweat, mucus, and saliva. Supple muscle affects the digestive system (diarrhea), eyes (miosis and inability to focus), bladder (involuntary), heart (bradycardia), and kidneys (involuntary urination)
Nicotinic(N1)	Autonomic	hypertension and tachycardia due to increased sympathetic drive
Nicotinic (N2)	Neuromuscular junction	Muscle fasciculation, followed by a loss of strength and paralysis

The main objectives of this study are:

1. Colorimetric and fluorometric biosensing of pesticides using recombinant Nus-OPH-mTFP fusion protein.

2. Colorimetric and fluorometric biosensing of pesticides using recombinant Nus-OPH and mTFP.

Table 1.2: Various other enzyme-based methods for pesticides detection in the environment

Enzyme	Target pesticides	Detection methods	References
Acetylcholinesterase (AChE)	Organophosphate (OP) and carbamate pesticides.	Colorimetric, fluorescent, and electrochemical assays	[22]
Butyrylcholinesterase (BChE)	Organophosphate and carbamate	Electrochemically and colorimetric assays	[23]
Alkaline phosphatase (ALP)	Organophosphates and organochlorines	Fluorescent and electrochemical assays	[24]
Tyrosinase	Fungicides and herbicides	Colorimetric and electrochemical	[25]
Lipase	Organophosphate pesticides	Fluorescent and electrochemically	[26]

Chapter 2

2.1. Materials and methods

2.1.1. Materials

PCR Purification, (Helix Biosciences) gel extraction kit, Agarose special low EED, and the plasmid extraction mini kit (HiMedia), Kanamycin (HiMedia), Luria Bertani Broth Miller (HiMedia), *Nde*I, *BamH*I, *Apo*I, *Xho*I, and *Spe*I (NEB). Ethyl paraoxon (Sigma Aldrich), $CaCl_2$, Bradford (HiMedia), dNTPs, Ligase, Taq DNA Polymerase, *Pfu* DNA Polymerase, EtBr, Lysozyme, Tris-Cl, NaCl, Imidazole, CHES buffer.

2.2. Methods

2.2.1. Plasmid isolation

E. coli having the required plasmid used as a vector was grown in LB. Bertani broth contains appropriate antibiotics. The plasmid was isolated from *E. coli* with a favor prep plasmid extraction kit. To pellet down the cells, the well-grown bacterial culture was centrifuged at 11,000xg for I minute. After that, the pellet obtained was mixed with FAPDI buffer containing RNase A and the cells were pipette back into suspension. The resuspended cells were combined with the FAPD2 buffer the cells were then lysed by gently inverting the tubes 5–10 times then kept the tube on room temperature for 5 minutes. After adding FAPD3 buffer to the lysed cells, it was promptly neutralized by gently inverting 5–10 times. The lysate was then removed by centrifuging the tube at its highest speed. WI was added to the FAPD column after the supernatant had been carefully transferred into the FAPD column, and The column was then again centrifuges at 11,000xg for 1 minutes. The flow-through obtained was then thrown away. After discarding the resultant flow through, add wash buffer and centrifuge for 30 seconds.

seconds at 11,000xg. After discarding the flow through, to dry the column, it was centrifuged at high speed for a further 3 minutes. The column was then kept into a fresh autoclaved microcentrifuge tube, and the plasmid product was eluted in 50ul of elution buffer.

2.2.2. Polymerase chain reaction (PCR)

Pfu DNA polymerase amplified the template DNA using forward and reverse gene-specific primers. Primer was used to prepare the master mix. Template DNA, 0.2mM dNTPs, $MgC1_2$, and *Pfu* DNA polymerases. PCR tubes were aliquoted with the master mix. Similar steps were taken to prepare the negative control, except it lacked template DNA. The thermos cyclers PCR reaction program was configured to include an initial denaturation at 95°C for 5 minutes, finial denaturation at 92°C for 30 seconds, respectively annealing at the temperature recommended by the primers for 30 seconds, and extension at 72°C for was set for a period of time-based on the length of the gene of interest. The last extension was set for 10 minutes at 72°C for a long period.

2.2.3. Colony PCR

Colony PCR was used to perform to check for the probable recombinant clones after ligation and transformation in the DH5α. Initially, a colony of bacteria was replicated and plated on a new plate, and after that instead of DNA. Consider this colony as a template for the master mix of PCR following the same procedure as described above.

2.2.4. Restriction digestion of the DNA

Using restriction enzymes as directed by the manufacturer, sequential digestion of the vector and double digestion of the target gene were carried out at 37°C for 3 hours. Then enzymes were heat inactivated as directed.

2.2.5. Gel elution of the digested DNA.

Using the Helix Biosciences gel purification kit, the digested DNA fragments were removed from the gel. Using a clean cutter, slices of the double-digested DNA fragment-containing agarose gel were put into newly weighed microcentrifuge tubes. The gel slice was weighed and then noted. The 300 mg of sliced gel is mixed with 500µl of FADF buffer. Gels were incubated at 55°C until completely dissolved, the tubes were then kept at room temperature for five minutes. After being transferred to the FADF column in a micro-centrifuged tube, the gel solution was then centrifuged at 11,000xg for 1 minute. After discarding the resulting flow through, the column was again centrifuged at 11,000 xg for 1 minute to wash it with the wash buffer (which also contained ethanol). To properly dry up the column, it was then centrifuged once more for an additional 3–4 minutes at high speed (18,000xg). Elution was carried out using 50µl of elution buffer, then stored at 4°C.

2.2.6. Competent cell preparation

Freshly prepared and autoclaved $CaCl_2$ were used to prepare Competent cells. Cells were cultivated for 16 hours without antibiotics in Luria Bertani broth. Once more inoculating overnight bacterial culture into 50ml of fresh LB broth then incubated at 37°C until the culture's optical density (OD) reaches 0.4–0.5 at 600 nm where the cells are in the log phase. Then, everything that followed took place on the ice. The culture was centrifuged for 10 minutes at 5000 rpm and 4°C maintained to pellet it down after being incubated on ice for 10 minutes. After that, cells were suspended in chilled 0.1M $CaCl_2$ (10 ml of $CaC1_2$ per 25 ml of cell culture) and incubated on ice for 45 minutes. Cells were pelleted down once more and then delicately suspended in 2 ml of cooled $CaC1_2$. Cells were then aliquoted into prechilled micro-Centrifuge tubes with 15% prechilled glycerol after being incubated for 4 hours on ice. Following that, the cells were then stored at -80°C

2.2.7. Ligation

T4 DNA Ligase (New England Biology) and 1x ligation buffer were used to ligate a restriction enzyme-predigested DNA vector and insert. The reaction mixture was incubated at 22°C for two hours, after which heat inactivation was carried out following the manual's instructions before ligation for transformation**.**

2.2.8. Transformation

The heat shock method was used to do the transformation. Thoughtful cells were put on hold for a while. A microcentrifuge tube was filled with the necessary quantity of DNA to be ligated, and the DNA was given heat shock treatment for being incubated at 42°C for 90 seconds. Followed by 120 seconds on ice four times of competence cells and then followed by 90 minutes of 37°C incubation in LB broth. Next, transformed cells were dispersed throughout the LB plate containing the desired antibiotic.

2.2.9. Agarose gel electrophoresis

By using an electric field, agarose gel electrophoresis is used to separate the DNA in an agarose matrix. In agarose gel, the shortest distance is covered by a larger molecule and the long distance is covered by a small molecule. Molten agarose was kept in the casting tray of the electrophoresis apparatus, and let to set then, the gel was stained with Ethidium bromide (EtBr). EtBr intercalates into the DNA and then can be detected under UV light. The sample was combined with loading dye and placed into the electrophoresis apparatus' wells. An IX TAE buffer was then added to the wells. Glycerol is included in the loading dye to indicate the sample density, and bromophenol blue tracks the sample entry into the reaction vessel.

2.2.10. Recombinant protein expression and purification.

A single colony of transformed cells as a primary culture was inoculated in LB broth that has been given the proper antibiotics and is allowed to grow at 12-14 hours at 37°C. LB broth was prepared and used for secondary culture then transfer 1% of primary culture into the fresh LB Broth and kept on an incubator shaker at 37°C till 0.8 OD was reached. Then IPTG was added in secondary culture and incubated at 20°C/16°C, 20/16 hours for Nus-OPH/ Nus-OPH-mTFP and mTFP on the shaking incubator at 180 rpm for the induction and expression of the protein. Then centrifuge the culture at 11,000rpm for 10 minutes to pellet down the cells. The pellet obtained was washed and resuspended with 500 mM NaCl, 50 mM tris (pH 8.0), and 10% glycerol. Lysozyme treatment was done to the cells and incubated for 20 minutes at 37°C then sonicate the cells to rupture the cell wall of bacteria until the solution gets cleared and centrifuged cell lysate for 15 minutes at 4°C and 14,000 rpm. Collected the supernatant and we expected it to be our protein in the soluble fraction. After performing SDS-Page induction was confirmed.

Protein was purified using affinity chromatography (Ni-NTA Sepharose beads). Prepared buffers for purification 50mM Tris pH 8.0, 300 mM NaCl, and various imidazole concentrations of 30, 50, 90, 150, and 300mM. The Column was washed with distilled water and stripping buffer then recharged the column with 0.1M $NiS0_4$. The Sample was equilibrated with 10mM imidazole and loaded into the column and incubated on ice for 30 minutes and centrifuged at 600xg for 10 minutes collected the flow through and then washed the column with wash buffer (50 mM Tris-Cl pH 8.0, 300 mM NaCl, and 30/50/90 mM imidazole) then centrifuged at 600 G for 5 minutes collect wash. The purified protein was eluted with (50 mM Tris pH 8.0, 300 mM NaCl, 150mM, and 300mM imidazole. The main principle of protein purification was based on affinity chromatography i.e., the

interaction between molecules in the mobile phase and stationary phase. Here the stationary phase has Ni-NTA-tagged Sepharose beads and the mobile phase are our proteins. Our protein has His-Tagged at its N-terminal that binds to the stationary phase and other proteins eluted out due to the weaker interaction by a lower concentration of imidazole. Targeted proteins bind with higher affinity to the column. An elution buffer of higher imidazole concentration elutes the protein of our interest.

2.2.11. De-Saltation and concentration of the purified protein

50 mM Tris (pH 8.0), 500 mM NaCl and 150 mM Imidazole were used to purify the protein. It was then concentrated to pH 8.0, 50 mM Tris, 500 mM NaCl, and 20 mM NaCl after being desalted. The purified protein was loaded into a 10 KDa amicon concentrator and centrifuged at 4000 x g till the protein volume in the concentrator was 10 times smaller than the volume of the loaded protein. Using 50 mM Tris with a pH of 8.0 and 50 mM NaCl, the volume was once more brought up to the starting level. Amicons were once again centrifuged at 4000 x g until the volume was ten times smaller than at the beginning. Throughout the de-salting and concentration process, the temperature was held at 4°C. The desalted and concentrated protein was then used for enzyme assay against ethyl paraoxon.

2.2.12. SDS-PAGE

Proteins are separated according to their size using a technique called Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE). Concerning the charge-to-mass ratio, SDS in the gel works as a surfactant and introduces a negative charge on the protein, covering the charge of the protein. The positive charge of the protein is reduced by the basic pH, and the inherent charge is also insignificant as compared to the SDS loaded. In a discontinuous SDS-PAGE, the protein passes through the pH 6.8 stacking gel first, followed by the pH 8.8 resolving gel. At the

the intersection of the stacking gel and the separating gel, the pH difference causes a stacking effect. Tris-glycine with SDS is largely present in the running buffer. At an alkaline pH, glycine maintained its deprotonated form and modest negative charge, acting as a zwitter ion at pH 6.8. The stacking gel contained the almost negative charge cl- advances first, sandwiching the protein between it and the positive glycinate ion. Glycinate is a negative charge in resolving gel, where it loses its slow-moving positive charge to become the leading ion and produce the bands after staining. Protein moves towards the positive electrode anode when an external field is applied with a varying rate of migration. The protein is separated as a result of the gel's function as a sieve. In comparison to larger proteins, tiny proteins go farther. The gel is stained with Coomassie dye after the SDS-PAGE electrophoresis. The intensity of the band on the gel can also be used to assess the amount of protein. Glass plates, a spacer, a comb, a casting tray, and a casting stand were used to cast the gel. Depending on the size of protein 1x loading dye was used to prepare the samples, and they were heated at 95°C for 5 minutes. After loading the sample, the gel was run in a 1x running buffer. For the necessary amount of time, the gel was dyed and then destained.

2.2.13. Activity assay of recombinant protein Nus-OPH.

1 µl of pure protein, pH 9.0, 0.1 mM $CoCl_2$, 50 mM CHES [2-(N-cyclohexyl amino) ethane-sulfonic acid] buffer and 1 mM ethyl paraoxon (Sigma) were combined to perform an activity assay for Nus- OPH. The final volume was made up to 200 µl with distilled water. The preparation of a blank sample was similar, except no enzyme was added. For 5 minutes, the reaction mixtures were incubated at 37^{o}C. By measuring the absorbance of PNP generated by the hydrolysis of ethyl paraoxon at 410 nm (ε 400 = 17,000/M/cm for PNP), the activity was estimated employing a plate reader, [26] (SYNERGY H1 microplate reader). In milligrams of total protein, specific activities were expressed as units (micromoles of ethyl paraoxon hydrolyzed per minute) per mg.

2.2.14. Biosensing of organophosphates.

The biosensing of ethyl paraoxon is based on the recombinant fusion protein Nus-OPH and mTFP. The pH-sensitive fluorophore molecule changes its fluorescence when the enzyme organophosphorus hydrolyzes the ethyl paraoxon due to a change in the pH during the reaction. The change in fluorescence was observed to detect the presence of ethyl paraoxon.

2.2.15. Colorimetric estimation of ethyl paraoxon

CHES buffer pH 9.0, 1 mM $CoCl_2$, and the recombinant protein Nus-OPH were mixed and incubated for five minutes at 37°C Resulting in colorimetric biosensing of paraoxon. The activity of Nus-OPH is defined as the quantity of enzyme required to hydrolyze 1µmole of ethyl paraoxon and is measured in units per milliliter (µmol min^{-1} ml^{-1}), while specific activity is measured in units per milligram. The following formula was used to compute the activity and specific activity:

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

$$\text{Specific activity} = \frac{\text{Activity}}{\text{Concentration of protein}}$$

2.2.16. Fluorometric estimation of Ethyl paraoxon

The Nus-OPH and mTFP (1:1 equimolar concentration) protein were mixed with 1 mM $CoCl_2$ in a fluorometric biosensing experiment, and the decrease in fluorescence intensity was measured after 5 minutes of

incubation at 37°C. Nus-OPH protein and mTFP were combined in an equimolar ratio of 1:1 (w/w) in fluorometric biosensing studies. The volume of the mixture of Nus-OPH protein and mTFP was then adjusted to 200 µl by adding 1 mM CoCl2, CHES buffer, paraoxon, and distilled water. After five minutes at 37°C, the reaction mixture's fluorescence intensity was assessed based on the fact that the excitation wavelength for mTFP was 462 nm and the emission wavelength was 491 nm [27].

2.2.17. Biosensing parameters of calibration curves

(a). Linearity: - The values that a straight line can graphically depict are a function of linearity.

(b). Limit of detection (LOD): - is the smallest amount of a substance or sample that can be distinguished from blank.

$$\text{LOD} = \frac{\text{Standard deviation of lowest point}}{\text{Slope/ sensitivity}}$$

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(c). Limit of quantification (LOQ): - The smallest amount of analyte that can be precise ly and quantitatively determined during an individual's analytical technique. The smallest amount of analyte that can be precisely and quantitatively determined during an individual's analytical technique.

LOQ= 3*LOD * = Multiply.

(d). Sensitivity: - Minimum detectable response generated by the change in the concentration. (slope)

(e). Resolution: - Minimum concentration difference detected by the analytical method.

$$\text{Resolution} = \frac{\text{Standard deviation of the lowest point}}{\text{Slope/ sensitivity}}$$

2.2.18. Effect of tap water on the fluorometric biosensing of ethyl paraoxon.

OPH: mTFP ratio (1:1 equimolar concentration) was mixed with different concentrations of ethyl paraoxon ranging from 5 µM to 800 µM to assess the influence of contaminated tap water on the biosensing of ethyl paraoxon. The experiment's findings show that tap water affects biosensing at lower ethyl paraoxon concentrations than at greater values. Fluorescence levels are seen to linearly fall even with contaminated tap water. With higher ethyl paraoxon concentrations fluorometric biosensing is more reliable.

2.2.19. Cloning of Nus-OPH-mTFP with/without linker sequence in pET28a Expression vector

To obtain the recombinant pET28-Nus-OPH-mTFP for the expression of Nus-OPH-mTFP we used the sequential cloning methods.

2.2.20. Cloning of mTFP with linker sequence in pET28a Expression vector.

Using primers PK946F and PK763R and *Pfu* DNA polymerase, the *mTFP* gene was amplified from the recombinant plasmid OPAA-FL-mTFP as a template. For the restriction digestion of pET28a *Bam*HI and *Xho*I restriction enzymes were used. The amplified *mTFP* gene was also simultaneously digested using *Bam*HI and *Xho*I enzymes before being cloned into a pET28a plasmid.

2.2.21. Cloning of mTFP with/without linker sequence in pET28-Nus-OPH vector.

To develop pET28-Nus-OPH-mTFP with or without linker sequence for the expression of Nus-OPH- mTFP. We used previously cloned pET28-Nus-OPH-mTFP and double digest with *Bam*HI and *Xho*I to remove the mTFP resulting in 710bp fragment release and then the remaining backbone pET28-Nus-OPH used as the vector for the cloning of mTFP. Using primers PK946F/PK762F and PK763R and *Pfu* DNA polymerase, the *mTFP* gene was amplified from the recombinant plasmid OPAA-FL-mTFP

as a template. The amplified *tfp* gene with linker sequence and without linker sequence was also simultaneously digested using *Bam*HI and *Xho*I enzymes before being cloned into a pET28-Nus-OPH vector. The T4 Ligase was used to ligate the double-digested mTFP and pET28-Nus-OPH. Restriction analysis using linker sequence-specific *Apo*1 restriction digestion validated the cloning of pET28-Nus-OPH-mTFP with linker sequence. For the restriction analysis of recombinant pET28-Nus-OPH-mTFP without linker sequence, we doubly digest with *Bam*HI and *Xho*I restriction enzyme.

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Chapter 3.

Colorimetric and fluorometric biosensing of Ethyl paraoxon using recombinant Nus-OPH and mTFP

3.1 Introduction

Colorimetric and fluorimetric biosensing approaches have emerged as powerful tool for detecting and measuring target analytes with high sensitivity and selectivity. These methods exploit the unique properties of biological molecules, such as enzymes and fluorescent proteins, to generate measurable signals in response to the presence or activity of the target analyte.

In this chapter, we used Nus-OPH and mTFP separately in one reaction for biosensing assays. A recombinant Nus-OPH protein was previously cloned in the lab in the pET28a expression vector by using Nus-OPH and mTFP in the equimolar ratio unique biosensing platforms can be created. This combination enables both colorimetric and fluorimetric detection of target analytes. The colorimetric detection relies on the enzymatic activity of OPH, which catalyzes the hydrolysis of OPs and generates a colorimetric signal i.e., p-Nitrophenol a yellow-colored product in the case of ethyl paraoxon and methyl parathion. This signal can be visualized in a simple colorimetric assay where the intensity of color change is proportional to the concentration of the target analytes. This colorimetric approach offers simplicity and ease of interpretation, making it suitable for rapid and on-site detection applications. But colorimetric detection has disadvantages as the change in color will not be observable at the lowest concentration of the analyte.

On the other hand, in fluorimetric approach, relies on the fluorescence intensity of fluorophore i.e mTFP as the decrease in fluorescence intensity due to a decrease in the pH of the reaction on account of increasing protons concentration on enzymatic reaction of ethyl paraoxon. Based on the fluorescence intensity of the fluorophore, a fluorometric assay

can be developed. Colorimetric and fluorimetric biosensing has been done on various substrate concentrations and also have checked the effect of tap water on the fluorimetric biosensing of ethyl paraoxon.

3.2. Results and Discussions

3.2.1. Expression and purification of Nus-OPH

E. coli Rosetta was transformed using the recombinant pET28a-Nus-OPH plasmid. Recombinant Nus-OPH was expressed in E. coli Rosetta (DE3) containing recombinant Nus-OPH vector using 0.1mM IPTG there was significantly more expression of recombinant Nus-OPH (**Fig. 3.2a, lane 4 and 5**). The induction of recombinant Nus-OPH protein was validated on an 8% SDS-PAGE gel using 0.1 mM IPTG concentration. **Fig. 3.2a** shows the results of Nus-OPH expression in both the pellet and supernatant. According to the results of this study, a concentration of 0.5 mM IPTG resulted in the highest expression of this protein. Around 70% of the Nus-OPH protein was expressed in the supernatant fraction at 0.5 mM IPTG concentration (**Fig 3.2a lane 5**).

The recombinant Nus-OPH fusion protein was purified using the affinity of a Ni-NTA resin. The purification was verified on an 8% SDS-PAGE gel. The binding Nus-OPH protein to the Ni-NTA resin is very significant, as seen in **Fig 3.2b**; Therefore, the flow-through didn't contain any amount of protein (**Fig. 3.2b, lane 3**). We observed 50-55% pure protein. We have used this protein for further colorimetric and fluorometric assays.

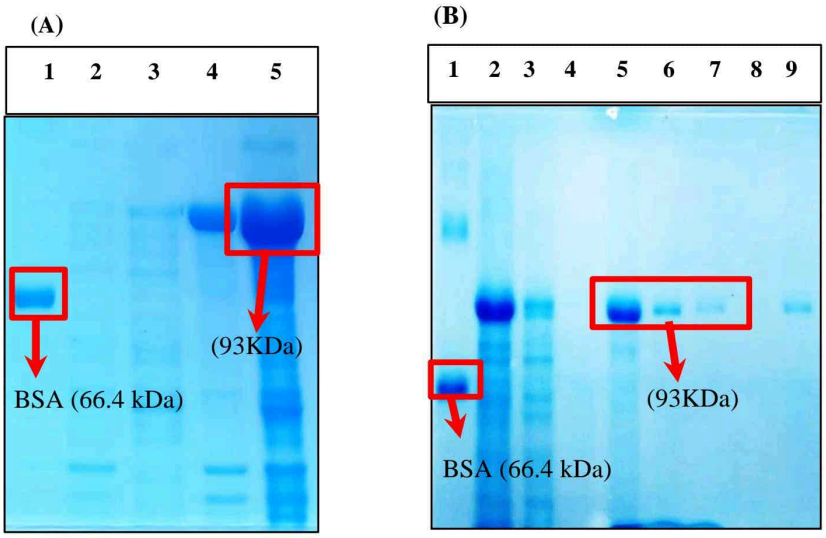


Fig. 3.2 (a) Expression and purification of Nus-OPH protein (a) induction of recombinant Nus-OPH protein. The induction of E. Coli Rosetta (DE3) with Nus-OPH expression vector was performed at 16^0C for 16 hrs. **Lane 1**: BSA 5ug, **Lane 2**: Uninduced pellet, **Lane 3**: Uninduced supernatant, **Lane 4**: induced pellet, **Lane 5**: induced supernatant**. (b) Purification of Nus-OPH protein**. **Lane 1**: BSA 5µg, **Lane 2**: cell lysate, **Lane 3**: flow through, **Lane 4**: 50mM imidazole. wash, **Lane 5**: 90mM imidazole wash, **Lane 6**: Elution 150mM imidazole, **Lane 7**: Elution 150mM imidazole. **Lane 8**: elution 150mM imidazole. **Lane 9**: elution 300mM imidazole.

3.2.2. Expression and purification of mTFP.

E. coli Rosetta was transformed using the recombinant pET28a-mTFP plasmid. E. coli Rosetta (DE3) containing recombinant mTFP vector was treated with 0.5mM IPTG to promote the expression of recombinant mTFP. there was significantly more expression of recombinant Nus-OPH (**Fig. 3.3a, lanes 4 and 5**). The induction of recombinant mTFP protein was validated on a 12% SDS-PAGE gel using 0.5 mM IPTG concentration. **Fig. 3.3a** shows the results of mTFP expression in both the pellet and supernatant. According to the results of this study, a concentration of 0.5 mM IPTG resulted in the highest expression of this protein. Around 60% of the mTFP protein was expressed in the supernatant fraction at 0.5 mM IPTG concentration (**Fig. 3.3a lane 5**).

The affinity of a Ni-NTA resin was used to purify the recombinant mTFP fusion protein. On a 12% SDS-PAGE gel, purification of the

recombinant mTFP protein was validated. The binding of mTFP protein to the Ni-NTA resin is very little significance, as seen in **Fig. 3.3b**; Therefore, the flow-through contained some amount of protein (**Fig. 3.3, lane 3**). We observed 50-55 % pure protein. We have used this protein as a pH-sensitive fluorophore along with the Nus-OPH enzyme for the fluorometric biosensing of ethyl paraoxon.

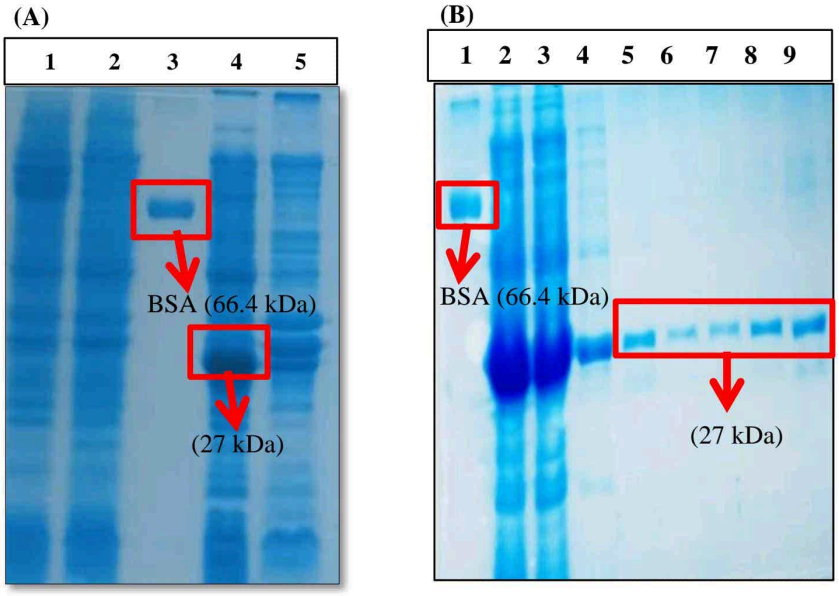


Fig. 3.3 (a) Expression and purification of mTFP protein (a) induction of recombinant mTFP protein. The induction of E. Coli Rosetta (DE3) with mTFP expression vector was performed at 20^0C for 20 hrs. **Lane 1**: Uninduced pellet, **Lane 2**: Uninduced supernatant Uninduced pellet, **Lane 3**: BSA 5ug, **Lane 4**: induced supernatant**, Lane 5**: induced pellet **(b) Purification of mTFP protein**. **Lane 1**: BSA 5µg, **Lane 2**: cell lysate, **Lane 3**: flow through, **Lane 4**: 30mM imidazole. wash, **Lane 5**: 50mM imidazole wash, **Lane 6**: Elution 70mM imidazole, **Lane 7**: Elution 90mM imidazole. **Lane 8**: elution 150mM imidazole. **Lane 9**: elution 150mM imidazole.

3.2.3. Specific activity assay of recombinant Nus-OPH against Ethyl paraoxon

To determine if the recombinant protein is truly enzymatically active, an enzyme assay was performed. Nus-OPH protein shows activity in both the lysate and the purified portion of the induced cell lysate. A cell lysate for ethyl paraoxon was then induced. Purified samples, however, displayed greater activity than induced cell lysate.

The specific activity of **Uninduced, induced**, **and purified** was found to be **3U/mg, 18U/mg,** and **50U/mg** respectively, for ethyl paraoxon. Using a **1mM** substrate concentration.

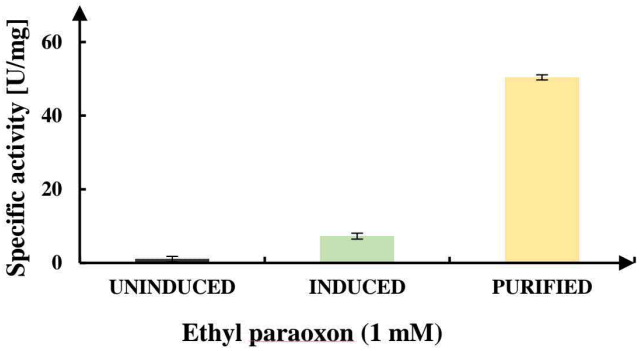


Fig. 3.4. Colorimetric biosensing assay of Nus-OPH with ethyl paraoxon Nus-OPH with ethyl paraoxon colorimetric biosensing assay. In the presence of CHES buffer at pH 9.0 and 37°C for 5 minutes, Nus-OPH with 1 mM substrate concentration. The standard deviation is shown by the error bar. The data were the average of duplicate samples.

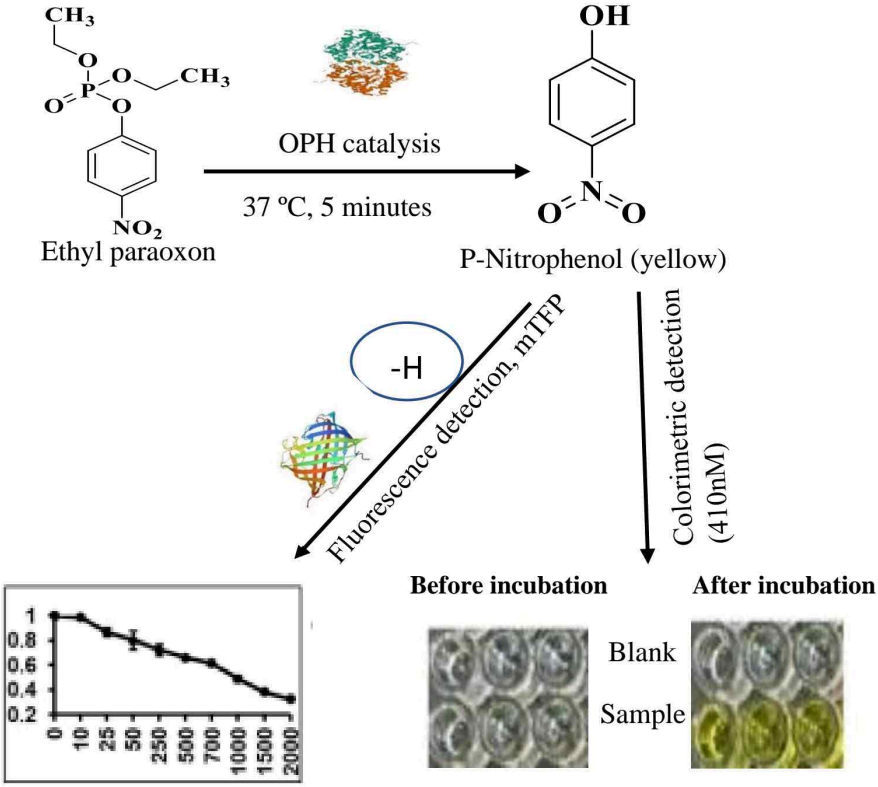


Fig. 3.1: Schematic illustration of chemical response of OPH & mTFP with ethyl paraoxon: OPH & mTFP sensing mechanism for fluorometric and colorimetric detection of organophosphate paraoxon. (*Adapted from Jain. et.al)*

3.2.4. Colorimetric biosensing of ethyl paraoxon with Nus-OPH and mTFP

Nus-OPH colorimetric biosensing was carried out with substrate concentrations in the range of **10µM to 1000 µM** and specific activity for this concentration was found to be **1,2,3,6,9,11,12,14,17 U/mg** respectively, and sensing, parameters were calculated in below Table 3.1.

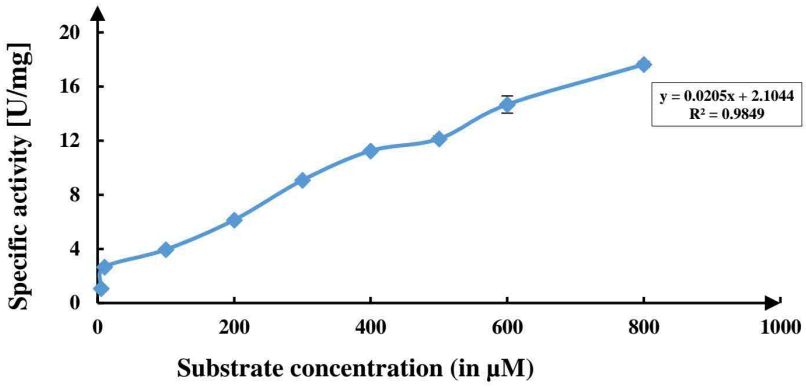


Fig 3.5. Colorimetric activity assay (a) Nus-OPH and mTFP with ethyl paraoxon in CHES buffer pH 9.0 at 37°C for 5 minutes. The average of the triplicate sample was used, while the error bar indicates the standard deviation.

Table: 3.1 Colorimetric biosensing parameters using Nus-OPH

Protein	Linearity (mM)	Sensitivity U/pm	Resolution (pm)	LOD(µM)	LOQ(µM)	Response time (minutes)
Nus-OPH	0.98	0.0205	3	9.9	29.7	5

3.2.5. Fluorometric biosensing of ethyl paraoxon with Nus-OPH & mTFP

Based on the fluorescence quenching of pH-sensitive fluorophores mTFP, a fluorescence assay was created. The hydrolysis of OP compounds by Nus-OPH gives yellow-colored molecules p-Nitrophenol and H^+ ions, a drop in the pH of the solution, fluorophore quenching, and a decrease in the

fluorescence intensity of mTFP. The reaction mixture was made with 10mM $CoCl_2$, and at various concentrations of ethyl paraoxon 100mM CHES buffer pH 9.0 was used, and the final volume was make up to 200µl the equimolar concentrations of Nus-OPH and mTFP were used in the process. After a 5-minute incubation at 37°C. The fluorescence intensity decreased was absorbed and biosensing parameters were calculated and described in below **Table 3.2.**

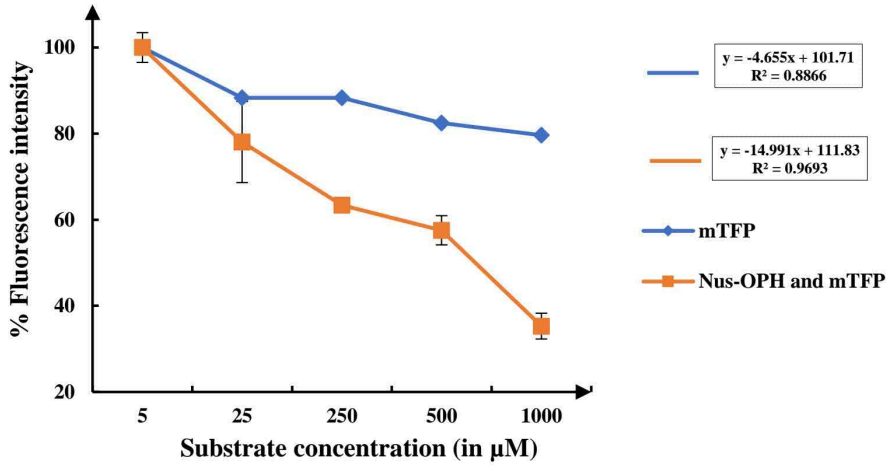


Fig. 3.6. Decrease in the fluorescence intensity at the increasing substrate concentration. Impact of pH on the fluorescence of mTFP with 25 mM CHES buffer pH 9.0, 10 mM $CoC1_2$ keep at 37°C for 5 min, and fluorescence change was observed. The average of duplicate samples is used, while the error bars indicate the standard deviation.

Table: 3.2 Fluorometric biosensing parameters using mTFP

Protein	Linearity (mM)	Sensitivity U/pm	Resolution (pm)	LOD(µM)	LOQ(µM)	Response time (minutes)
mTFP	0.96	14.99	0.22	0.74	2.24	5

3.2.6. Effect of tap water on the fluorometric biosensing of ethyl paraoxon.

OPH: mTFP ratio 1:1 was mixed with different concentrations of ethyl paraoxon ranging from 5 µM to 800 µM to assess the influence of contaminated tap water on the biosensing of ethyl paraoxon. The experiment's findings show that tap water affects biosensing at lower ethyl paraoxon concentrations than at greater values. Fluorescence levels are seen to linearly fall even with contaminated tap water. Fluorometric biosensing is more reliable on higher ethyl paraoxon concentrations.

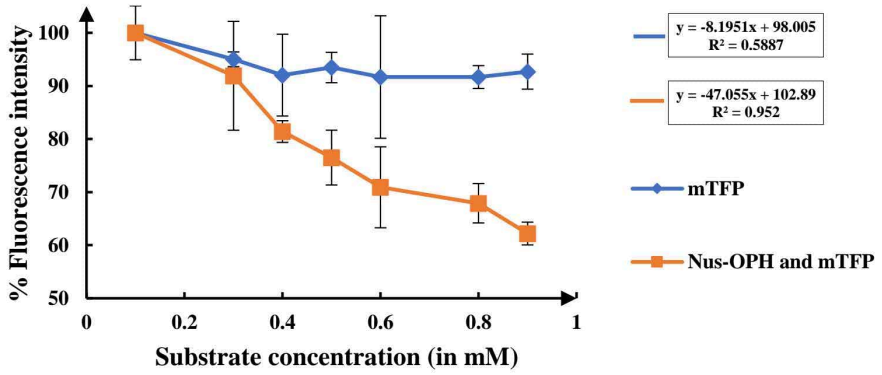


Fig. 3.7: Effect of tap water on the fluorescence biosensing: OPH protein: mTFP ratio 1:1 was analyzed in distilled water using varying concentrations of paraoxon at 37°C for 5 minutes with CHES buffer (pH 9.0). The average of duplicate samples is used, while the error bars indicate the standard deviation.

Chapter 4

Colorimetric and fluorometric biosensing of Ethyl paraoxon using recombinant fusion protein Nus-OPH-mTFP.

4.1 Introduction.

In an ongoing effort to investigate the creation of sensing platforms, a fusion protein Nus-OPH and mTFP1 fluorescent protein have been used. There are several widely used fluorescent proteins as a fusion partner, mTFP is a true monomer and possibly does not interfere with its fusion partner's function. mTFP as a fusion protein has several advantages like repeatability, stability, and easy purification and provides improved fluorescence outputs for analytical and sensing applications without the need for additional labeling or complicated processing.

In the previous chapter, we used Nus-OPH and mTFP recombinant protein in equimolar ratio for colorimetric and fluorimetric assays. In this chapter, our focus is to use recombinant Nus-OPH-mTFP fusion protein for biosensing assays. Initially, we used previously cloned Nus-OPH-mTFP fusion protein in the pET28a expression vector (*Cloning done by Vinay Singh*) and have done induction expression, purification and proceed for enzyme-specific activity assays, and fluorescence assays. After doing a fluorescence assay, we found that mTFP as a fusion partner is not showing any fluorescence, in a specific activity assay we found that the enzyme is active. In the current chapter, we have used two different strategies like cloning of *mtfp* gene along with Nus-OPH in the pET28a expression vector with a linker sequence that may help in the proper folding of mTFP protein in the fusion protein and simultaneously we did cloning of *mtfp* gene without linker sequence in pET28a-Nus-OPH vector. Details of Cloning, expression, and purification are discussed in **section 4.2.**

4.2. Results and Discussions

4.2.1. Expression and purification of Nus-OPH-mTFP

E. coli Rosetta was transformed using the recombinant pET28a-Nus-OPH-mTFP plasmid. 0.1mM IPTG was used to induce the expression of recombinant Nus-OPH-mTFP in *E. coli* Rosetta (DE3) carrying recombinant Nus-OPH-mTFP vector; there was significantly more expression of recombinant Nus-OPH (**Fig. 4.2a, lane 4 and 5**). The induction of recombinant Nus-OPH protein was validated on an 8% SDS-PAGE gel using 0.1mM IPTG concentration. **Fig. 4.2a** shows the results of Nus-OPH-mTFP expression in both the pellet and supernatant. According to the results of this study, a concentration of 0.1mM IPTG resulted in the highest expression of this protein. Around 50% of the Nus-OPH-mTFP protein was expressed in the supernatant fraction at 0.1mM IPTG concentration (**Fig. 4.2a lane 5**).

The recombinant Nus-OPH-mTFP fusion protein was purified using the affinity of a Ni-NTA resin. The purification of the recombinant Nus-OPH-mTFP protein was verified on an 8% SDS-PAGE gel. The binding of Nus-OPH-mTFP protein to the Ni-NTA resin is very less significant, as seen in **Fig. 4.2b**; Therefore, the flow-through contained a significant amount of protein (**Fig. 4.2, lane 3**). With ethyl paraoxon, we examined the colorimetric enzyme activity of recombinant Nus-OPH protein; the fusion protein was found to be enzymatically active. We have used this protein as an enzyme for further fluorometric sensing.

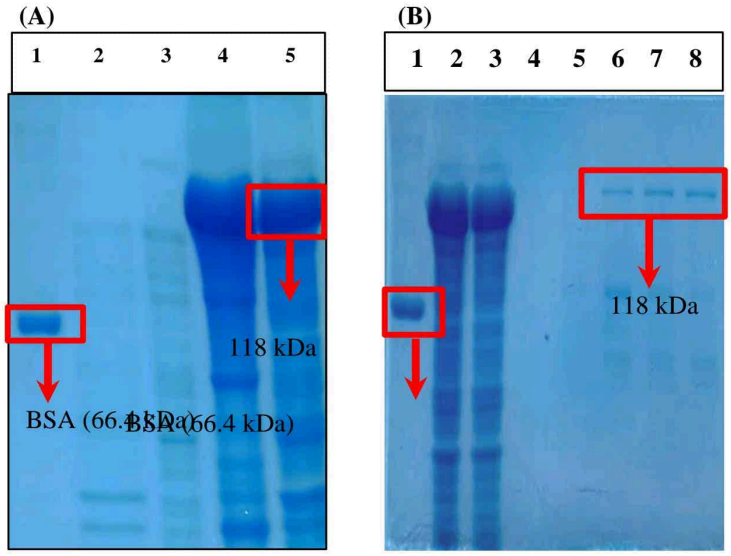


Fig. 4.2 (a) Expression and purification of Nus-OPH-mTFP protein (a) induction of recombinant Nus-OPH-mTFP protein. **Lane 1**: BSA 5ug, Lane **2**: Uninduced pellet, Uninduced pellet, **Lane 3**: Uninduced supernatant **Lane 4**: induced pellet **Lane 5**: induced supernatant**, (b)Purification of Nus-OPH-mTFP protein**. **Lane 1**: BSA 5µg, **Lane 2**: cell lysate, **Lane 3**: flow through, **Lane 4**: 50mM imidazole. wash, **Lane 5**: 70mM imidazole wash, **Lane 6**: Elution 90mM imidazole, **Lane 7**: Elution 150mM imidazole. **Lane 8**: elution 300mM imidazole.

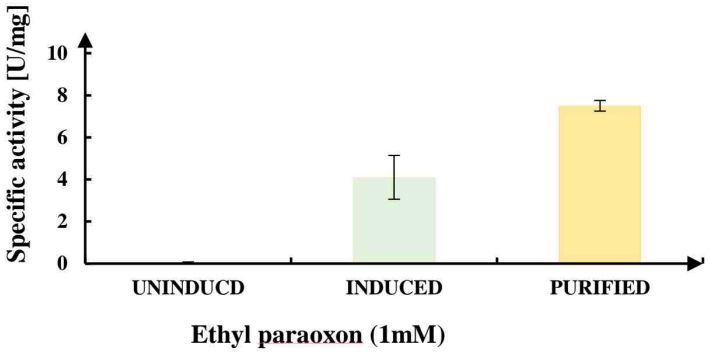


Fig. 4.3. Colorimetric biosensing assay of Nus-OPH with ethyl paraoxon. Nus-OPH with 1mM substrate concentration in CHES buffer, pH 9.0 at 37 °C for 5 min. values were the average of duplicate samples and the error bar indicates the standard deviation.

4.2.3. Fluorometric biosensing of Ethyl paraoxon with Nus-OPH-mTFP.

In the fluorometric biosensing of fusion protein Nus-OPH-mTFP. We had seen that the enzyme Nus-OPH was active and fusion partner mTFP was not showing any fluorescence at any pH, which might be due to improper folding of both the protein. Then we come up with a strategy to clone the mTFP gene with flexible linkers again in pET28a expression along with the Nus-OPH gene that will help with the proper folding of two proteins. Simultaneously we have tried to clone *mtfp* gene without a linker sequence.

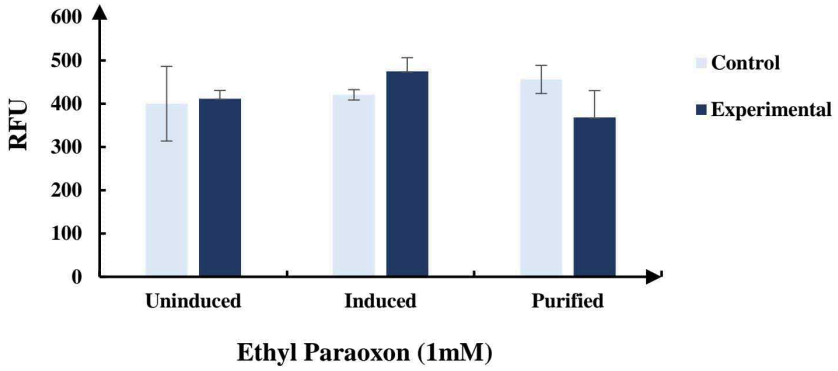


Fig. 4.4. Fluorescent assay fusion protein Nus-OPH-mTFP with ethyl paraoxon in CHES buffer pH 9.0 at 37 ^{0}C for 5 minutes. The average of the triplicate sample was used, while the error bar indicates the standard deviation.

Fig. 4.1. Schematic illustration of cloning of *mtfp* gene with linker sequence in pET28a expression vector results into pET28-mTFP Plasmid.

4.2.4. Cloning of mTFP gene with linker sequence into pET28a Expression vector

We were trying to clone the mTFP gene with a linker sequence into the recombinant pET28a vector downstream of the strong T7 promoter. The cloning was performed to obtain protein of interest codes for an N- terminal His-Tag. The *Bam*HI and *Xho*l enzymes were used to digest the pET28a plasmid (**Fig. 4.5a, lane 2**). Using Primers PK946F and PK763R and *Pfu* DNA polymerase. pET43-OPAAFL-mTFP was used as a template for the PCR amplification of the *mtfp* gene (**Fig. 4.6a, lane 2,3,4**). The *Bam*HI and *Xho*l were used to double digest the PCR-generated mTFP gene. Ligation was done with the T4 DNA ligase enzyme. The ligated was then transferred into E. coli (DH5α). Colony PCR was performed and the recombinant clones were screened after running agarose gel (**Fig. 4.6c, lane 4**). We get the probable recombinant clone after that we have to isolate pET28-mTFP recombinant plasmid (**Fig. 4.7a, lane 2**). and have done recombinant plasmid PCR and further restriction analysis for the confirmation of positive clone (**Fig. 4.7b, lane 3**)

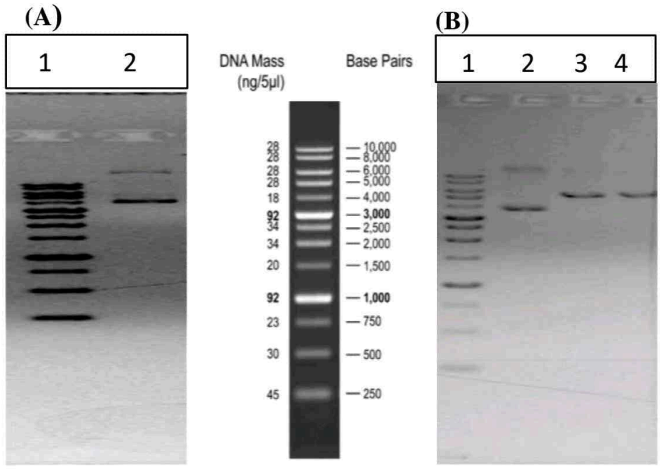


Fig. 4.5 Gel image: **(a)** Recombinant pET28a- **plasmid vector isolation**. **Lane 1**: 1kb DNA ladder, **Lane2**: pET28a- plasmid **(b)** Double digested plasmid: **Lane 1**: 1kb DNA ladder, **Lane 2**: supercoiled plasmid, **Lane3**: single digestion using *Bam*Hl; **Lane 4**; double digested using *Bam*HI and *Xho*l.

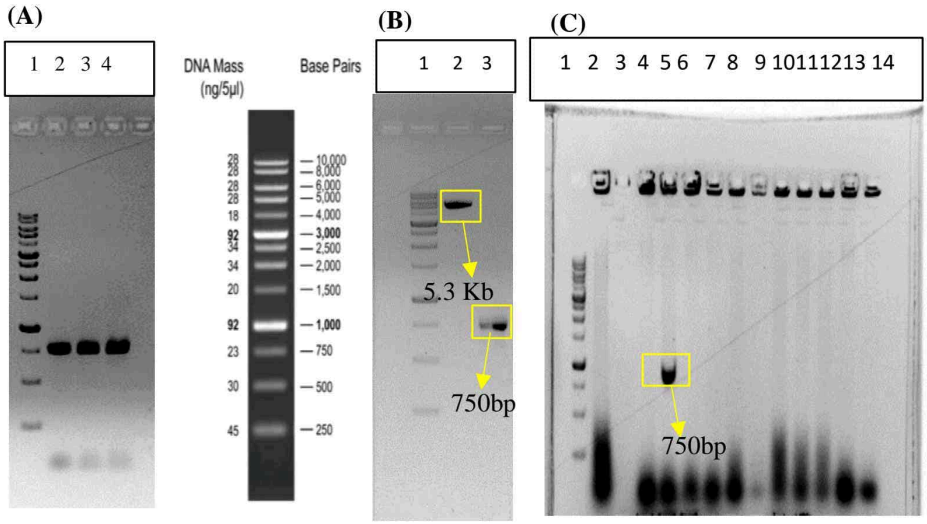


Fig. 4.6 Gel image: **(a)** Insert PCR: **Lane 1**: 1kb DNA ladder, **Lane2-4**: mTFP PCR Product **(b)** Analytical gel: **Lane 1**: 1kb DNA ladder, **Lane 2**: Double digested vector **Lane3:** Double digested insert mTFP Using *Bam*Hl & *Xho*l**;(c)** Colony PCR: - **Lane 1**; 1kb DNA ladder, **Lane 2:** positive control**, Lane 3:** Negative control, **Lane 4-14;** Clone 1-10.

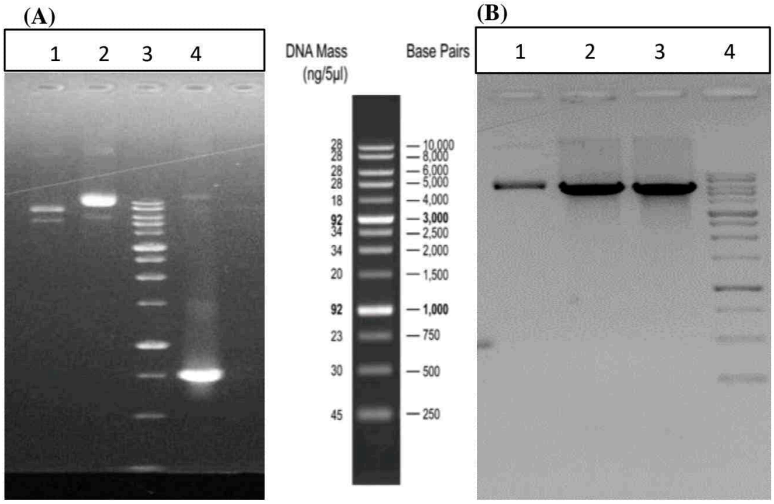


Fig. 4.7 Gel image: **(a)** Recombinant plasmid isolation and r-Plasmid PCR. **Lane 1**: pET28a Null vector, **Lane2**: pET28a-mTFP C-2, **Lane3**: 1kb DNA ladder, **Lane4;** Recombinant plasmid PCR (**b)** Restriction analysis of recombinant clones: **Lane 1**: Supercoiled pET28a **Lane 2**: Single digested, **Lane3**: double digestion; **Lane 4**; 1kb DNA ladder.

4.2.5. Expression and purification of pET28-mTFP (with linker sequence).

E. coli Rosetta was transformed using the recombinant pET28a-mTFP plasmid. Induction was done with 0.5mM IPTG to express the recombinant pET28-mTFP in *E. coli* Rosetta (DE3) carrying recombinant pET28-mTFP vector; there was significantly more expression of recombinant pET28-mTFP (**Fig. 4.8a, lane 4 and 5**). The induction of recombinant pET28-mTFP protein was validated on a 12% SDS-PAGE gel using 0.5 mM IPTG concentration. **Fig. 4.8a** shows the results of pET28-mTFP expression in both the pellet and supernatant. According to the results of this study, a concentration of 0.5mM IPTG resulted in the highest expression of this protein. Around 60% of the pET28-mTFP protein expressed in the supernatant fraction at 0.5 mM IPTG concentration (**Fig 4.8a lane 5**).

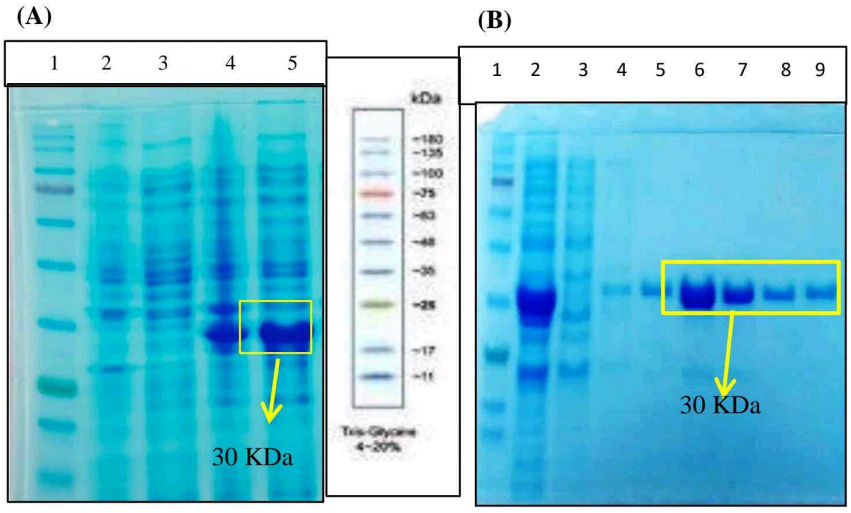


Fig. 4.8 (a) Expression and purification of mTFP protein (a) induction of recombinant mTFP protein. The induction of E. Coli Rosetta (DE3) with Nus-OPH-mTFP expression vector was performed at 20^0C for 20 hrs. **Lane 1**: BSA 5ug, **Lane 2**: Uninduced pellet, **Lane 3**: Uninduced supernatant **Lane 4**: induced pellet **Lane 5**: induced supernatant**, (b) Purification of mTFP protein**. **Lane 1**: BSA 5µg, **Lane 2**: cell lysate, **Lane 3**: flow through, **Lane 4**: 50mM imidazole. wash, **Lane 5**: 70mM imidazole wash, **Lane 6**: Elution 90mM imidazole, **Lane 7**: Elution 150mM imidazole. **Lane 8**: elution 300mM imidazole.

4.2.6. Cloning of mTFP gene with linker sequence into pET28-Nus-OPH Expression vector

We were trying to clone the mTFP gene with a linker sequence into the recombinant pET28-Nus-OPH vector downstream of the strong T4 promoter. We have used previously cloned pET28-Nus-OPH-mTFP and double digest with *Bam*HI and *Xho*I to remove the mTFP resulting in 711bp fragment release and then the remaining backbone pET28-Nus-OPH used as a vector for the cloning of mTFP with linker sequence. The cloning was performed to obtain protein of interest codes for an N- terminal His-Tag. The *Bam*HI and *Xho*l enzymes were used to digest the pET28-Nus-OPH-TFP plasmid to release 750bp of *Tfp* gene (**Fig. 4.9a, lane 4**). Using Primers PK946F and PK763R and *Pfu* DNA polymerase. pET43-OPAAFL-mTFP was used as a template for the PCR amplification of the *mtfp* gene (**Fig. 4.9b, lane 2,3,4**). The *Bam*HI and *Xho*l were used to double digest the PCR-generated mTFP gene. Ligation was done with the T7 DNA ligase enzyme.

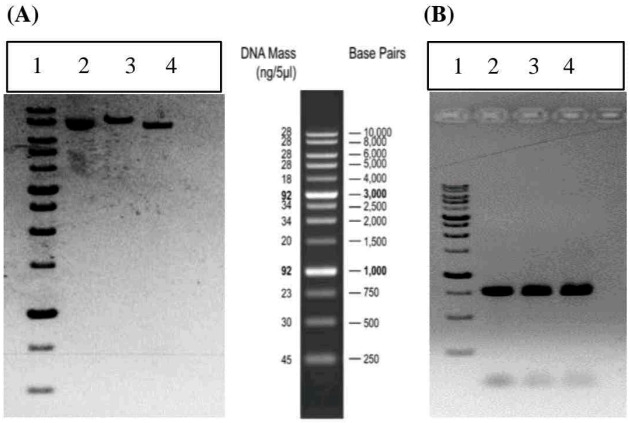


Fig. 4.9 Gel image: **(a)** Vector preparation: **Lane 1**: 1kb DNA ladder, **Lane2**: pET28-Nus-OPH-mTFP plasmid supercoiled **Lane3**: Single digested *(Bam*H1*)* **Lane3**: Double digested *(Bam*H1) & (*Xho*1) **(b)** Insert mTFP **Lane 1**: 1kb DNA ladder, **Lane 2-4**: Insert PCR Product.

The ligated product was then transferred into *E. coli* DH5α. Colony PCR was performed using Vector specific primers and the recombinant clones were screened after running agarose gel (**Fig. 4.10b,**). We get the probable recombinant clone after that we isolate pET28-Nus-OPH recombinant plasmid with linker sequence and do recombinant plasmid PCR

and further restriction analysis for the confirmation of positive clone (**Fig. 4.11b, lane 5,9,11**).

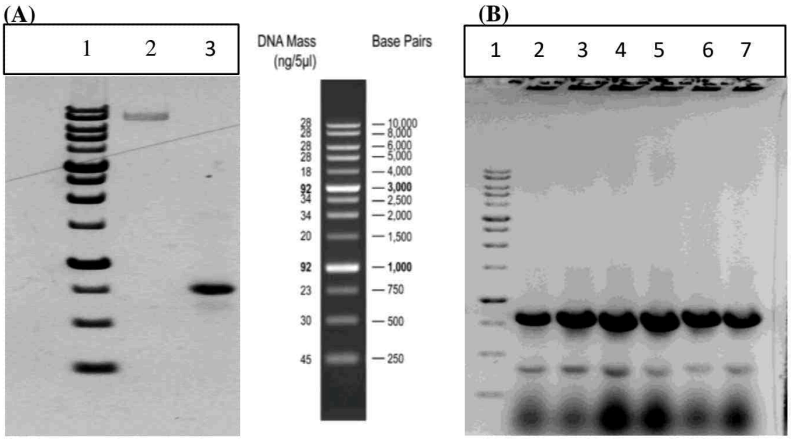


Fig. 4.10 Gel image: **(a)** Analytical gel for the concentration determination of insert and vector after gel elution: **Lane 1**: 1kb DNA ladder, **Lane2**: Double digested vector pET28-Nus-OPH-mTFP **Lane3**: Double digested **insert (b)**Colony PCR (vector specific): **Lane 1**: 1kb DNA ladder, **Lane 2-7**: Clone 1-6.

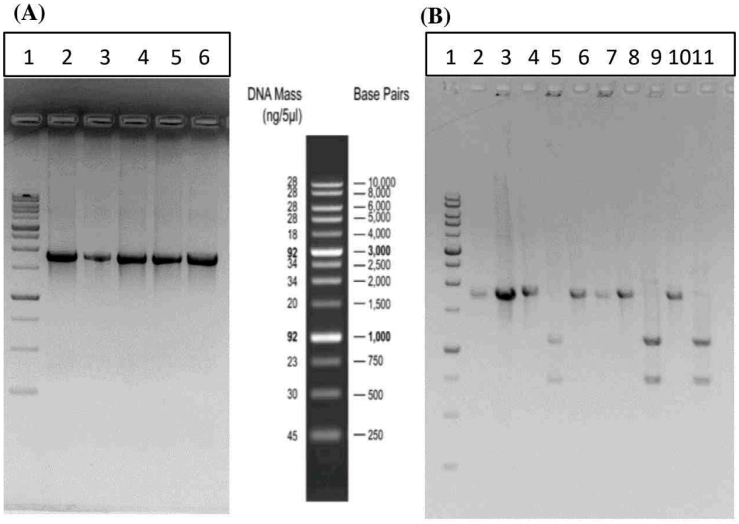


Fig. 4.11 Gel image: **(a)** Plasmid PCR (Vector specific): **Lane 1**: 1kb DNA ladder, **Lane2**: positive control, **Lane3-6:** clone 1-4 **(b)** Restriction analysis: **Lane 1**: 1kb DNA ladder, **Lane 2,4,6,8** Undigested plasmid PCR product of a null vector and clone 1-4: **Lane3:** *Apo*1 digested PCR product of null vector **Lane 5,7,9,11**; *Apo*1 digested PCR product of clone 1-4**.** *Apo*1 restriction used here is specific to the linker sequence and it digests the recombinant plasmid PCR product having a linker sequence. It didn't cut the PCR product of a null vector.

4.2.7. Expression and purification of Nus-OPH-mTFP (With linker sequence)

E. coli Rosetta was transformed using the recombinant pET28a-Nus-OPH-mTFP plasmid. 0.1mM IPTG was used to induce the expression of recombinant Nus-OPH-mTFP in *E.coli* Rosetta (DE3) carrying recombinant Nus-OPH-mTFP vector; there was significantly more expression of recombinant Nus-OPH-mTFP, expected size of our protein should be 122KDa and we get only band at about 93KDa (**Fig. 4.12a, lane 4**). The induction of recombinant Nus-OPH-mTFP protein was validated on an 8% SDS-PAGE gel using 0.1mM IPTG concentration. **Fig. 4.12a** shows the results of Nus-OPH-mTFP expression in both the pellet and supernatant. According to the results of this study, a concentration of 0.1 mM IPTG resulted in the highest expression of this protein. Around 50% of the Nus-OPH-mTFP protein was expressed in the supernatant fraction at 0.1 mM IPTG concentration (**Fig 4.12a lane 4**).

The recombinant Nus-OPH-mTFP fusion protein was purified using the affinity of a Ni-NTA resin. The purification of Nus-OPH-mTFP protein was verified on an 8% SDS-PAGE gel. The binding of Nus-OPH-mTFP protein to the Ni-NTA resin is very less significant according to the binding capacity of column, as seen in **Fig. 4.12b**; Therefore, the flow-through contained some amount of protein (**Fig. 4.12b, lane 3**). With ethyl paraoxon, we examined the colorimetric enzyme activity of recombinant Nus-OPH-mTFP protein and examined the fluorescence of fusion partner mTFP; the fusion protein was found to be enzymatically active, and fusion partner mTFP was also showing fluorescence in cell lysate.

4.2.8. Specific activity assay of recombinant Nus-OPH-mTFP against Ethyl paraoxon.

To determine if the recombinant protein is truly enzymatically active, a Nus-OPH-mTFP enzyme assay was done. The protein Nus-OPH-mTFP demonstrated activity in both the lysate and the purified portion of the induced cell lysate. For ethyl paraoxon, uninduced cell lysate was shown

to be significantly less active than purified samples, however, displayed greater activity than induced cell lysate.

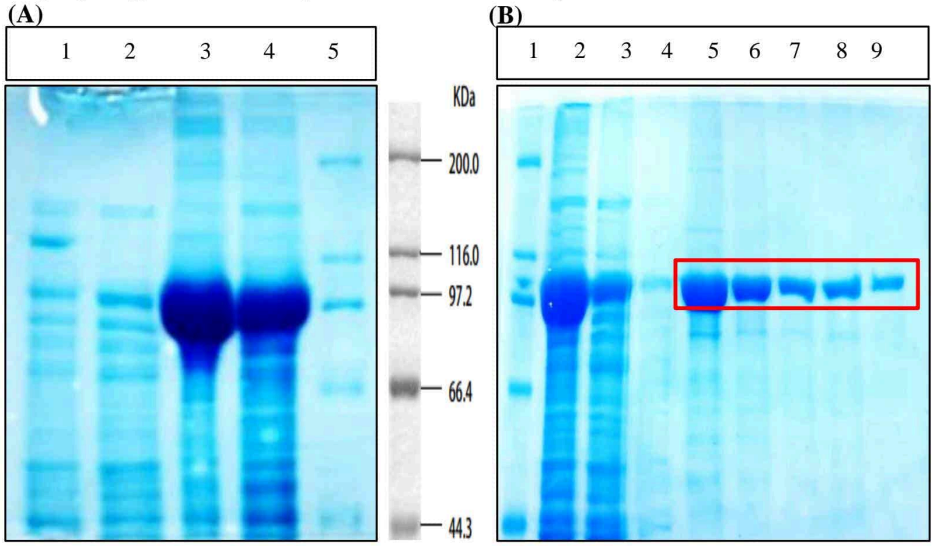


Fig. 4.12 (a) Expression and purification of Nus-OPH-mTFP protein (a) induction of recombinant Nus-OPH-mTFP protein. The induction of *E.Coli* Rosetta (DE3) with Nus-OPH-mTFP expression vector was performed at 20^0C for 20 hrs. **Lane 1**: Uninduced pellet**, Lane 2**: Uninduced supernatant **Lane 3**: induced pellet **Lane 4**: induced supernatant**,** Lane **5:** Unstained protein ladder (**b) Purification of Nus-OPH-mTFP protein**. **Lane 1**: Unstained protein ladder**, Lane 2**: cell lysate, **Lane 3**: flow through, **Lane 4**: 50mM imidazole. wash, **Lane 5**: 90mM imidazole wash, **Lane 6**: Elution 150mM imidazole, **Lane 7**: Elution 150mM imidazole. **Lane 8**: elution 150mM imidazole. **Lane 9**: elution 300mM imidazole.

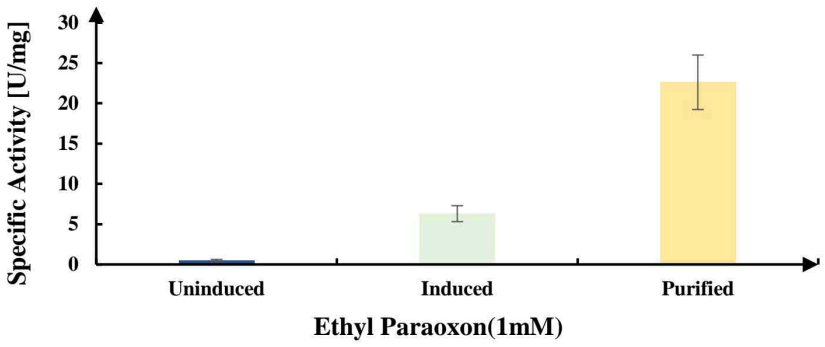


Fig. 4.13. Colorimetric biosensing assay of Nus-OPH with ethyl paraoxon. Nus-OPH with 1mM substrate concentration in CHES buffer, pH 9.0 at 37 °C for 5 min. values were the average of duplicate samples and the error bar indicates the standard deviation.

4.2.9. Fluorometric biosensing of Ethyl paraoxon with Nus-OPH-mTFP

The fluorometric estimation of Nus-OPH-mTFP both with cell lysate and purified was done. The decrease in fluorescence intensity was shown in the cell lysate as compared to the control where the substrate was not added and the purified protein was not showing any fluorescence. According to the sequencing results, there is a frameshift mutation due to the incorporation of a nucleotide after the restriction enzyme site in the linker sequence. This frameshift resulted in the formation of 2 stop codon in the linker region.

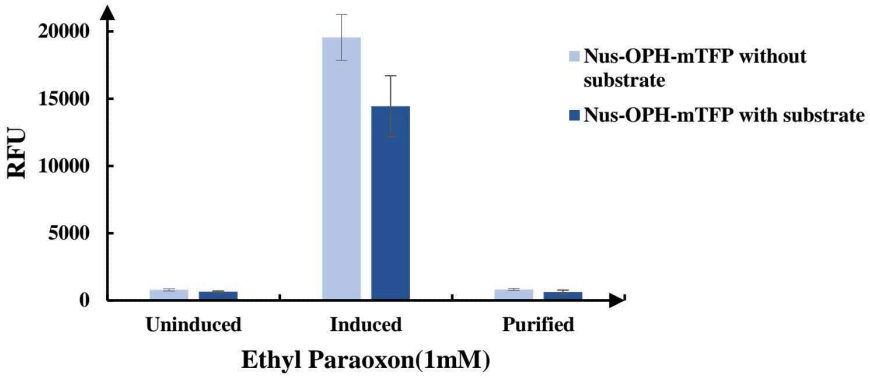


Fig. 4.14. **Fluorescent assay fusion protein Nus-OPH-mTFP** with ethyl paraoxon in CHES buffer pH 9.0 at 37ºC for 5 minutes. The average of the duplicate sample was used, while the error bar indicates the standard deviation.

4.2.10 Cloning of mTFP gene into pET28-Nus-OPH Expression vector

The *mtfp* gene was cloned into the recombinant pET28-Nus-OPH vector downstream of the strong T7 promoter. We have used previously cloned pET28-Nus-OPH-mTFP and double digest with *Bam*HI and *Xho*I to remove the mTFP resulting in 711bp fragment release and then the remaining backbone pET28-Nus-OPH used as a vector for the cloning of *mtfp* gene. The cloning was performed to obtain protein of interest codes for an N- terminal His-Tag. The *Bam*HI and *Xho*l enzymes were used to digest the pET28-Nus-OPH-TFP plasmid to release 711 bp of *tfp* gene (**Fig. 4.15b, lane 4**). Using Primers PK762F and PK763R and *Pfu* DNA polymerase. pET43-OPAAFL-mTFP was used as a template for the PCR amplification of the *mtfp* gene (**Fig. 4.15c, lane 3**).

The *Bam*HI and *Xho*l were used to double digest the PCR-generated *mtfp* gene. Ligation was done with the T7 DNA ligase enzyme. The ligated product was then transferred into *E. coli* DH5α. Colony PCR was performed using Vector specific primers and the recombinant clones were screened after running agarose gel (**Fig. 4.16b,**). After doing colony PCR we get some positive clones then we isolated the pET28-Nus-OPH-mTFP recombinant plasmid **(Fig. 4.17a)**. The recombinant plasmid was at its appropriate size. Then proceeded for recombinant plasmid PCR and further restriction analysis was done using *BamH*I and *Xho*I restriction enzymes **(Fig. 4.17c)**.

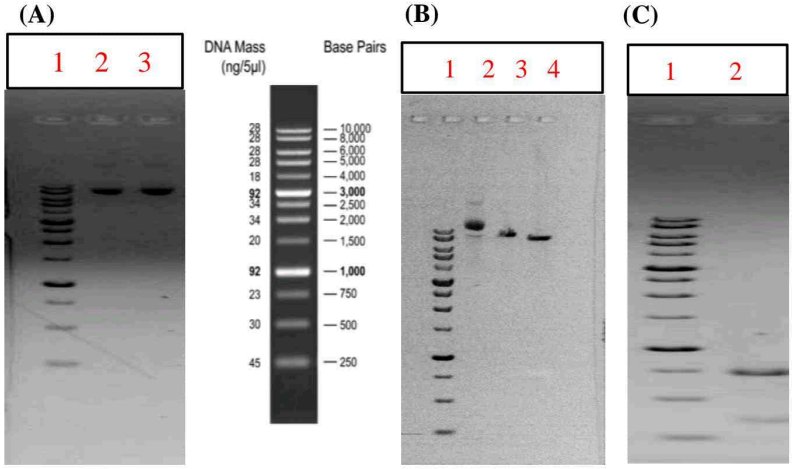


Fig. 4.15 Gel image: **(a)** pET28a-Nus-OPH-TFP **plasmid isolation**. **Lane 1**: 1kb DNA ladder, **Lane2-3**: pET28a-Nus-OPH-TFP plasmid **(b)** Double digested plasmid: **Lane 1**: 1kb DNA ladder, **Lane 2**: supercoiled plasmid, **Lane3**: single digestion using *Bam*Hl; **Lane 4**; double digested using *Bam*HI and *Xho*l**.(c)** Insert preparation: **Lane 1**: 1kb DNA ladder, **Lane 2**: *tfp* gene PCR product.

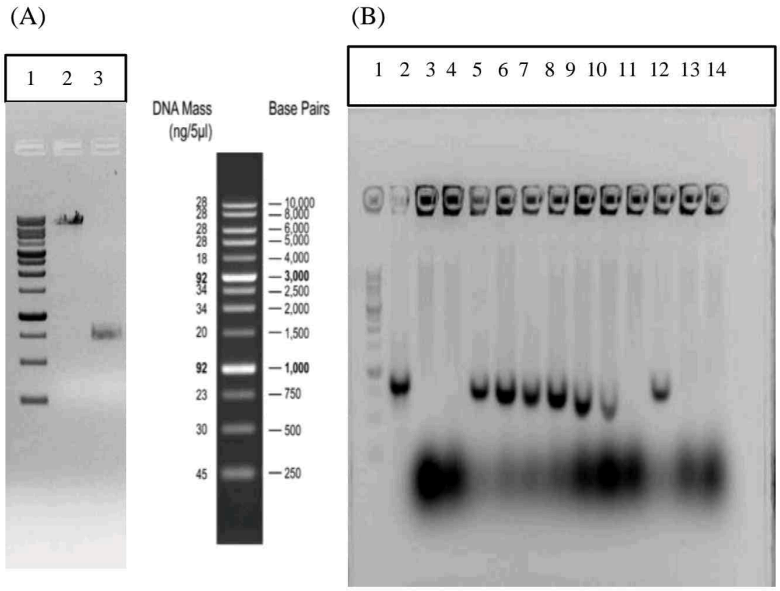


Fig. 4.16 Gel image: **(a)** Analytical gel for the concentration determination of both double-digested insert and vector after gel elution. **Lane 1**: 1kb DNA ladder, **Lane 2**: Double digested pET28-Nus-OPH Vector, **Lane 3**: Double digested *tfp* gene. **(b)** Colony PCR, **Lane** 1: 1kb DNA ladder, **Lane 2**: positive control, **Lane 3-13**: clone 1-11 **Lane 14**: Negative control.

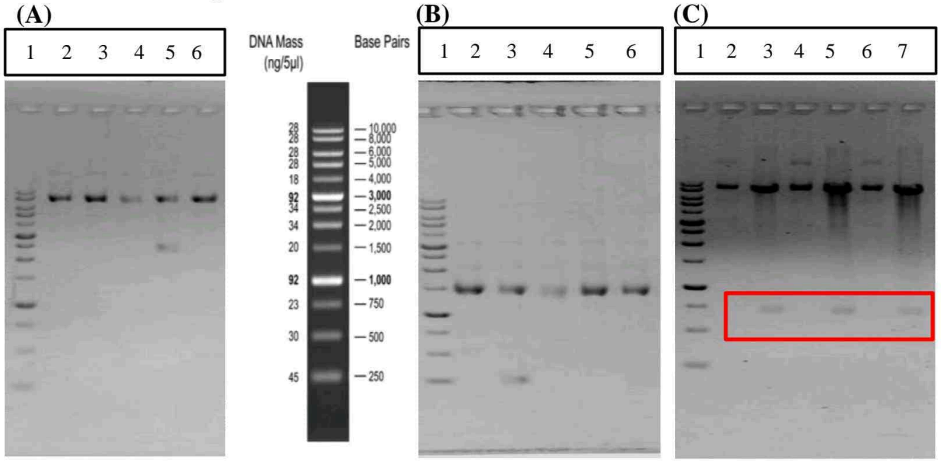


Fig. 4.17 Gel image: **(a)** Recombinant pET28a-Nus-OPH-TFP **plasmid isolation**. **Lane 1**: 1kb DNA ladder, **Lane2-6**: C1,C2,C3,C4,C5,C10 **(b)** Recombinant plasmid PCR: **Lane 1**: 1kb DNA ladder, **Lane 2-6** PCR of C1,C2,C3,C4,C5,C10 **(c)** Restriction analysis of positive recombinant clones **Lane 1**: 1kb DNA ladder, **Lane 2,4,6** : Supercoiled plasmid of C3,C4,C10, **Lane3,5,7**: double digested using *Bam*HI and *Xho*l OF C3,C4,C10.

4.2.11. Expression and purification of Nus-OPH-mTFP

E. coli Rosetta was transformed using the recombinant pET28a-Nus-OPH-mTFP plasmid. 0.1mM IPTG was used to induce the expression of recombinant Nus-OPH-mTFP in *E. coli* Rosetta (DE3) carrying recombinant Nus-OPH-mTFP vector; there was significantly more expression of recombinant Nus-OPH-mTFP, The induction of recombinant Nus-OPH-mTFP protein was validated on an 8% SDS-PAGE gel using 0.5mM IPTG concentration. **Fig. 4.18a** shows the results of Nus-OPH-mTFP expression in both the pellet and supernatant. According to the results of this study, a concentration of 0.5 mM IPTG resulted in the highest expression of this protein. Around 50% of the Nus-OPH-mTFP protein was expressed in the supernatant fraction at 0.5 mM IPTG concentration (**Fig 4.18a lane 5,7**).

The recombinant Nus-OPH-mTFP fusion protein was purified using the affinity of a Ni-NTA resin. The purification of Nus-OPH-mTFP protein was verified on an 8% SDS-PAGE gel. The binding of Nus-OPH-mTFP protein to the Ni-NTA resin is less significant according to the binding capacity of column, as seen in **Fig. 4.18b**; Therefore, the flow-through contained some amount of protein (**Fig. 4.18 b, lane 3**). With ethyl paraoxon, we examined the colorimetric enzyme activity of recombinant Nus-OPH-mTFP protein and examined the fluorescence of fusion partner mTFP; the fusion protein was found to be enzymatically active, and fusion partner mTFP was also showing fluorescence in cell lysate.

4.2.12. Specific activity assay of recombinant Nus-OPH-mTFP fusion protein against Ethyl paraoxon.

Specific activity is used to determine whether the recombinant protein is enzymatically active. A specific activity assay of Nus-OPH-mTFP was done. Specific activity is shown to be both in induced cell lysates and purified samples, however purified samples show higher activity as compared to induced cell lysates.

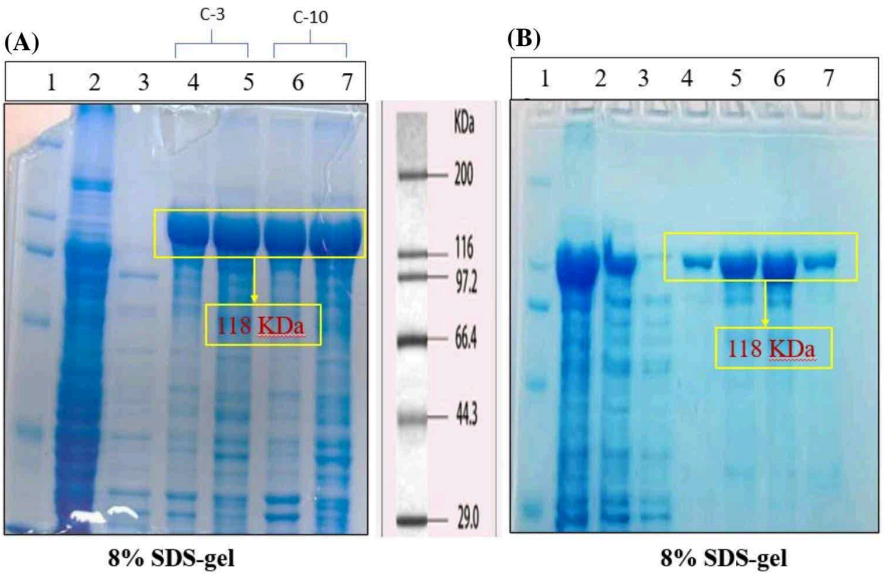


Fig. 4.18 (a) Expression and purification of Nus-OPH-mTFP protein (a) induction of recombinant Nus-OPH-mTFP protein. The induction of *E.Coli* Rosetta (DE3) with Nus-OPH-mTFP expression vector was performed at 20ºC for 20 hrs. **Lane 1**: Unstained protein ladder **Lane 2**: Uninduced pellet**, Lane 3**: Uninduced supernatant **Lane 4**: induced pellet c-3 **Lane 5**: induced supernatant c3**, Lane 6:** induced pellet c-10, **Lane 7** induced supernatant c10 (**b) Purification of Nus-OPH-mTFP protein**. **Lane 1**: Unstained protein ladder**, Lane 2**: cell lysate, **Lane 3**: flow through, **Lane 4**: 50mM imidazole. wash, **Lane 5**: 90mM imidazole wash, **Lane 6**: Elution 150mM imidazole, **Lane 7**: Elution 150mM imidazole. **Lane 8**: elution 300mM imidazole.

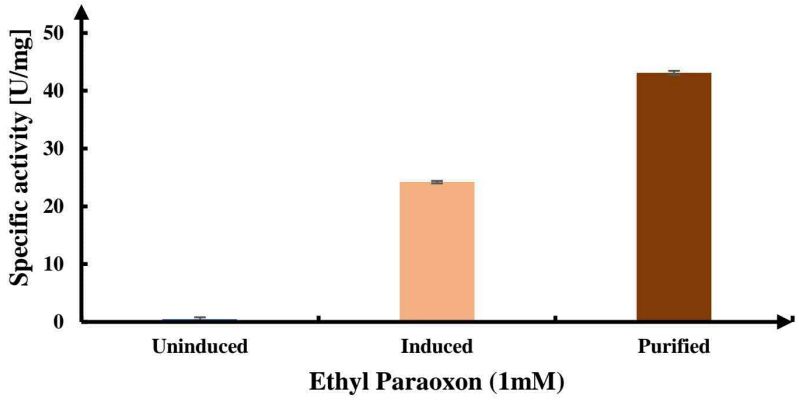


Fig. 4.19. Colorimetric biosensing assay of Nus-OPH with ethyl paraoxon. Nus-OPH with 1mM substrate concentration in CHES buffer, pH 9.0 at 37ºC for 5 min. values were the average of duplicate samples and the error bar indicates the standard deviation.

4.2.13. Fluorometric biosensing of Ethyl paraoxon with Nus-OPH-mTFP

Fluorometric assay of Nus-OPH-TFP With Ethyl paraoxon was done with both cell lysates and purified samples the decrease in fluorescence intensity was observed in both cell lysates and purified samples as compared to the control where the substrate was not added. However, purified samples show little less fluorescence intensity.

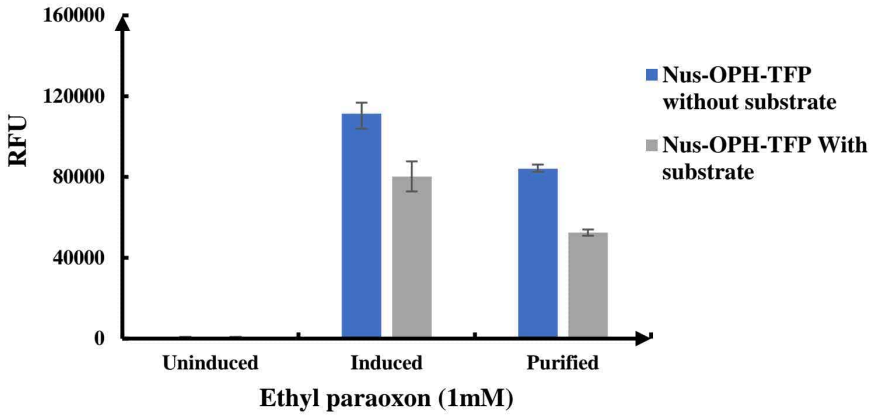


Fig. 4.20. **Fluorescent assay fusion protein Nus-OPH-mTFP** with ethyl paraoxon in CHES buffer pH 9.0 at 37°C for 5 minutes. The average of the duplicate sample was used, while the error bar indicates the standard deviation.

Chapter 5

5.1. Summary and future perspectives

As pests develop resistance to the present pesticides and new chemicals are developed, the use of pesticides is rising. Due to circulation and runoff, pesticides cause harm to groundwater, rivers, and soil by transferring chemicals from their initial location. The used pesticide is poisonous to the bugs and targeted insects. it is intended to control, but it also unintentionally harms non-target animals, including humans. Due to the overuse of hazardous chemicals, animal pollinators are severely impacted. Because they pollinate over 90% of plants, pollinators also play a critical role in maintaining ecological and economic equilibrium in the environment. Since persistent pesticides persist in the environment for a longer period and can bioaccumulate in the food chain, and cause pesticide poisoning of the aquatic system that is a serious concern. Because conventional pesticide monitoring methods are ineffective and expensive, they are unsuited for real-time control, which makes biosensors more alluring, especially for field applications and unskilled users. The present field analytical sensors are difficult in manipulating and handle, have low specificity, need specialist equipment, and trained personnel, and take a long time to analyze. It is urgently necessary to develop biosensors that can identify harmful chemical pesticides. This research describes the cloning, production, and purification of enzymes that break down OPs as well as developments in fluorescent-tagged fusion proteins-based biosensing of the Organophosphorus compound.

The fluorescence-based biosensing parameters were optimized for OPH and mTFP in a 1:1 ratio. In chapter 4 of the thesis where the objective was to use fusion protein Nus-OPH-TFP for biosensing of organophosphorus compound, Cloning, expression, and purification of Nus-OPH-TFP fusion protein with and without linker sequence was done simultaneously. After purification Nus-OPH-TFP fusion protein was not showing any

fluorescence at any pH, according to the Sanger sequencing results there is incorporation of nucleotide in the linker region after the restriction enzyme site due to that there is a frameshift mutation. However, the fusion protein without a linker sequence is showing fluorescence even after purification. Further, stability study, kinetics study, and other characterization of fusion protein Nus-OPH-TFP need to be done. The LOD for fluorometric biosensing of ethyl paraoxon using OPH:mTFP ratio 1:1 was 0.74 µM. We studied the effect of tap water in the fluorescence-based biosensing of ethyl paraoxon with concentrations varying from 5µM to 800µM. It was observed that the detection of paraoxon was more robust at higher concentrations, and there was less impact of contaminations of tap water on the detection of paraoxon at higher concentrations compared to lower paraoxon concentrations.

Enzymatic approaches for the detection of pesticides are compared to the conventional method. The detection limit of conventional methods is lower than that of enzyme-based strategies. Traditional procedures can detect pesticides in the nanogram range (ng L-1) level, whereas enzymatic approaches reach detection limits in the µg L -1 range, depending on the specific methodology utilized. This is not to say that enzymatic methods are not helpful they can aid in the quick, in-place screening of a lot of cases, which can then be confirmed otherwise with chromatographic methods to precisely identify and quantify. In this way, two approaches for the detection and measurement of pesticides could complement one another. The development of a low-cost, dependable, stable, user-friendly, and environmentally friendly sensor that can identify the presence of dangerous chemical pesticides is made possible by our study on fluorescent-based biosensors. However, the enzymes that break down OPs are non-human sources and present in 55 pharmacokinetic and immunogenicity problems. Pesticides and nerve agents might be detoxified within the bloodstream as a result of the creation of these enzymes in a varied production system that

involves surface modification. As a result, they meet the criteria for optimal bioscavangers that have prolonged circulation without cytotoxicity.

6. Annexure

6.1. List of primers

Primer	Sequence (5'-3') [a,c]	Restriction site[b]
Primers for cloning of mTFP[linker] into pET28a expression vector		
PK946F	5'GAA**GGATCCggcagtgcaggcagcgcagcgggtagtg gtgaattt**atggtgagcaagggcgaggag 3'	*Bam*H1
PK763R	ATGC**CTCGAG**ttacttgtacagctcgtccatgcc	*Xho*I
Primers for cloning of mTFP[linker] into pET28a-Nus-OPH vector		
PK946F	5'GAA**GGATCCggcagtgcaggcagcgcagcgggtagtg gtgaattt**atggtgagcaagggcgaggag 3'	*Bam*HI
PK763R	ATGC**CTCGAG**ttacttgtacagctcgtccatgcc	*Xho*I
Primers for cloning of mTFP [without linker] into pET28a-Nus-OPH vector		
PK762F	ATGC**GGATCC**atggtgagcaagggcgaggag	*Bam*HI
PK763R	ATGC**CTCGAG**ttacttgtacagctcgtccatgcc	*Xho*I

[a] Nucleotides in the small case are complementary to the genome sequence.

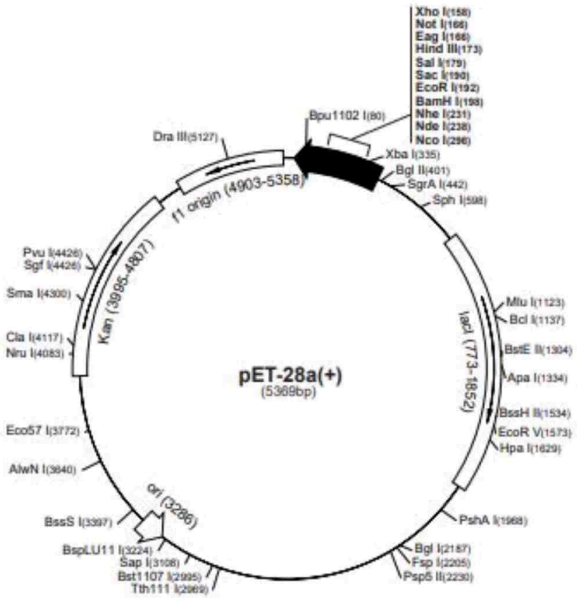
[b] Underlined (bold case) in the corresponding sequence.

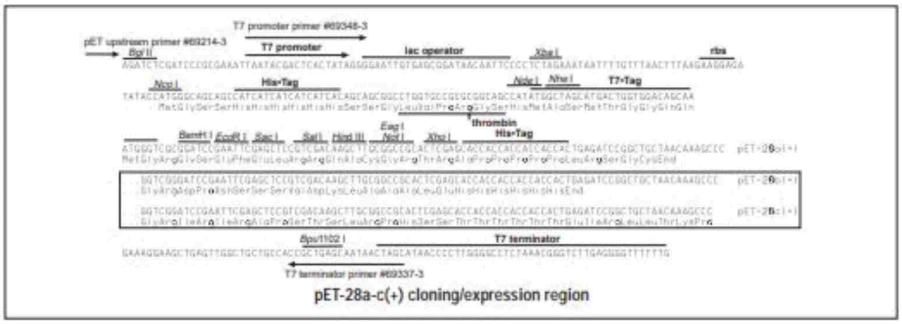
[c] Nucleotides in a small case and bold letters corresponds to the linker sequence.

6.2. Plasmid map of pET28a

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*BamH* I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.





Chapter 6

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