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Review

Organophosphate pesticides an emerging environmental contaminant: Pollution, toxicity, bioremediation progress, and remaining challenges

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ABSTRACT

Organophosphates (OPs) are an integral part of modern agriculture; however, due to overexploitation, OPs pesticides residues are leaching and accumulating in the soil, and groundwater contaminated terrestrial and aquatic food webs. Acute exposure to OPs could produce toxicity in insects, plants, animals, and humans. OPs are known for covalent inhibition of acetylcholinesterase enzyme in pests and terrestrial/aquatic organisms, leading to nervous, respiratory, reproductive, and hepatic abnormalities. OPs pesticides also disrupt the growth-promoting machinery in plants by inhibiting key enzymes, permeability, and trans-cuticular diffusion, which is crucial for plant growth. Excessive use of OPs, directly/indirectly affecting human/environmental health, raise a thoughtful global concern. Developing a safe, reliable, economical, and eco-friendly methods for removing OPs pesticides from the environment is thus necessary. Bioremediation techniques coupled with microbes or microbial-biocatalysts are emerging as promising antidotes for OPs pesticides. Here, we comprehensively review the current scenario of OPs pollution, their toxicity (at a molecular level), and the recent advancements in biotechnology (modified biocatalytic systems) for detection, decontamination, and bioremediation of OP-pesticides in polluted environments. Furthermore, the review focuses on onsite applications of OPs degrading enzymes (immobilizations/biosensors/others), and it also highlights remaining challenges with future approaches.

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Introduction

The term pesticide is a common term used to characterize several classes of fungicides, rodenticides, wood preservatives, garden chemicals, and household disinfectants used to either destroy the pest or gain resistance (Vinet and Zhedanov, 2010). Plenty of pesticides with different functional groups are used worldwide to cultivate crops. The residues of pesticides are regularly detected in air, soil, and water because of their widespread use (Golfinopoulos et al., 2003; Kumar et al., 2018). Coinciding with the changes in farming practices and developments in intensive agriculture, the use of pesticides has increased worldwide over the past couple of decades. The use of pesticides and the way they have gained importance in agriculture can be summarized by the following analogy "the saucer is to the cup and hence are the pesticides to the modern agriculture." Organophosphates (OPs) form the most widely and majorly used group of pesticides worldwide, among the various pesticides currently in use, accounting for 45% of the total world market (Mali et al., 2022a; Müller et al., 2007). OPs are the esters of phosphoric acids , including aliphatic, phenyl, or heterocyclic derivatives as a part of their complex chemical structures. Organochlorines were substituted with OPs to a greater extent as one of the major groups of pesticides to improve crop yield and used to guard the crops against the losses incurred due to the attacks from the pests. Chlorpyrifos, monocrotophos, quinalphos, malathion, parathion, and dimethoate are leading agricultural organophosphate pesticides (Fig. 1). Several ecosystems in the world have been contaminated with OPs due to their excessive and continuous usage. Removal of these chemopollutants is essential, considering their toxic effects caused due to their interactions with the biological system. The microbial activity in the environment decides the fate of the pesticide (Bose et al., 2021). Many pesticides are recalcitrant in nature, but microorganisms may degrade some of them easily. Bioremediation is an efficacious tool to restore contaminated soil and manage the polluted environment. Recent developments in environmental biotechnology where use of microbes for detoxification, degradation, and removal of toxic compounds from contaminated soil are emerging as efficient, effective, and cheap biotechnological approaches for cleaning up the polluted environment. Active research is being carried out on OPs as their residues are persistent in nature and contaminate the environment. Advancements in bioremediation technology, microbial degradation of OPs have allowed for a better understanding of the mechanisms for effective on-site therapization of the contaminated environment (Sidhu et al., 2019).

This comprehensive review emphasizes the current scenarios of OPs pollution, mode of toxicity, and promising bioremediation tools (by soil-borne microbes or natural/engineered enzymes), highlighting the practical applications of microbes or microbial enzyme systems, immobilizations, biosensor developments, bioremediation, and therapeutic detoxification. Based on the available literature and progress in OPs bioremediation, in the review, the current challenges and future approaches toward OPs bioremediation and therapeutic detoxification has been discussed.

1. Organophosphates

The standard structure of OPs compounds is $O{=}P(OR)_3$. OPs occur in a diverse range like most other functional groups. OPs are naturally found in biomolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and adenosine triphosphate (ATP). They are present in chemically synthesized compounds such as pesticides (glyphosate, chlorpyrifos, ethion, malathion, dichlorvos, fenthion, diazinon, malathion), herbicides (tribufos (DEF), merphos), anti-helminthic (trichlorfon), ophthalmic agents (echothiophate, isofluorate), industrial chemicals (tricresyl phosphate), households (phosmet), flame retardants in the electrochemical industry (tri-*n*-butyl phosphate), and chemical warfare agents (venomous agent X (VX), tabun, soman, and sarin) (Foong et al., 2020). Out of the voluminous use of pesticides globally, OPs are widely used as pesticides in the agricultural sector. OPs are typically divided into four subgroups based on their molecular structures: (1) phosphates, (2) phosphothioates, (3) phosphorodithioates, and (4) phosphorothiolates. In Fig. 1, the widely used OPs pesticides and chemical warfare agents (CWFAs) with their structures, half-life, and toxicity have been mentioned.

2. Current scenario of OPs pollution

Due to their toxicity and carcinogenicity, the potential inimical effects of OPs pesticides on the environment and living system have always been a matter of debate and concern. India contributes to one-thirds of total poisoning cases worldwide due to pesticides pollution, soil and water (surface and groundwater) are being contaminated. Many researchers across the globe have reported that glyphosate, an OPs herbicide widely used in agriculture, contaminates the ocean ecosystem through submarine groundwater discharge (exchange of groundwater between the land and ocean) (Welch et al., 2019). It was reported in the literature survey that these pesticides pose acute toxicity to non-targeted animals and adversely affect environmental health (Jain et al., 2019; Singh, 2009). The chemical warfare agents (CWFAs) are another primary form of OPs compound. It has been evaluated that about 2,00,000 tons of OPs compounds have been stored worldwide by many nations and terrorist organizations (Iyer et al., 2015). Several studies have reported that the OPs pesticides are persistent in soil from 10 to 360 days; for instance, parathion remains in the soil for more than 180 days (Singh et al., 2006).

Over the last few years, a high concentration of OPs pesticides (above the threshold limit, as per European and US environmental protection agencies EPA) has been detected in the environment, including agricultural, forestry regions, and surrounding water, across the globe. For instance, several studies have been reported on OPs contamination in the China such as Li et al. (2021) reported a total of 8 OPs pesticides with high pollution (parathion, methyl parathion, phorate, and disulfoton 18.70, 4.14, 19.20, and 17.30 ng/g, respectively) . Similarly, OPs pollution was reported from the agricultural soil of China (delta region of Yangtze River). It detected more than

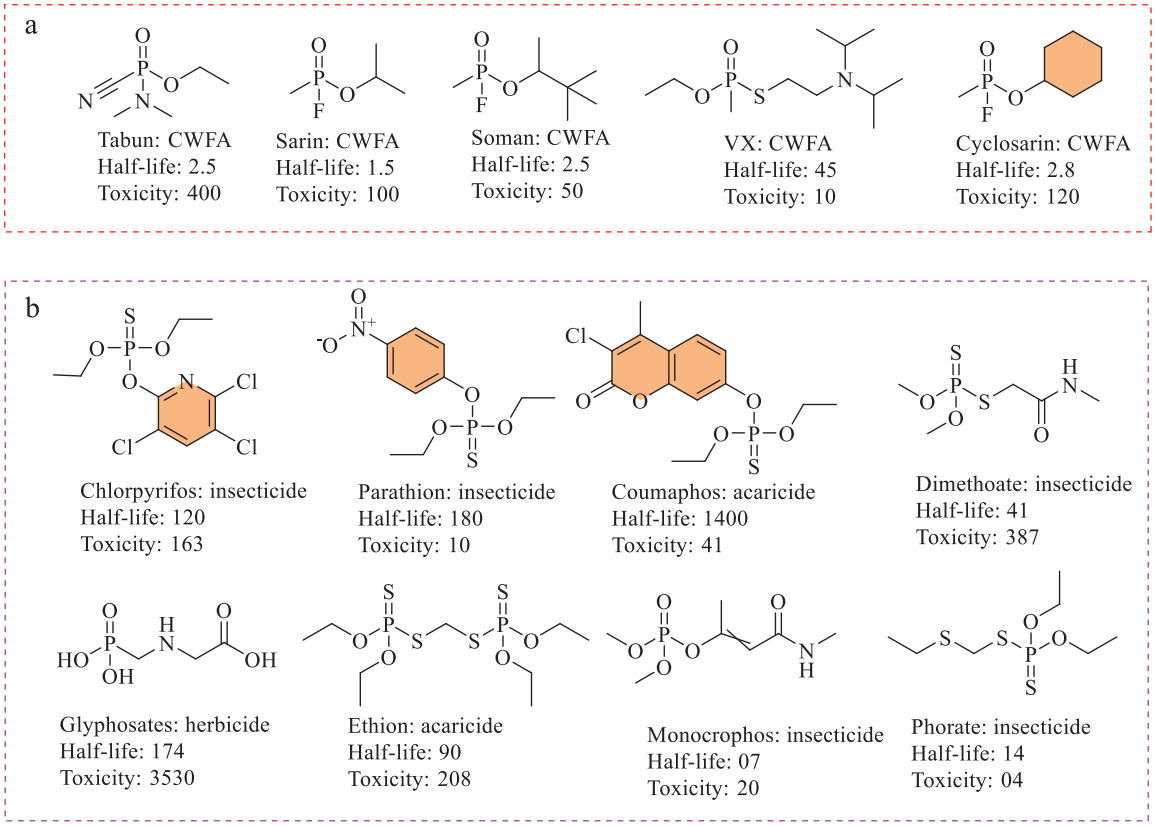

Fig. 1 – Schematic representation of organophosphates (OPs) compounds. (a) Chemical warfare agents (CWFA), mammalian toxicity (LD_{50} values: concentration of ingested mg/kg of animal weight, where the half-life of the tested animal die) CWFA are vaporized and deaths occurred by ingestion, inhalation or dermal contact (mg/min), all CWFA are lethally effecting within 10 to 15 min. (b) Molecular structure of different OPs pesticides with their toxicity and half-life in days (Mali et al., 2022a).

9 OPs with high concentrations (phorate, dimethoate, methyl parathion, parathion, thionazin, and sulfotep 82.90, 395.00, 87.00, 89.00, 49.00, and 16.60 ng/g, respectively) (Pan et al., 2018). Bhandari et al. (2020) have been detected and reported the distribution of OPs pesticides residues (1.23 to 239 ng/g) from the Nepal agricultural soil. The predominant contaminates were chlorpyrifos (177 ng/g) and 3,5,6-trichloro-2-pyridinol (57 ng/g) (Bhandari et al., 2020). Apart from the agricultural soil and surrounding environment, OPs pesticides were also detected frequently in the water bodies (pond, tubewell, and river). Lari et al. (2014) have reported OPs pesticides (chlorpyrifos, dichlorvos, phorate, and methyl parathion) with high concentrations (0.06 to 0.39 ng/mL) from the rivers (Amravati, Bhandara, and Yavatmal) near Mumbai, India. Pesticide contamination has been well documented from the agricultural soil, surrounding water, and industrial areas worldwide. However, contamination of OPs pesticides has been reported first time from the dense forest area of Brazil, and predominant OPs were glyphosate (66.38 ng/g) and amino-methylphosphonic acid (26.03 ng/g) (da Silva et al., 2021).

2.1. *Mode of action and effects of OPs on humans and animals*

OPs poisoning is a globally severe clinical problem: OPs alone in any form leads to about 3 million poisonings and 3,00,000 human deaths per year (Katyal et al., 2020). OPs pesticides can enter into the human/animal body through primarily three pathways: (1) inhalation, (2) ingestion, and (3) dermal contact. It covalently binds with the acetylcholinesterase (AChE) enzyme of the nervous system (AChE hydrolyses acetylcholine into choline and acetic acid). Acetylcholine, a neurotransmitter, is an essential component of the nervous system as it transmits nerve impulses in the brain, muscular and skeletal systems. OPs interfere with the breakdown of acetylcholine at synapses and red blood cell membranes (Farnoosh and Latifi, 2014). OPs are potent inhibitors of AChE; when OPs inactivate AChE, the concentration of acetylcholine builds up at nerves, making them overactive. The schematic mechanism of AChE enzyme, which irreversibly binds with OPs pesticides, has been represented in Fig. 2. Victims of OPs poisoning typically die because of a short-

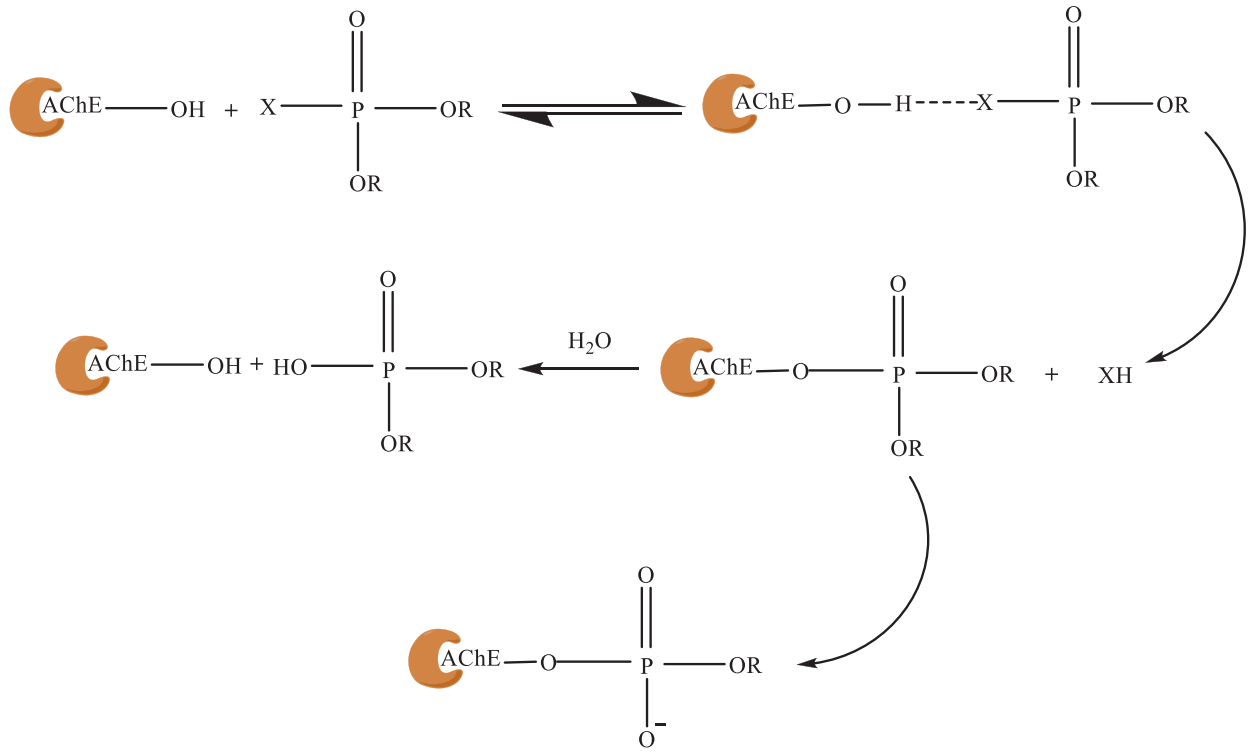

Fig. 2 – Schematic mechanism of acetylcholinesterase (AChE) enzyme irreversibly binds with OPs pesticides. Structures and mechanism were drawn by ChemDraw 19.1.

age of breath (Chambers and Levi, 1992; Pundir et al., 2019). OPs not only act as inhibitors of acetylcholinesterase but also bind to other proteins and enzymes and inhibit by covalently binding with serine containing enzymes such as proteases, esterases, carboxylesterases, neuropathy target esterase (NTE), plasma pseudocholinesterase, and A-esterases (Berent et al., 2014; Naughton and Terry, 2018). Along with these, a few non-cholinesterase targets of OPs and their downstream effects have been listed in Appendix A Table S1.

Organophosphate toxicity can result from intentional or unintentional contamination of food sources, household or occupational exposure, terrorist or military action, etc. Advancements in the risk assessment (environment and human) and preparedness are ongoing for chronic, low-level OPs exposure from food sources, although no clinical effects have been reported so far. Toxic effects of OPs can be broadly classified under three categories; acute effects or short-term effects, delayed or intermediate effects, and delayed polyneuropathy. OPs inhibit the AChE enzyme, resulting in the accumulation of acetylcholine, which causes acute effects. Signs and symptoms of acute OPs poisoning can be seen in; Central nervous system (CNS) due to muscarinic and nicotinic effects (Naughton and Terry, 2018). Similarly, in Appendix A Table S2, other effects of OPs on nervous system-receptors, site, and post symptoms, have also been mentioned.

Intermediate effects were also recognized, characterized by muscle weakness involving the neck, limb, and respiratory muscles starting from 1 to 4 days after an OPs poisoning incident and lasting for about 5-18 days; this may result from muscle necrosis (Ray, 1998; Senanayake, 1998).

Other than inhibition of AChE or neuropathy target esterase, the following are some of the putative mechanisms of OPs toxicity: (1) Phosphorylation of proteases, esterases, or proteins involved in cell signaling, (2) Interaction with cytoskeletal proteins, (3) Excessive calcium influx in cells at nerve endings, (4) Prolonged receptor stimulation at nerve endings leading to muscle fasciculation and necrosis, and (5) Hypoxic brain damage (Baker and Sedgwick, 1996; Berent et al., 2014; Gupta, 2004; Gupta and Abou-Donia, 1995; Naughton and Terry, 2018; Senanayake and Karalliedde, 1987). Following are the signs and symptoms of patients who were believed to have been exposed to OPs for a long time; headache, fatigue, anxiety, confusion, impaired concentration, sleep disorders, muscle spasms, muscular pains, incoordination, irritability, depression (including suicidal thoughts in severe cases), intolerance to alcohol and other chemicals, memory loss, nightmares, nausea, respiratory disease, numbness of the extremities, increased sensitivity to repeated exposure to OPs and other psychiatric disorders (Bonvoisin et al., 2020; Gupta, 2004).

When a person is exposed to a different class of OPs, there is a risk of potentiation. In the presence of an OPs mixture, the enzymatic detoxification of one compound is inhibited, leading to potentiation. Potentiation is rare but might get significant when one of the compounds is a potent inhibitor of enzymes involved in detoxification, resulting in increased toxicity. Such interactions of OPs have been comprehensively re-

viewed by Gallo and Lawryk (1991) and Rajmohan et al. (2020). They have shown that limited potentiation occurs when the level of OPs compounds is either high or equal to the enzymes involved in detoxification.

2.2. *Effects of OPs on plants*

OPs affect not only animals but also plants at different levels. They have been known to alter several pathways and mechanisms; plant mineral uptake (Krenchinski et al., 2017), photochemical reactions (Vivancos et al., 2011), photosynthesis (Zobiole et al., 2012), carbon metabolism (Zobiole et al., 2011), amino acid synthesis, nitrogen metabolism, fatty acid metabolism (Zobiole et al., 2010), oxidative stress (Filimonova et al., 2016; Stauber et al., 2016; Vagi et al., 2017; Yanniccari et al., 2012) and chlorophyll metabolism (Serra et al., 2013). OPs interfere with photosynthesis reactions by interacting with intermediate compounds (organic and inorganic) at different stages of photosynthesis, hindering the plants biochemistry. OPs induce Fe^{2+} deficiency in plants, leading to the inhibition of syntheses of enzymes like catalase, δ-aminolevulinic acid (δALA), and peroxidase, the major components of the chlorophyll biosynthetic pathway (Yanniccari et al., 2012). When plants are exposed to the herbicide glyphosate, the activity of ribulose 1,5-biphosphate carboxylase oxygenase (RuBisCO) is affected by reducing the levels of 3-phosphoglyceric acid (PGA) and ribulose-1,5-biphosphate (RuBP) (Jain et al., 2019). Mishra et al. (2009) showed that when *Vigna radiata* was treated with phorate (OPs insecticides), the emergence of seedlings was delayed . Parween et al. (2011) reported that chlorpyrifos reduced the plant growth and nitrogen metabolism in *V. radiata* and enhanced lipid peroxidation.

Glyphosate and its degraded product, the amino-methyl phosphonic acid (AMPA), altered the plant growth; glyphosate (phosphonic acid) chelates the metal ions, and AMPA leads to leaf injuries. Glyphosate has an inhibitory effect on δALA - a precursor of chlorophyll biosynthesis, due to which the chlorophyll content gets disturbed (Krenchinski et al., 2017). It directly influences PSII (system of photosynthesis) associated proteins-D1, D2, which happens due to the inhibition of the synthesis of aromatic amino acids by the shikimate pathway (Gomes et al., 2017). The metal chelating activity of glyphosate influences the pigment formation and water splitting complex depending on Mn^{2+} (Mertens, 2018). These effects ultimately culminate in the inhibition of ATP synthesis, which occurs due to the inhibition of electron transport via photosynthetic machinery and lack of photon gradient generation. Different plant processes affected by OPs pesticides are schematically represented in Appendix A Fig. S1a-c. As glyphosate exposure influences the shikimate pathway, the biosynthesis of plastoquinones (PQ) from the intermediates of the shikimate pathway is affected, showing the effect on photosynthesis on a broader scale (Gomes et al., 2017). Due to the inhibition of the shikimate pathway, the concentration of carotenoids decreases, and as a chain reaction, it affects the synthesis of zeaxanthin, and carotenoids are used as precursors in the xanthophyll cycle. The xanthophyll cycle is involved in photoprotection, and its inhibition leads to over activation of chlorophyll. The over activation of chlorophyll leads to the inhibition of CO_2 fixation due to the accumulation of H_2O_2, which oxidizes the thiol groups of several Calvin cycle enzymes, as shown in Appendix A Fig. S1b.

Dimethoate was observed to induce detrimental effects in soybean and cotton, inhibiting photosynthetic efficiency and photosynthetic pigments (Murthy et al., 2005). It was reported that dimethoate inhibits δALA dehydratase and protochlorophyllide reductase, leading to decreased chlorophyll content. Dimethoate and other OPs pesticides also produce reactive oxygen species (ROS) (Sidhu et al., 2019). They produce ROS, accumulate pesticides in the leaves of higher plants, and increase lipid peroxidation and enzymes like peroxidase, catalase, and superoxide dismutase (Mishra et al., 2009).

Considering the current scenario, OPs pollution poses a risk to flora and fauna of terrestrial as well as aquatic ecosystems which is a severe environmental concern to the public. Several research groups across the world are actively working on robust remediation systems for removing toxic OPs pollutants from the environment. The physicochemical methods such as physical decontamination (burying, incineration, washing, and sorbent materials) (Janos et al., 2014) and chemical decontamination (oxidation, reduction, and hydrolysis) methods have been systematically reviewed in (Jacquet et al., 2016).

3. Microbial bioremediation of OPs pesticides

Bioremediation provides a fast, economical, and environmentally friendly way to remove toxic compounds from the environment. OPs pesticides can be remediated biologically through microbial bioremediation, where hydrolysis, rearrangement, conjugation, and degradation reaction could occur. Several environmental factors (pH and temperature) and native soil microbes affect the fate of the pesticides released into the environment. There are two pathways (abiotic and biotic) known to degrade pesticides in the environment. Researchers exploring microbial diversity, specifically in contaminated environments, search for native microbes that can utilize or mineralize a broad range of toxic pollutants. Microbes significantly affect the tenacity of the pesticides in the soil as microbes are the primary source of pesticide degradation and detoxification in agricultural soil (Iranzo et al., 2001; Kumar et al., 2018). However, the use of microbes for bioremediation requires a better understanding of ecological, physiological, microbiological, biochemical, and molecular aspects involved in the transformation of the pollutant. The degradation pathway of OPs pesticides (through P-O, P-S, C-P, and P-F linkages) are shown in Appendix A Fig. S2.

3.1. *Myco-degradation*

Several fungal strains have been reported to possess the intrinsic capability to degrade OPs pesticides in the environment, such as filamentous fungi, *Aspergillus terreus, Aspergillus fumigatus,* and *Tricholoma giganteum,* which have frequently been isolated from the OPs polluted environmental soils (Kumar et al., 2019; Rudakiya et al., 2020). Various other such as *Trichoderma viride, Aspergillus niger, Aspergillus* (EM8),

Aspergillus oryzae, and *Penicillium nototum* have been reported to degrade OPs pesticides. Fungi such as *Aspergillus, Rhizoctonia,* and *Penicillium* were reported to degrade malathion, producing non-toxic metabolites, which were identified as phosphorodithioates, malathion monoacid, dimethyl phosphate, and dimethyl phosphorothioate (Kumar et al., 2019). An enzyme was isolated from the fungus *Aspergillus niger* ZHY256, capable of hydrolyzing phosphorothiolester group (P-S linkage) of OPs pesticides, including dimethoate and malathion (Liu et al., 2001). Several fungi have been reported to utilize malathion as a source of phosphorus. *Aspergillus sydowii* and *Aspergillus flauvs* can use 5-7 mmol/L of malathion as a source of phosphorus. However, the release of inorganic phosphorous during the degradation process was not detected. It could have been assimilated as soluble phosphorous in fungal biomass (TM and Masmali, 2016). Recently Lacasse enzyme was purified from the fungi *Tricholoma giganteum* AGDR and biochemically characterized to enhance the degradation of OPs pesticides (100 mg/L); profenofos (87%), methyl thiophanate (70%), and chlorpyrifos (29%) within 15 hr (Rudakiya et al., 2020). All fungi do not have metabolic machinery and catalytic activity to hydrolyze P-S, P-F, and P-O linkage of OPs pesticides (Appendix A Fig. S2). However, a promising fungal strain has not been identified from the environment, possessing all the characteristics to degrade these pesticides in the environment. Therefore, it is necessary to continue discovering suitable OPs degrading enzymes from fungal strains and modify them to improve catalytic properties against different classes of OPs pesticides.

3.2. *Phyco-degradation*

Blue-green algae or cyanobacteria present an economically efficient tool for bioremediation of pesticides, textile dyes, heavy-metals, and water contaminated with organic compounds (Wei et al., 2015). Few cyanobacteria have been reported to degrade OPs pesticides which produce nontoxic metabolites. The first time reported algae was *Gonium pectoral* for bioremediation of OPs pesticides, which demostrated 57% degradation of malathion (1 mg/L) within 4 days. After that, several algae have been reported to degrade OPs pesticides viz. *Nostoc muscorum, Spirulina platensis, Chlorella vulgaris, Scenedesmus quadricuda* and *Anabaena oryzae* (Ibrahim et al., 2014; Kumar et al., 2019; Vijayan et al., 2020). The algae are routinely used in wastewater treatments to remove phosphorous and nitrogen. Several algal species like *N. muscorum, S. platensis,* and *A. oryzae* have been reported to degrade malathion. Among all, *N. muscorum* was observed to degrade more than 90% of malathion and tolerate a high concentration of malathion (10-100 mg/L). A high concentration of protein and carbohydrates was found in *N. muscorum* compared to *S. platensis* and *A. oryzae*. These algae species were further grown under phosphorous stress conditions in the presence and absence of malathion (+/-) to check whether algae can utilize malathion as a source of phosphorous or not. The algal growth was accelerated with high phosphorous concentrations in algae *N. muscorum* (Ibrahim et al., 2014). Results revealed that the algae could utilize OPs pesticides (malathion) as a sole source of carbon and phosphorous. Algae were also reported to degrade OPs pesticides chlorpyrifos, dimethoate, and malathion as a sole source of carbon and phosphorous. Recently Nimisha et al. (2020) have isolated several algal species from the paddy field; among all, *Coleofasciculus chthonoplastes* showed 90% degradation of chlorpyrifos (4 µL/mL) within 21 days, and nontoxic metabolites were identified as 1, 1, 2, 3, 3 pentachloro propane and 3, 5, 6-trichloro two pyridinol (Vijayan et al., 2020). The application of algae for OPs bioremediation offers a significant advantage over the other microbes because it also acts as a bio-fertiliser which improves soil fertility.

3.3. *Bacterial degradation*

OPs degrading bacteria have the potential to mineralize a broad range of aromatic, aliphatic, and heterocyclic toxic pollutants as a sole source of nitrogen, phosphorous, carbon, and energy. As shown in Appendix A Fig. S2, the degradation of OPs pesticides through hydrolysis of P-O, P-S, P-F, and C-P linkages is considered the most vital step in bioremediation (Kumar et al., 2018; Rayu et al., 2017). Bacteria have been studied for both bio-mineralization and co-metabolism of OPs pesticides. A list of recently identified potent OPs degrading bacteria are described in Appendix A Table S3. The first OPs degrading bacterium was isolated from the paddy field in Philippians, which was identified as *Flavobacterium* sp. (ATCC 27551) could degrade 95% of diazinon (540 µg/L), 66% of parathion (540 µg/L) within 24 hr and the degraded product was identified as 2-isopropyl-6-methyl-4-hydroxypyrimidine (IMP), *p*-nitrophenol, respectively. Evidently, bacteria possess enzymes that can cleave diazinon and parathion pesticides (Sethunathan and Yoshida, 1973). Since then, several OPs degrading bacterial species have been isolated and identified across the world; among them, few bacteria can utilize these OPs as a source of carbon, a few as phosphorous, some as nitrogen sources, and few can make exclusive use of the organophosphate (Rayu et al., 2017). Contaminated sites contain a widespread microbial population which have adapted themselves to the contaminants (Rajmohan et al., 2020; Richins et al., 1997). Thus, these sites would be the best ecological niches to isolate bacteria that can degrade contaminants. Recently, the new *Arthrobacter* sp. HM01 has been reported to degrade high concentration (200 mg/L) of pesticides chlorpyrifos and their toxic metabolite 3,5,6-trichloro-2-pyridinol (TCP) within 10 hr and also shows high tolerance against chlorpyrifos (1000 mg/L) (Mali et al., 2022b). Rayu et al. (2017) isolated several potent OPs degrading bacteria from OPs contaminated sugarcane farm soils. Three bacterial strains, *Pseudomonas* sp., *Rhizobium* sp., and *Xanthomonas* sp., were used to make a consortium for chlorpyrifos (10 mg/L) biodegradation which showed complete degradation within 6 days and TCP detected as a primary metabolite. *Pseudomonas* sp. and *Xanthomonas* sp. could degrade TCP as a source of carbon and nitrogen (Rayu et al., 2017). Recently Haque et al. (2020) isolated *Leuconostoc mesenteroides* WCP307 from OPs pesticide contaminated vegetable field and detected a high concentration of chlorpyrifos in both field and vegetables (cabbage and *yeolmu-kimchi*). They isolated chlorpyrifos degrading, or OPs degrading gene (*opd*A and *opd*E), which play a key role in chlorpyrifos (50 µg/mL) degradation and 83% of degradation after 6 days (Haque et al., 2020). Similarly, few OPs

degrading genes were isolated from the contaminated environment and had the ability to hydrolyzed toxic pesticides, such as methyl parathion hydrolase from *Agrobacterium tumefaciens* RBS01, parathion hydrolase from *Flavobacterium* sp., OPs hydrolase from *Arthrobacter* sp. HM01 (Mali et al., 2022c).

The ability of diverse bacterial species to degrade OPs pesticides, either partially or completely, could be a promising tool for in-situ bioremediation. However, the microbial bioremediation process is limited by several factors such as soil type, the concentration of microbes, soil pH and temperature, nutrients, and concentration of pollutants. It also requires a better understanding of ecological, physiological, microbiological, biochemical, and molecular aspects involved in transforming the pollutant. These natural soil-borne microbes have poor catalytic activities, are substrate selective, require a favorable environment (viz. temperature and pH) to degrade OPs pesticides. Most microbes work efficiently at low temperature (20-25°C) and in alkaline soil (pH 8-9). Therefore, scientific communities actively seek to isolate or discover prominent OPs degrading enzyme systems (capable of degrading multiple OPs pesticides simultaneously with high catalytic activity under extreme environmental conditions) from potent microbes. In this context, we explore the biocatalytic system as a promising approach toward OPs bioremediation.

4. Enzymatic bioremediation of OPs pesticides

Cell-free enzyme systems emerged as promising candidates for bioremediation because they provide better efficiency over the microbial systems, rapidly degrade/detoxify high concentrations of OPs, and are functionally active in harsh environmental conditions. Several cell-free OP-degrading biocatalytic systems were discovered and purified from soil microbes. Here we explore a few prominent and robust biocatalytic systems for OPs bioremediation. Recently, a novel OPs hydrolase (opdH) enzyme was isolated from *Arthrobacter* sp. HM01 and shows high catalytic activity (k_{cat}/K_m 1.2×10^7/(mol•sec)), degrades multiple OPs pesticides (paraoxon, methyl parathion, parathion, chlorpyrifos, and malathion) in contaminated water (Mali et al., 2022d).

Phosphotriesterases (PTEs) are OPs degrading enzymes capable of hydrolyzing a broad range of OPs pesticides. PTE enzymes were discovered in *Flavobacterium* sp. ATCC 27551 (Sethunathan and Yoshida, 1973) and *Pseudomonas diminuta* MG (Benning et al., 2001). Because of its ability to hydrolyze OPs, it is also known as organophosphate hydrolase (OPH). PTEs are well categorized from bacteria and are also found in other micro-organisms and animals (Farnoosh and Latifi, 2014). PTE/OPH exhibits high catalytic activity, catalyzing the hydrolysis of P-O, P-F, C-P, and P-S bonds from a broad range of OPs compounds (Katyal et al., 2020; Thakur et al., 2019; Wang and Sun, 2021). The kinetic characteristics of PTE (k_{cat}/K_m 1.1×10^8/(mol•sec)) and the mechanism of OPs hydrolysis are well known (Schenk et al., 2016). The hydrolysis step is essential in detoxifying OPs, making the compound susceptible to further biodegradation (Singh and Walker, 2006). It implies that PTE activity is specific and depends on the orientation of the phosphoric acid group (PO_4), which defines how OPs would be hydrolyzed (Iyer et al., 2013). PTE enzymes belong to the metallohydrolase superfamily of proteins; several PTE enzymes have been reported from different microbes for the last few decades.

Few well-known biocatalytic systems are as follows; organophosphorus acid anhydrases (OPAA), methyl parathion hydrolases (MPH), diisopropyl fluorophosphatase (DFpase), and paraoxonase (PON1), and more as described in Appendix A Table S4.

4.1. *Catalytic mechanism of phosphotriesterase (PTE)*

The crystal structure and functional properties of PTE enzymes from different microbes were described in Appendix A Text S1. The PTE enzymes have high catalytic activity and a broad range of OPs substrates. Phosphotriesterase can hydrolyze insecticides, including phosphorothioesters, phosphotriesters, and thiophosphotriesters (Benning et al., 2001; Fan et al., 2019). Phosphotriesterases can hydrolyze halide bonds, thiol linkages, and substrates with electron-withdrawing phenolic (C_6H_5OH) leaving groups. The non-specific nature of the enzyme-substrate binding site/pocket makes this enzyme a promising candidate for environmental bioremediation of OPs with a broad range of substrates (pesticides). The substrate interacts with the active site of hydrophobic pockets. PTE is a dimeric metallo-hydrolase. Each monomer has two divalent metal ions in the active site. The conserved metal ion residues are ligated to α-strand residues of the C-terminal end. The functional loops that arrange to form C-terminal ends of the barrel constitute substrate binding site. The active site consists of H55, H57, D301, H201, H230, K169 residues, as shown in Appendix A Fig. S3a. One of the metals which is more buried is ligated in a trigonal bipyramidal fashion to H55, H57 residues of β-strand 1, D301 residue of β-strand 8, and the other solvent-exposed metal is ligated to H201 of β-strand 5 and H230 of β-strand 6. Both the metals are bridged by carboxylate lysine (K169) residue of β-strand four and a hydroxide ion (Wang and Sun, 2021). PTE enzymes have native Zn^{2+} ion and are also functionally active in the presence of different divalent ions such as Cd^{2+}, Mn^{2+}, or Ni^{2+} (Benning et al., 2001).

The catalytic mechanism of PTE was probed by employing physical, structural, kinetic, and probe-labelling methods to trace each part of the mechanism. The catalytic mechanism of OPs hydrolysis by PTE enzyme was demonstrated by utilizing O^{18} labeled water, where the nucleophilic attack was directed at the phosphorous center of OPs substrate rather than the leaving group (Bigley et al., 2020). Studies revealed that water molecules directly interacted with substrate instead of forming an intermediate complex (covalent enzyme-bound intermediate). The X-ray structure of the PTE active site showed a bridging water molecule or hydroxide between the metals. Kinetics (pH rate) profiling of the PTE enzyme confirmed that this bridging hydroxide made a nucleophilic attack. The bridging hydroxide also interacts with D301 by hydrogen bonds. Crystal structures of PTE showed that this residue is involved in the deprotonation of the hydroxide (OH^-) as the nucleophilic attack takes place. The released proton (H^+) is shuttled to the solvent via D233 residue (Chen et al., 2007). This speculation was confirmed by SDM (D233A), where mutated residue (D233) of PTE enzyme lost and disrupted the proton

shuttle and notably reduced the rate of OPs hydrolysis. Several groups of researchers probed the mechanism of paraoxon hydrolysis computationally, employing different levels of theory (Fan et al., 2019). A top-level Density Functional Theory (DFT) projected a 2-transition state system coupled with high energy intermediate, involving only hydroxide (OH^-), divalent metal ions, and an enzyme side chain that ligated the metals (Aubert et al., 2004). The projected first transition state includes substrate binding and distortion of phosphorous center; therefore, this center comes in proximity of hydroxide (OH^-), and intermediate complex predicted to be pentavalent species. Further, in a second transition state, proton (H^+) is released from the hydroxide (OH^-) and transferred to active site residue D301, breaking the bond of leaving group. The complete catalytic mechanism of PTE enzymes for OPs are shown in Appendix A Fig. S3.

PTE enzyme isolated from the overproducing bacterial strain of *Pseudomonas diminuta* carries out the cleavage of organophosphate molecule by specific hydrolysis of phosphoester bond, thereby reducing the toxicity of the compound almost by 100-folds (Latip et al., 2019). Enzymatic hydrolysis might not result in the same products as that of abiotic hydrolysis. Several OPs degrading enzymes discovered and purified from environmental microbes possess inherent properties to catalyze the hydrolysis of OPs pesticides or nerve agents (Appendix A Fig. S2). Recently two organophosphate degrading enzymes from *Leuconostoc mesenteroides* WCP307 were reported. The novelty of their study lies in the fact that the organophosphate degrading enzymes OpdA and OpdE do not belong to earlier known esterolytic and lipolytic protein families. These enzymes exhibit a catalytic action involving serine amino acids for histidine or aspartate amino acids as in canonical OPH or PTE enzymes (Despotović et al., 2019).

Mishra et al. (2012) has attempted to detoxify OPs contaminated water using the PTE enzyme. The pilot-scale test demonstrated that the PTE enzyme (500 UI) could detoxify OPs contaminated water in the column (Mishra et al., 2012). These results show that the cell-free enzyme systems can remove toxic OPs compounds from the environment. However, laboratory-based efforts have achieved success in OPs bioremediation to a good extent. However, the true challenge for researchers or scientists lies in scaling lab-based success (deploying OPs degrading microbes or microbial enzymes) to the field level. The main challenge in scaling up microbes or microbial enzymes is that they may exhibit un-predicated or volatile behaviors when employed with native microflora of contaminated soil or soil containing different chemicals that may inhibit OPs degradation (partially/fully). Therefore, there is an urgent need for research that may include bioengineering techniques (directed or rational genetic modification) to improve catalytic activity (in a racemic mixture of toxic chemicals), stability (in environmental conditions), and substrate selectivity (against poor substrates) of deployed enzymes.

5. Bioengineering approaches to improve OPs degrading bio catalytic systems

Despite the discovery of broad-spectrum OPs degrading enzymes, not all the native enzymes are able to degrade OPs pesticides. Nonetheless, the OPs degrading enzymes (PTEs/OPH) offer a great starting point for improving these bio-catalytic systems for environmental bioremediation in an economical way. Classically, bioengineering approaches (enzyme modification) have been used to enhance the catalytic properties of PTE enzymes. Mutagenesis involves studying the role of conserved residues (of the catalytic domain) in enzyme evolution. Currently, protein engineering approaches have been exploited in the industrial sector to produce a broad range of products for the betterment of humankind. The biocatalyst market has been expected to grow 5 to 10-folds during 2025-2030. Based on the extent of the knowledge of protein sequence, structure, and active function of the selected enzyme, three types of bioengineering approaches are being utilized to improve enzyme catalytic properties, namely rational designing (site-directed mutagenesis), directed evolution, and semi-rational approaches.

5.1. *Rational approach*

Rational designing is the oldest and well-established approach for protein engineering. This technique was introduced after the expansion of rDNA technology. Rational design (site-directed mutagenesis; SDM) requires complete knowledge of the protein structure with the conserved catalytic domain, which plays an active role in catalysis. Based on the preliminary information of protein structure and functional relationship, site-specific residues are selected for SDM (Appendix A Fig. S4a). There are three ways to identify the target residues for SDM: (1) analyzing the protein structure, (2) multiple sequence alignment, and (3) computational method. It has become easier to derive information about the structure and functions with improved X-ray crystallography. This would allow for a holistic understanding of the protein, leading to focusing on a specific area of a protein to develop desirable characteristics, such as improving the catalytic activity by targeting the conserved catalytic domain. SDM can modify protein sequences by exchanging the secondary/tertiary structure elements, replacing the whole functional domain, or generating chimeric proteins. Rational design can be applied to a well-characterized protein and a protein whose structure is yet to be determined. The rational design allows for building a structure to a protein with the help of previously determined closely related proteins. This is known as homology modelling. Homology modelling helps to understand the structure-function relationships with the help of closely related proteins. Once it is determined, it would be easy to focus on the character of a protein to improve by targeting the specific portion of the protein. However, the complete knowledge of most known protein structures and catalytic mechanisms is not fully explored or available on the protein database. The advancements in computation tools and techniques in rational designing have led researchers to examine crucial residues of the catalytic or substrate binding site in 3D protein structure.

Indeed, several residues located on the outer rim of a conserved site often contribute to essential properties. Several mutagenesis approaches have been applied to improve OPH/PTE enzymes to effectively degrade OPs pesticides (Wang and Sun, 2021). The mutations created at the active site directly affect the catalytic activity of the enzymes. Little was

known about the mutations at sites other than active sites until whole gene randomization mutagenesis or error-prone PCR and DNA shuffling were developed. Data suggests that the mutations occurring in areas remote from active sites have much less to contribute to the catalysis directly (Katyal et al., 2020). Mutations in such areas affect the thermostability, and stability of the enzyme on the whole and also affect how electrostatic interactions take place. Sometimes such modifications might help form an intramolecular disulfide bridge, which leads to an increase in the thermostability of the enzyme. Hence, one of the answers for a long-sought question – "what role does most parts of the protein other than the active site have to play?" could well be in increasing the stability of the enzyme or improving the intramolecular and intermolecular interactions. Recently discovered or improved variants of PTE/OPH enzymes are described in Table 1.

It has been observed that most OPs pesticides are bulkier in size; therefore, it is difficult to fit in the active site pocket. This could explain the low degradation rates of these pesticides by various organophosphate hydrolases. Most studies involving site-directed mutagenesis that have targeted the binding pockets to allow for the perfect entry or open a gate for OPs pesticides into the active site have shown high binding affinity with substrates, resulting in rapid catalysis (Iyer and Iken, 2015). Structural and functional study of PTE/OPH reveals that the His-254 and His-257 have been the main targets for mutagenesis. The bulky imino side chain of histidine has been substituted with arginine or leucine; results show several-fold improvements in OPs hydrolysis. These substitutions increase the size of the binding pocket such that OPs pesticides fit into the active site. The substitutions at positions 254 and 257 for histidine have been so prominent that they can even be observed in error-prone PCR. The variants with substitutions at positions 254 and 257 had been observed to exhibit relatively high catalysis than other variants (Schenk et al., 2016).

5.2. *Directed evolution*

This potent approach has been used for generating tailor-made enzymes for several biocatalytic applications such as detection, therapeutic detoxification, and environmental bioremediation. Unlike the rational design approach, directed evolution enables rapid uncharacterized enzymes and does not require in-depth knowledge of enzyme structure (Appendix A Fig. S4b). Therefore, directed evolution is in practice or employed to improve the enzyme properties such as catalytic, substrate specificity, and thermostability. Directed evolution can be carried out through whole gene randomization (error-prone PCR) or DNA shuffling. Error-prone PCR includes mutated primers, altering the DNA polymerase with low fidelity, or increasing the salt content during the PCR (Iyer and Iken, 2015). This method will enable the generation of a library with several mutants. From this library, screening and selection would enable the selection of better mutants among the library with desired characteristics.

On the other hand, DNA shuffling involves using restriction enzymes to cleave the genes at random sites and then their shuffling. This can be done in two ways, first through gene amplification with low-fidelity DNA polymerase to introduce random nucleotides in the DNA sequence. Several rounds of amplification would result in the same gene with mutations at random locations. These DNA sequences are subjected to restriction digestion followed by shuffling. Shuffling is followed with overlap PCR, resulting in a reconstituted gene library with mutations at random locations and screening to select the potential mutant. In the second method, homologous genes from related organisms are subjected to restriction digestion. The gene segments are shuffled and reconstituted by overlap PCR. This method of DNA shuffling might combine better qualities of homologous proteins into a single protein improving its characteristics. However, a challenging task is to develop a robust and precise screening tool to evaluate mutant libraries for selecting high-throughput mutants. The larger the number of gene variants or gene pool/library, the greater is the chance of getting potential mutants with desirable characteristics. Extensive research has been carried out to enhance OPs degrading enzymes (PTE/OPH) by bioengineering approaches to degrade OPs pesticides rapidly. Because of their unique features, these enzymes are appealing for therapeutic detoxification and environmental bioremediation of OPs pesticides.

Recently many researchers reported the potential of enzyme engineering in OPs degradation. **(1) Bioengineering in the catalytic domain:** A PTE variant (I106G/S308G/H257Y and L303/H257Y) demonstrated a preference, increased to as high as 460-folds, towards toxic enantiomer of sarin and soman nerve agent analogs, compared to wild-type PTE enzyme (Katyal et al., 2020). Raushel and co-workers created a mutant library at two different sites in addition to random mutagenesis at loop regions and screened potent PTE variant (VRN-VQFL) with 25-folds enhancement in catalytic activity toward the VX analogs DXVX (Bigley and Raushel, 2019). Tsai et al. (2012) used error-prone PCR coupled with site-directed mutagenesis approach to generate mutant libraries and identified the best OPs hydrolyzing variants with significant improvement in substrate specificity towards nerve agents, including Sarin, Soman, Tabun, and Cyclosarin. One variant (H257Y/L303T) shows a 13,000-folds rise in an activity specifically towards Sarin, Soman and Cyclosarine as compared to wild-type PTE (Tsai et al., 2012), several other studies are described in Table 1. **(2) Bioengineering in the outer rim of the catalytic domain:** Recent research has shown that the residues located outside the active site play an important role in enzyme functions. Grimsley and Carletti demonstrated that EBP (diethyl-4-methyl-benzyl-phosphonate) is a competitive inhibitor of the PTE enzyme, but it acts as a non-competitive inhibitor in a few PTE variants (Carletti et al., 2009; Katyal et al., 2020). These studies suggest that the PTE enzyme has an allosteric site that plays a critical role in enzyme activity. Although this site has not been explored much, it opens a new gateway to understand the role of the allosteric site in the functioning of the PTE enzyme. However, this finding demonstrates that residues outside the catalytic or substrates binding pockets could be suitable for mutagenesis. Cho et al. (2006) targeted loop-7 of PTE enzyme for mutagenesis. This loop is distally located on the protein surface near the leaving group pocket. This study demonstrated one powerful variant, 22A11 (A14T/K185R/I274N/A80V/H257Y), with ~25-folds improvement in the hydrolysis of methyl-parathion and 10-folds improvement in paraoxon, coumaphos, and

Table 1 – Improved variants of OPs degrading enzymes, targeted site, and their significance.

Amino acid substitution	Enzyme	Organism	Pesticide degraded	Function	Reference
Binding pockets					
H254R/H257L	OPH	*Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551	Demeton S, Soman	Achieved a 2-30-folds increase in substrate specificity (k_{cat}/K_m) for demeton S and 11-18-folds increase in specificity for NPPMP (analog of Soman)	Singh, 2009b
H254R	OPH	*Pseudomonas diminuta*	DFP, Demeton S	4-folds increase in the hydrolysis of demeton-S (VX analog), a 14-folds decrease with paraoxon , and a 183-folds decrease with DFP (sarin analog)	Grimsley et al., 1997
H254G/ H257W/L303T	PTE	*Flavobacterium* sp.	Chemical warfare agents (Soman)	H254G- results in a cavity that allows for the introduction of a third metal-binding site; three substitutions enhanced the turnover rate for an analog of the most toxic stereoisomer of soman by nearly 3 orders of magnitude	Hill et al., 2003
H254Q/H257F	PTE	*Flavobacterium* sp.	Demeton S	10-folds enhancement in $k_{cat}$and a 3-folds enhancement in k_{cat}/K_m; displayed a 9-folds enhancement in k_{cat}/K_m for the hydrolysis of the VX analog and demeton-S.	Katyal et al., 2020
Y257H and F272L	OpdA	*Agrobacterium* sp. P230	Diisopropyl fluorophosphate	Increased activity of OpdA	Wang and Sun, 2021
Remote sites from active site					
L136Y	OPH	*Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551	VX	Displayed a 33% increase in the relative VX hydrolysis rate compared to wild type enzyme	Hawwa et al., 2009
S59C and S227C	OPH	*Flavobacterium* sp. ATCC 27551 and *Pseudomonas diminuta*		Affect metal content and catalytic activity	Schenk et al., 2016
H250I/ I263W	PoOPHM2	*Pseudomonas oleovorans*	Methyl-parathion and Ethylparaoxon	Displayed 6962 and 106-folds improvements in catalytic efficiency	Luo et al., 2014
P76D/P78K	MPH	*Ochrobactrum* sp. M231		Could generate an ionic bond and increase the structural electrostatic energy, which could then increase the stability of the protein	Su et al., 2011
D71G/E101G/V235L; G207D	DrOPH	*Deinococcus radiodurans*	Ethyl paraoxon and Methyl paraoxon	Largest catalytic efficiency, reduced k Proton relay and catalytic increase in activity	Hawwa et al., 2009
Error prone PCR					
(A14T, A80V, K185R, H257Y, I274N) variant 22A11	OPH	*Flavobacterium* sp. ATCC 27551 and *Pseudomonas diminuta*	Methyl parathion	Improvement not only in the hydrolysis of methyl parathion but also the overall hydrolysis rate of all other substrates tested	Goldsmith and Ashani, 2018
DNA shuffling					
A14T, L17P, A80V, V116I, K185R, A203T, I274N, P342S (variant B3561)	OPH	*Flavobacterium* sp. ATCC 27551 and *Pseudomonas diminuta*	Chlorpyrifos	725-folds increase in the k_{cat}/K_m value for chlorpyrifos hydrolysis as well as enhanced hydrolysis rates for several other OPs compounds	Thakur et al., 2019

OPH: organophosphate hydrolase; PTE: phosphotriesterase; OpdA: organophosphate hydrolase MPH: methyl parathion hydrolase.

parathion. The same variant was selected as a template for gene shuffling, and large mutant libraries were generated. One potent OPH variant (B3561) was identified, which exhibited ~700-folds improvement in chlorpyrifos hydrolysis (Wang and Sun, 2021).

Substantial bioengineering techniques have been employed to generate efficient PTE variants with enhanced physicochemical properties. The catalytic efficiency of the PTE enzyme for paraoxon (k_{cat}/K_m 1.2 × 10^8/(mol•sec)) has been found to approach the diffusion limit (Singh, 2009). However, the catalytic efficiency of the PTE enzyme notably decreases (k_{cat}/K_m 2.1 × 10^5/(mol•sec)) with other OPs substrates like malathion and G-series or VX nerve agents. For biotherapeutic application, it is advisable to have catalytic efficiency more than k_{cat}/K_m 1.0 × 10^7/(mol•sec) for OPs prophylaxis. Thus, protein engineering approaches have continually tried to improve catalytic activity and stability of the PTE enzyme against toxic pesticides.

5.3. *Efficacy and cost of engineered biocatalytic systems in the actual project*

The enzyme-based green bioremediation approach has several advantages over the existing decontamination methods and researchers across the globe demonstrate the undeniable power of enzyme engineering in the improvement of public and environmental health. In laboratory trials, several researchers reported that the engineered biocatalytic system could efficiently degrade not only OPs pesticides but also chemical warfare agents within limited periods under different environmental conditions, making this system promising candidates for bioremediation/elimination of toxic OPs from the environment. Salient features of the improved biocatalytic system: (1) high catalytic activity against different stereo-selective substrates (enantiomers R/S), (2) act on multiple substrates, simultaneously, (3) rapidly degrade/detoxify hazardous pesticides in the environment, (4) highly stable in harsh environmental conditions, (5) compatible with sensing devices for detection of contamination in the environment, and (6) efficiently detoxify pesticides in animal models (*in-vivo/vitro*) and can replace chemical- drugs (atropine) with improved enzymes system, in the near future.

The actual cost of these approaches to develop a viable product in the real-world: Most of the studies were limited to laboratories due to high cost, low post-recovery, and less stability in the environment. The researchers are trying to enhance the stability of enzymes by immobilizing them on solid supporting material and also demonstrated detection and degradation of OPs pesticides, simultaneously. The scaling up enzymes production to make it commercially viable or utilized in actual projects has been impeded by low recombinant enzymes/products recovery, high processing cost. Thus, a few barriers need to be addressed in the near future: (1) high manufacturing cost, (2) low product recovery rate, (3) required skilled workforce for culture maintenance and downstream processing, (4) required sophisticated instruments, (5) required additional supportive material to extend stability in a heterogeneous environment, (6) required high-cost fabrication materials for the development of a biosensor, and (7) compromise catalytic activity in the unpredictable environment. Despite several limitations, few commercial entities specifically Gingko Bioworks and Novozymes, have developed cost-efficient engineered enzyme systems to remove OPs insecticide from the sheep by sheep-dip formulation in Australia.

6. Applications of biocatalytic systems

OPs compounds have been employed in two highly distinct fields in human society: (1) used as a pesticide in agricultural fields to improve crops productivity and as plasticizer/flame retardant in electrochemical industry; (2) on the other end, they become a core form of chemical warfare agents. OPs compounds adversely affect human and environmental health in dramatically different ways. The prolonged usage of these toxic pesticides has made them recalcitrant, which are present at high concentrations in various environments. Therefore, developing rapid, safe, and reliable systems to detect and decontaminate these pollutants is relevant to combatant and non-combatant populations. The cell-free bio-catalytic system offers a new avenue for the detection and bioremediation of toxic OPs pesticides. The advancement of enzyme engineering with computational tools has played a significant role in enhancing catalytic efficiency, substrate specificity (stereoselectivity), and stability of enzymes for accurate bio-catalytic applications.

6.1. *Immobilization*

Most biocatalytic systems are limited because of their stability. Once removed or extracted from the microbial cell, they lose their functional properties by altering the enzyme structural conformation, commonly induced by environmental factors (pH, temperature, and buffer). Scientists, commercial and industrial researchers struggle to develop highly stable biocatalytic systems by approaching advanced computational bioengineering tools and techniques. The rapid rate of hydrolysis, the efficiency of biodegradation, environmentally friendly, and biological compatibility of OPs degrading enzymes (PTEs/OPH) are emerging as ideal candidates for developing commercially viable products. As mentioned earlier, adsorbing the enzyme on solid materials improves the stability of bio catalytic systems and longevity.

To improve the hydrolysis of OPs by the PTE, researchers immobilized PTE on Cd-Se nanoparticles using a C-terminal His_6-tag sequence. The hydrolysis of paraoxon was monitored at 405 nm by comparing the hydrolysis of free enzyme and immobilized PTE nanoparticles (Pundir et al., 2019). The PTE immobilized on the nanoparticle, or quantum dots was taken in two sizes - a 525 nm quantum dot and a 650 nm quantum dot. They observed that the PTE immobilized on quantum dot showed better production of PNP, implying efficient hydrolysis of paraoxon. The 625 nm quantum dot PTE showed the highest hydrolysis of PTE than the other two (Samanta et al., 2018).

A DNA tetrahedral assembly that was previously designed was modified into a three-way double-stranded DNA junction with the integration of chimeric His-5 peptide DNA conjugate. This three-way junction was converted into a DNA cage by a 24 hr annealing system such that they presented the histidine for the assembly of quantum dots. 540 nm quantum

dots were assembled on these cages, and PTE was immobilized on these quantum dots. Hydrolysis of paraoxon was then compared with the free enzyme. It was found that the former had enhanced the rates up to 12.5-folds. This again suggests a strong synergy between nanoparticles and DNA which should have enhanced the enzyme activity by increasing the stability (Alves et al., 2018).

Similarly, *E. coli* releases fragments of its outer membrane encapsulating nucleic acids, proteins, and biomolecules as proteoliposomes. They were utilized to construct outer membrane vesicles (OMVs). OMVs are the mimics of the bacterial outer membrane encapsulated with proteins. A recombinant protein was made in which the PTE was anchored to the inner wall of the outer membrane of *E. coli* enabling the loading of protein into nascent OMVs. The encapsulated PTE showed better stability, and when these PTE filled OMVs were tested at unfavorable environmental conditions such as elevated temperatures, high salt content, and range of pH, its enzyme activity was preserved. These PTE filled OMVs were easy to purify and showed a better rate of hydrolysis of paraoxon in water samples (Alves et al., 2018; Thakur et al., 2019).

6.2. Biosensors

Biosensors are analytical devices considered highly sensitive and specific in detecting OPs compounds onsite (Mishra et al., 2012). They work with the help of (1) a bio-recognition element to recognize an analyte producing a physical, chemical, or biological response and (2) a transducer to convert the generated response into a measurable signal. Based on the transducer, the OPH biosensors are broadly categorized into three classes: (1) microbial biosensors, (2) optical biosensors, and (3) electrochemical biosensors.

Several physicochemical approaches have been developed to fabricate sensors devices for pesticides detections and have several limitations to implement in actual projects, such as being expensive, time-consuming, unportable, laborious, requiring skilled human resources, and not even eco-friendly (Hondred et al., 2018). Enzyme-based biosensors have emerged as an attractive technique for detecting target analysts (qualitatively or quantitative) in agricultural, food quality control, biomedicine, pharmaceutical industry, and environment. The enzymatic biosensor has several advantages over the physicochemical electric sensory devices (Kaur and Singh, 2020). It offers an undeniable benefit, such as use/operate, eco-friendly, economical, high sensitivity, specificity, miniaturization, real-time detection capability, required minimum samples, high throughput, and portability. Several enzyme-based biosensors have been developed and commercially available for public health management.

6.2.1. Application of optical biosensors

Optical biosensor applications are well established in biomedical fields (detection and monitoring of cholesterol, glucose, sorbitol, glutamate, and pyruvate) (Nguyen et al., 2019). However, optical biosensor applications are less reported for pesticides detection in agri-products and environment. Here we highlighted the best applications of enzyme based optical biosensors for OPs pesticides detection in the environment. The lysine residue of the OPH is covalently linked with sulfo-N-hydroxy succinimide modified gold nanoparticles in an OPH based optical biosensor. When a change in distance occurs between fluorophore and gold nanoparticles, it brings about a variation in fluorescence that directly detects OPs pesticide paraoxon with high sensitivity (Kumar and D'Souza, 2010; Pundir et al., 2019). Methyl parathion was detected using another optical biosensor in which *Sphimgomoas* sp. with OPH was immobilized onto a microplate and onion membrane leading to its detection with a limit of 4-80 μmol (Khaksarinejad et al., 2015). This was further improved concerning its selectivity and stability in which the *Sphingomonas* sp. cells were integrated with poly-ethyleneimine (PEI) functionalized silica nanoparticles (Si-NP) with enhanced stability for 180 days to detect methyl parathion at a LOD (limit of detection) of 0.1-1.0 mg/L. In another study, an optical biosensor was developed to detect paraoxon in which OPH was conjugated to nano-magnet-silica core-shell with coumarin as a fluorescence-generating molecule with LOD of 5×10^{-6} μmol (Kaur and Singh, 2020).

6.2.2. Application of electrochemical biosensors

The enzyme based electrochemical biosensors are extensively used commercially viable sensors because of their simplicity, feasibility, rapidity, and reusability. Commonly used transducers in electrochemical biosensors are potentiometry, amperometry, impedimetric, and conductometry (Pundir et al., 2019). The applications of electrochemical biosensors are well established in the biomedical sector (measuring/detecting glucose, alcohol, lactate, and many more by oxidases enzymes), but limited applications have been reported for pesticides. Here we highlighted the recent application of electrochemical biosensors for OPs pesticides detection. The PTE/OPH hydrolyses pesticides like methyl parathion, paraoxon, fenitrothion, parathion to generate an electro-active 4-nitrophenol species which further oxidizes and produces a net current under applied voltage which can be directly recorded as a biosensor response. This is also directly proportional to the concentration of pesticide in the sample. Electrochemical biosensors can be amperometric OPH biosensors if they measure electric current, while those measuring the potential difference are called potentiometric OPH biosensors. Tang et al. (2014) reported a new application of an amperometric biosensor consisting of OPH, which is entrapped in the carbon electrode and could effectively detect paraoxon and methyl parathion with a LOD of 9×10^{-14} μmol and 7×10^{-14} μmol, respectively . The application and fabrication of potentiometric biosensor, the OPH enzyme was immobilized using physical adsorption, cross-linking with glutaraldehyde, entrapment, and covalent linkage on matrices (such as membrane, microsphere, screen printed electrode, or nanoparticles) which formed a potentiometric OPH enzyme electrode to detect OPs pesticides (Breger et al., 2015).

6.2.3. Application of microbial biosensors

Various physical or chemical techniques could immobilize the microbes onto the transducer. A dissolved oxygen electrode is used to detect the changes that occur due to the activation or inactivation of microbial respiration in the form of the generation of electroactive metabolites (Tang et al., 2014).

Microbial biosensors have several advantages over the enzyme-based biosensor: (1) reduced the prime cost of enzyme production, purification, labor, and analysis time; (2) enzyme isolation and immobilization on transducer may inactive the functional properties of an enzyme (if the active/substrate binding site of enzymes were disarranged on transducer). The microbial biosensor minimizes this complication; and (3) genetically modified microbes can simultaneously express desirable enzymes on cell-surface to detect and degrade pesticide. Here we highlighted an important application of microbial biosensors for OPs pesticides detection in the environment.

For instance, the expression of OPs degrading enzyme phosphotriesterase (PTE) on genetically modified *E. coli* to detect and detoxify pesticides coumaphos (Mansee et al., 2000). Also improved the stability of microbial biosensor by co-expressing *Vitreoscilla* haemoglobin (VHb) along with phosphotriesterase on the cell-surface of engineered *E. coli*, to rapidly detect OPs pesticide paraoxon as compared to only expressed PTE on *E. coli* cell-surface (Kanugula et al., 2011). *E. coli* was genetically engineered to display mutant OPH on its surface for onsite detection of p-nitrophenyl substituted OPs (Latip et al., 2019). When this engineered bacteria were immobilized on a glass carbon electrode (GCE) modified with ordered mesopore carbons (OMCs), it could detect paraoxon, parathion, and methyl parathion with LOD of 9.0 nmol, 10 nmol, and 15 nmol, respectively (Simonian et al., 2005).

For pesticide detection, all three biosensors share a similar type of mechanism but differ in terms of sensitivity. For instance, pesticides detection in agri-products (fruit, vegetable, milk, honey, etc.) required a highly sensitive biosensor with a minimum limit of detection (LOD 0.001 μmol) hence amperometric biosensors can be a better choice. For field level applications, expected to find higher concentrations of pesticides. Therefore, optical or microbial biosensors can be preferable.

6.4. *Medical applications*

Organophosphate hydrolases or PTE enzymes apply in medicine in two ways, pre-exposure prophylactic treatment and post-exposure antidote applications. However, prophylactic treatment is more challenging; it is preferred because most of the acetylcholine esterase is inhibited before the appearance of the symptoms. As OPs circulate quickly throughout the body upon exposure, PTE, in the form of intramuscular injections, would be effective for detoxification but fails to deliver PTE into the blood (Novikov et al., 2010). To overcome these shortcomings, PTE was modified by high molecular weight polyethylene glycol, which was shown to increase the mean retention time of PTE in the blood to 47 hr compared to the wild type, which could be retained only for an hour in the blood (Iyer and Iken, 2015; Singh, 2009).

When bacterial enzymes are used in human treatment, they must address two problems, the immunological reactions that occur in the body owing to their injection and the inactivation of blood proteases (Sogorb et al., 2004; Wang and Sun, 2021). Thus, formulating the OPs degrading enzymes in a carrier that provides an environment that is permeable to OPs pesticides and their hydrolysis products while compromising its immunogenicity should be a better option for their use (Katyal et al., 2020). Nanotechnology encapsulated PTE in liposomes, enhancing PTE activity and circumventing immune defenses (Alejo-González et al., 2018; Goldsmith and Ashani, 2018).

Likewise, erythrocytes and liposomes were used in several experiments inside mice to carry PTE (Nachon et al., 2013; Thakur et al., 2019). The enzyme was encapsulated using several techniques showing that its activity was maintained up to 45 hr and later started decreasing beyond its half-life (Alejo-González et al., 2018; Singh, 2009). For practical applications, PTE/OPH enzymes should be functionally active and stable for a prolonged period while evading the immune system. There is also a requirement for a safe and effective nanoparticle delivery or transport system. That should be the focal point of the research in the coming years.

7. Concluding remarks and future prospect

Here, we have comprehensively summarized the mode of action and current scenario of OPs pesticides in geographically different world regions while highlighting the environmental consequences. The sequential increase in manufacturing and consumption of OPs pesticides in the world results in terrestrial and aquatic ecosystems contamination. They pose a severe threat to civilians as well as the military population. Bioremediation coupled with microbes or microbial enzymes emerged as an attractive, cost-effective, and eco-friendly approach to remediate the neurotoxic OPs compounds (pesticides/nerve agents). Enzymatic bioremediation, especially PTE/OPH enzymes, offers a tremendous potential to decontaminate OPs compounds. Developments in protein engineering and computational biology have led researchers to generate active and stable variants of an enzyme (PTE/OPH) that exhibit high catalytic activity, stereoselectivity against OPs pesticides. Immobilization techniques have improved enzyme stability to some extent, and this approach is being used to develop enzyme-based biosensors for detecting and monitoring OPs pesticides in the environment.

Scientists or industrial entities are searching for potential bioremediation systems (microbes/enzymes) from the environmental isolates. Researchers are continuously exploring advanced bioengineering techniques to develop significantly advanced or robust bioremediation tools for detecting and decontaminating OPs pesticides and other toxic pollutants in the environment. Advancements in computational tools and the availability of 3D protein structures on PDB database which lead researchers to acquire information and understand similar structures and OPs catalytic mechanisms evolutionarily conserved in all OPs degrading enzyme families. This fundamental study allows researchers to reduce the cost and time involved in conducting future enzyme engineering studies. However, new tools and technologies are being integrated into science laboratories. The rational and directed evolution and combinatorial mutagenesis techniques may become prevalent in the future to generate remarkable variants with efficacy which are much needed in medical applications (OPs prophylaxis). However, the variants of PTE, OPH, OpdA, and PON1

enzymes showed a promising result to some extent in in-vitro conditions and preliminary in-vivo model studies.

Moreover, the current transport or delivery systems need modifications to reduce the loss of enzyme efficiency, which are crucial for medical applications. Despite their great success in in-vitro detoxification of OPs pesticides through microbial or enzyme systems, it is important to evaluate these systems in an animal or in vivo conditions to check their compatibility in real-world conditions. While considering the significant efforts or attempts towards this goal, the industrialization of these biocatalyst systems is still uncertain due to their high production, manufacturing costs, and complications in downstream processing to recover enzymes. These challenges pose a considerable barrier for making them commercially viable products. Considering the above challenges, developing a robust, cost-effective, eco-friendly, and commercially viable biocatalyst system for therapeutic use or environmental bioremediation is necessary. Therefore, it is essential to continuously enhance biocatalyst systems to improve activity, stability, and stereoselectivity against OPs compounds. Soon biocatalyst systems will replace the chemical-based OPs medicine like atropine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jes.2022.04.023.

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