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UNDERSTANDING THE ROLE OF BACTERIAL GENES AND ENZYMES IN ORGANOPHOSPHATE DEGRADATION: A STEP TOWARDS ENHANCED BIOREMEDIATION

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Abstract: The use of Organophosphates (OPs) as pesticides and insecticides increased after world war II, although organophosphates (OPs) are comparatively less persistent in the environment, but are highly toxic to animals. OP toxicity is causing a threat to biodiversity, hence it becomes essential to deal with the degradation of such compounds. Various techniques like photolysis, and chemical degradation have been used for OP degradation but these techniques are not cost-effective and require *ex-situ* treatment, hence bioremediation is considered a potential alternative for OP degradation. Understanding the degradation pathways followed by different bacteria, genes and enzymes involved in such pathways can act as a step towards the development of an effective bioremediation technique for OP degradation. Recombinant biotechnology and protein engineering are used to develop designer bacteria, biocatalysts and enzymes with enhanced activity for OP-degrading bacteria. The present review highlights the bacterial degradation pathways, genes and enzymes involved in bioremediation pathways and new approaches for the development of OP bioremediation techniques.

Keywords: Bioremediation, Biotechnology, Organophosphates, Pesticides, Protein engineering.

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INTRODUCTION

The use of organophosphorus compounds as pesticides, plasticizers, petroleum additives, and chemical warfare agents has increased substantially throughout the world after World War II. These pesticides are mainly grouped into four categories of chemicals that are carbamates, organophosphorus, organochlorides, and pyrethrins, out of these organophosphorus compounds constitute about 38% of the total pesticides. First organophosphorus compound tetraethyl pyrophosphate came into existence in 1973 (Singh, 2009), and after that many other such compounds have been commercialized.

Organophosphate (OP) compounds such as chlorpyrifos, methyl parathion, paraoxons, diazinon, etc. are used as fungicides, insecticides, pesticides and others like tabun and sarin are used as chemical warfare agents. OP's

can be degraded easily by sunlight and water exposure, have a small half-life and are immobile in the soil, hence posing less threat of groundwater contamination (Ragnarsdottir, 2000), but continuous and persistent use of OP's has contaminated many ecosystems in the world. The OP's are toxic to humans as well as other organisms and they can be absorbed from contaminated food and water, due to runoff from pesticide-treated land, pesticides leach into water bodies which causes a threat to amphibians, fishes and other non-targeted organisms (Coupe *et al*., 2000; Schipper *et al*., 2008; Prakash, 2018; Kaur and Mishra, 2019; Prakash and Verma, 2021).

All OP's have a somewhat similar structure and hence they function similarly. OP's inhibit the activity of acetylcholine esterase and hence decrease the breakdown of acetylcholine. Organophosphate binds covalently to the active site and phosphorylates the enzyme making regeneration very slow which results in over-accumulation of acetylcholine at synapses and this in turn over stimulates the receptors in the nervous system. Agitation, convulsion, hypersalivation, paralysis, and respiratory failure due to accumulation of acetylcholine lead to death in insects and other animals. OP toxicity is an anthropogenic act that causes a threat to biodiversity hence it becomes important to deal with the degradation of organophosphate (Prakash and Verma, 2020; Masih, 2021; Prakash and Verma, 2022). The OP's like chlorpyrifos can be degraded by both biotic and abiotic means which include physical methods, biochemical methods, and microbial degradation (Prakash and Verma, 2014). A list of organophosphate (OP)-degrading bacteria is presented in Table 1 along with the mode of degradation followed by them.

It has been shown that microorganisms at laboratory conditions (pH-7, 25^{O}C) degrade OP's faster than chemical hydrolysis and photolysis (Ragnarsdottir, 2000), so bioremediation can be considered as one of the potential alternatives for the degradation of organophosphate compounds. The first OP-degrading bacteria was isolated from the soil sample in 1973 and identified as *Flavobacterium* sp. ATCC2755 by Sethunathan and Yoshida. Since then research is going on to isolate and characterize bacteria that are involved in OP degradation and to understand the role of different microbial genes and enzymes in the degradation of OP's (Singh and Walker, 2006). This article reviews the role of bacteria in the degradation pathway of OP's, bacterial genes and enzymes involved in biodegradation, and further approaches for utilizing the potential of OP-degrading bacteria in bioremediation.

Table 1: List of OP-degrading bacteria along with the mode of degradation.

Compound	Bacteria	Degradation mode
Chlorpyrifos	*Enterobacter* sp.	Catabolic (C, P)
	Pseudomonas diminuta	Co-metabolic
	Micrococcus sp.	Co-metabolic
	Flavobacterium sp. ATCC27551	Co-metabolic
Parathion	*Flavobacterium* sp. ATCC27551	Co-metabolic
	Pseudomonas stutzeri	Co-metabolic
	Pseudomonas diminuta	Co-metabolic
	Agrobacterium radiobacter	Co-metabolic
	Bacillus spp.	Co-metabolic
	Pseudomonas sp.	Catabolic (C, N)
	Arthrobacter sp.	Catabolic (C)
	Xanthomonas sp.	Catabolic (C)
Monocrotophos	*Pseudomonas mendocina*	Catabolic (C)
	Arthrobacter spp.	Catabolic (C)
	Bacillus megaterium	Catabolic (C)
	Bacillus megaterium	Catabolic (C)
	Pseudomonas aeruginosa F10B	Catabolic (P)
	Clavibacter michiganense SBL11	Catabolic (P)

Chlorpyrifos degradation by bacteria

Chlorpyrifos (CP) - o, o diethyl o (3, 5, 6 trichloro 2, pyridyl phosphorothioate) is one of the widely used insecticides. It was introduced by Dow's chemicals in 1965 in the USA as pest and insect control for agriculture as well as domestic use (John and Shanavas, 2015). According to 'India for safe food', 7163 metric tonnes of chlorpyrifos were consumed in India between 2005-06 and 2009-2010.

Many bacteria have been reported that can degrade chlorpyrifos, for example- *Pseudomonas acidovorans, Enterobacter* sp. *Flavobacterium* sp. *Pseudomonas* sp. etc. Microorganisms can degrade CP by oxidation and hydrolysis which lead to the formation of byproducts like 3, 5, 6 trichloro-2-pyridinol (TCP), trichloro-2-methoxy-pyridine, diethyl phosphate (DETP), desethyl chlorpyrifosoxon, chlorpyrifos oxon. Many studies show that the degradation of CP is slow at low pH (acidic soil) and significantly increased when pH was shifted from low to high. At alkaline pH, microbes can degrade CP by the co-metabolic pathway and release TCP as a byproduct. TCP has 3 choline atoms on a pyridinol ring, to break the ring choline atom needs to be removed first which is toxic for the microbial community. *Flavobacterium sp., Pseudomonas diminuta* co-metabolically degrade CP in liquid medium but do not utilize it as a sole source of carbon or sulfur.

On the other hand, *Enterobacter* sp. isolated from Australia shows enhanced degradation of CP and utilizes TCP, DETP byproduct of CP hydrolysis as a sole phosphorus and carbon source. TCP can be hydrolyzed by *Pseudomonas* sp. in a liquid medium (Singh and Walker, 2006). Various studies showed that bacteria can degrade CP more effectively. Bacteria like *Pseudomonas fluorescens, Bacillus subtilis, Brucella melitensis,* and *Pseudomonas aeruoginosa* can degrade 89, 85, 87, and 92%, of CP respectively which is relatively high as compared to 34% degradation at control in the 30 days. TCP was further inoculated with *P. aeruginosa* strain for 20 days, which resulted in TCP degradation into an unknown metabolite(Vidya Lakshmi *et al.*, 2008).

Parathion degradation by bacteria

Parathion formulated as o, o-dimethyl-o-p nitrophenyl phosphorothioate, first introduced in 1994 since then it is widely used as an insecticide for controlling agriculture pests and household insects (Chapalamadugu and Chaudhry, 1992). According to India for safe food, 8408 metric tonnes of Methyl Parathion (insecticide) was used between 2005-06 and 2009-10 in India. Degradation of such insecticides is important, these are not much persistent in the environment, but still have high contamination due to continuous use. Research on microbial degradation of parathion is going on and many bacteria have been identified that can metabolize parathion and methyl parathion completely.

It was observed by Nelson in 1982 that the population of bacteria in soil increased with an increase in parathion concentration (Nelson, 1982). *Flavobacterium* sp. the first OP compound degrading bacteria can also degrade parathion. *Pseudomonas* sp. hydrolyze parathion to form p-nitrophenol and it can further utilize p-nitrophenol as a carbon source. Most of the microbes degrade parathion by the first hydrolysis step, but some can follow different pathways *Bacillus* sp. reduces the nitro group first and later hydrolyzes the intermediate to form p –aminophenol. Parathion and methyl parathion can be mineralized by oxidation at the first step, but the detailed biochemistry of this pathway is not known yet (Chapalamadugu and Chaudhry, 1992).

Monocrotophos degradation by bacteria

Monocrotophos (3-hydroxy-N-methyl-cis-crotonamide) (dimethyl phosphate)-MCP, is a frequently used hazardous insecticide, it is 100% soluble in water and mostly used to protect crops from pests (Bhadbhade *et al.*, 2002). Many bacteria have been isolated, which can metabolize MCP as a sole source of carbon and nitrogen.

Paracoccus sp. M1 degrades 79.92% of MCP in 6 hours initially under aerobic conditions and later degradation can be carried out anaerobically. This species of bacteria can also degrade other amide herbicides and organophosphate

pesticides. Other bacterial species like *Bacillus* sp., *Pseudomonas medocina, Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423, etc are also efficient in the degradation of MCP. *Bacillus megaterium* and *Arthrobacter atrocyaneus* degraded 82.53% and 78.94 % of MCP respectively. MCP degradation also includes hydrolysis of the P-O-C bond at the first step, which makes the compound available for further degradation.

The hydrolysis produces dimethyl phosphate and N-methyl acetoacetamide intermediates in MCP degradation. *Bacillus megaterium* and *Arthrobacter atrocyaneus* produce esterase and phosphatise. These two are required to break the dimethyl phosphate and N-methyl acetoacetamide into phosphate as an end product. It clearly indicates that these bacteria degrade MCP by releasing phosphate (Bhadbhade *et al*., 2002).

GENES INVOLVED IN THE DEGRADATION OF OP's

OP removal by microbes is the best method to choose because microorganisms have the complement of degradation pathways and enzymes that are required for OP degradation. The molecular base for OP degradation is evolving field for scientists and a lot of research is going on in this field. Our understanding of genetic and molecular base OP degradation is increasing dramatically, with the discovery of so many new genes in different bacterial species that degrade OP's (Sun *et al*., 2016).

Many studies pay special attention to the role of catabolic genes in OP degradation. These genes can be used for recombinant DNA technology (RDT) to develop special bacterial strains which can degrade a large number of OP with high efficiency, or use these recombinant bacteria to produce OP degrading enzymes which can be applied directly in the contaminated site in a cell-independent manner (Chapalamadugu and Chaudhry, 1992). Earlier most of the studies are dependent on the isolation of bacteria on culture medium, as we know that less than 1% of microorganisms can grow on the culture medium so this acts as a hurdle in further research. But now the advancement in metagenomics and the development of culture-independent PCR techniques have paved the way for amplification and sequencing of functional genes directly from the environment (Singh, 2009). Degradation of OPs tends to have the same characteristics not all bacteria can metabolize OPs completely, some can only degrade them up to some extent and can produce intermediate toxic compounds. The bacteria that can completely mineralize OP, have a closely linked cluster of genes involved in the OP degradation pathway. These clusters of genes are flanked mostly with the transposable element, which suggests that these pathways are mobile and can be horizontally transferred between the species (Siddavattam *et al*., 2003). The two large families of hydrolase enzymes involved in OP metabolism are *mpd* metallo-β-lactamase and *opd* phosphotriesterase (PTE) family, the understanding of the molecular and genetic levels of these families can help to develop new techniques for bioremediation.

Opd gene structure and origin

The first organophosphate degrading gene was *opd*, it is encoded by a large plasmid isolated from *Sphingobium fuliginis* ATCC 27551 (*Flavobacterium* sp. ATCC27551) and later this plasmid was transferred to other soil bacteria. The sequence of *opd* gene-encoded amino acid isolated from *Flavobacterium* sp. ATCC27551 and *P. diminuta* showed 100% similarity, but they exist on the non-homologous plasmid, this shows that *opd* gene is distributed through horizontal gene transfer (HGT) added by mobile genetic element (MGE's).

Sequencing of pPDL2 plasmid of *Flavobacterium* sp. ATCC27551 shows that *opd* genes are flanked by upstream IS elements which code for ISF1sp1 and downstream TnR and TnA which are Tn3 like elements and code for resolvase and transposases respectively. pMCS1 plasmid of *B. diminuta* shows similarity to pPDL2 plasmid in having flanked IS's element and linked transposable elements like resolvase and transposases (Siddavattam *et al*., 2003). pMCS1 and pPDL2 plasmid have the size of 70 and 39 kb respectively, the *opd* gene in both the plasmid is present within 5.1kb highly conserved region. The homologous region in the plasmid extends from 2.6kb upstream of *opd* gene and 1.7 kb

downstream to that of *opd* gene (Chapalamadugu and Chaudhry, 1992). Both the plasmid have some unique characteristics like pMCS1 plasmid contains the *tra* gene responsible for the transfer of plasmid in the new host by conjugation (Pandeeti *et al*., 2011) and pPDL2 possesses the gene encoding for protocatechuate dioxygenase, an aromatic transporter protein, and intergrase which helps in lateral integration of plasmid on the chromosome in the host which can not replicate the plasmid (Pandeeti *et al*., 2011).

An *opd* gene homolog (*opdA*) was isolated from *Agrobacterium radiobacter* P230 which is a coumaphos-degrading bacteria. This gene is present on the chromosome of the bacteria and shows 88% similarity in the nucleotide sequence of the *opd* gene found in *B. diminuta* GM and *S. fuliginis* ATC 27551 (Fig.1). The *opdA* gene isolated from *A. radiobacter* has an upstream *tnpA* gene and a downstream IS element that shows homology with *Mycobacterium fortuitum*'s Tn*610* transposon (Horne *et al*., 2003). *OpdA* is also transposable, this further establishes the link between *opdA* and the plasmid-borne *opd* gene.

Mpd gene structure, origin, and evolution.

Mpd (methyl parathion degradation) genes are involved in different OP degradation pathways, These genes impart a special ability in bacteria to degrade chlorpyrifos, methyl paraoxon, and methyl parathion. Very few microbes can degrade these OP'S. *Pseudomonas* sp. A3, can utilize methyl parathion as a carbon source and this bacteria degraded methyl-parathion into *p*-nitrophenol, which can be further metabolized into maleyl-acetate via the formation of 1,2,4-benzenetriol, and hydroquinone as intermediate (Rani and Lalithakumari, 1994).

Diethylthiophosphate is utilized as the sole phosphorus by a yet unknown mechanism. *M*pd gene has less than 20% similarity with *opd* genes or other OP degrading genes. Most of these genes are present on the chromosome with one exception that is *Pseudomonas putida* WBC-3 have *mpd* gene on 70 kb plasmid (Liu *et al*., 2005). This plasmid *mpd* gene shows similarity with the chromosomal *mpd* gene, which also indicates towards the HGT. Sequencing of chromosomal DNA, showed that downstream to *mpd* gene sequence, 880-nucleotide insertion sequence is present. These insertion sequences consist of transferases and are flanked by the inverted repeats. Other than this 3 associated ORF's having similarity with permease components having ABC-type transport are present on complementary strands. These ORF's play role in the secretion of methyl parathion hydrolase and its transport in bacteria (Zhang *et al*., 2006).

NCBI database reveals that multiple putative *mpd* homologous genes having conserved beta lactase domain, are present in different soil bacteria, these bacteria may act as pathogens. The role of *mpd* genes in OP degradation is yet undiscovered and needs further research. Sequencing of more MGE can add up more information about such genes.

Other OP metabolizing genes

A family of promiscuous and unrelated OP-metabolizing genes discovered recently encode the OPAAs (Organophosphate acid anhydrases) enzymes. These genes are isolated from many organisms such as squid, humans, and the bacterial *Alteromonas* genus. The enzyme encoded by them can catalyze the hydrolysis of the same substrate as that of MPH and OPH enzymes but these genes do not share much sequence similarity with *opd* genes and are far more efficient in degrading chemical agents such as soman and sarin.

It is now thought that these OPAA had evolved from prolidase (Vyas *et al*., 2010). Other such genes possibly include the transposable *ophc2* gene and *opdB* gene isolated from bacteria like *Stentrophomonas*, *Pseudomonas*, and *Lactobacillus brevis* respectively. These are named after the original *opd* gene, they neither have homology with *opd* gene sequence and nor share any structural similarity. The *ophc2* is a novel gene from the metallo-β-lactamase domain-containing family and *opdB* is related to one of the serine-dependent hydrolases. This provides reliable proof for the contribution of the β-lactamase superfamily in the bioremediation of OP (Gotthard *et al*., 2013). Another PTEencoding gene *hocA* is isolated from *P.monteilli* encode for 19 kDa enzyme, which hydrolyzes parathion indicating towards the evolution of OP degrading

enzymes from mono-esterase or phosphotriesterase (Horne *et al*., 2006).

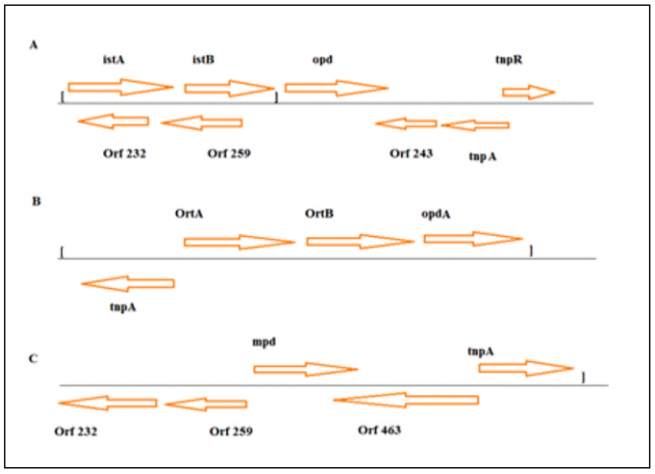

Fig. 1: Gene map for clusters of OP degrading genes. Arrows show the directionality and location of each gene and brackets represent inverted repeat sites of insertion elements A. Cluster of Opd genes arrangement on the plasmids of *B. diminuta* Gm and *S. fuliginis* ATCC27551 along with the associated flanking elements, B. Cluster of opdA gene of *A. radiobacter* P230 along with the associated transposase and insertion elements. C. mpd gene clusters along with associated ORF's.

ENZYMES INVOLVED IN OP DEGRADATION

The first step of OP degradation is hydrolysis in most cases, other than hydrolysis OP degradation also involves oxidation, reduction, etc. Lots of enzymes are involved in OP degradation, but most of the studies focus on organophosphorus acid anhydrolase (OPAA) organophosphorus hydrolase (OPH), and methyl parathion hydrolases (MPH), because these are most widely used by bacteria and show a wide range of activity towards substrates having P-F, P-S, P-O bond.

Studies have been done on the activity enhancement of these enzymes, although many new enzymes are also isolated from different bacterial species (Table 2). These studies can help in the improvement of OP degradation techniques so continuous efforts are required to isolate new enzymes and increase the catalytic efficiency of existing enzymes (Singh and Walker, 2006).

Table 2: Different OP-degrading bacterial enzymes.

Degrading enzyme	Origin	Structure	Molecular weight (kDa)
OPH	*Pseudomonas diminuta*	Dimer	72
OPAA	*Alteromonas* spp.	Monomer	50-60
OPDA	*A. radiobacter*	Dimer	70
ADPase	*Nocardia* sp.	Monomer	43
AMPP	*Escherichia coli*	Tetramer	52
HOCA	*Pseudomonas monteilli*	Monomer	19
SC-OPH	SC strain	Tetramer	67
NS-OPH	*Nocardiodes simplex*	Monomer	45
PEH	*Burkholderia caryophilli*	Tetramer	58

VARIOUS APPROACHES FOR BIOREMEDIATION OF OP's

Bioremediation is one of the promising techniques for the removal of various contaminants from the environment. Naturally growing bacteria can degrade various OP's and use them as an energy source, but microbes show very slow growth and some condition in the environment inhibit their growth hence significant degradation of such compounds is not achieved. Bioaugmentation (using laboratory-grown bacteria) and biostimulation can be used to overcome these problems. Two major bioremediation approaches are:

1. Use of bioreactors for bioremediation, the best example is a filter bioreactor developed by mulbry et al with a consortium of many microorganisms which can be used for the degradation of 15,000 litres of coumaphos in a single batch (Mulbry *et al*., 1998). Scientists investigated the bioremediation of CP by *Pseudomonas* (Iso 1) sp. in a bioreactor made up of pieces of polyurethane. It was shown that more

than 91% of steady-state efficiency was achieved for an inlet load of 300 mg /L/d, although accumulation of TCP can reduce the efficiency of the reactor with time (Yadav *et al*., 2014). It was shown by Saiguang Xue and his colleagues in 2019 that when purified His tagged organophosphohydrolase (*opdA*) enzyme was immobilized on MIL88A (metal-organic framework) and showed 5 times higher activity than free enzyme. Immobilization improved tolerance to organic solvent, thermal stability, SDS tolerance. When used to degrade OP on grapes and cucumber this immobilized enzyme showed 66 and 61% initial activity even after reuse (Xue *et al.*, 2019).

2. *In-situ* bioremediation provides more wide range of applications. Many bacteria have been isolated and characterized which can degrade OP in laboratory conditions but the application of such bacteria in contaminated sites poses several challenges like the requirement of nutrition, the nonavailability of oxygen, and the presence of more than one contaminant can inhibit the growth of particular bacteria, etc. The use of enzyme extract can be a possible alternative.

Thus, the *Streptomyces phaeochromogenes* immobilized the whole cell and cell extract both show similar degradation of OP compound, use of cell extract can reduce handling problems (Santillan *et al*., 2020).

So due to the presence of several challenges, new approaches like the use of recombinant technology to develop bacteria, which can grow in all environmental conditions and have genes for multiple OP degradation, enhancement of the catabolic activity of various enzymes and using them directly at a contaminated site, etc are getting more importance from the scientific community. The figure 2 shows different bioremediation methodologies.

Use of recombinant organisms

The increasing knowledge of the genetic and molecular level of OP degradation developed the way for use of genetically modified organisms. Genes of OP degradation are mostly present on plasmids, isolation of such plasmid followed by transformation into another organism that can amplify the plasmid more rapidly and later introduction of these transformants with altered stability and activity to contamination site can help to overcome the problem faced by wild type bacteria. Various efforts are being made in this direction (John *et al*., 2020). *Opd* gene containing recombinant plasmid, when transferred into an *E. coli* was poorly expressed under its promoter, but when expressed under *E. coli*'s promoter (lac promoter) increased expression was achieved (Mulbry and Karns, 1989). Cloning of parathion hydrolase gene present on 66-kb plasmid isolated from *Pseudomonas diminuta* in various organisms like *E. coli* (Serdar *et al.,* 1989), *Streptomyces lividans* (Steiert *et al.,* 1989), and insect cells (Dumas *et al*., 1989) have been successfully achieved.

Anchoring of OPH on *E. coli*'s surface with the help of an Lpp-OmpA 46-159 (a fusion system), is a new approach towards the detoxification of organophosphorus compounds. The immobilized living biocatalysts on the solid support can provide a cost-effective alternative. Although these recombinants were effective in parathion degradation but are unstable (Richins *et al*., 1997). This problem was resolved by the construction of a new plasmid that can express OPH on the surface of a cell under the control of tac promoter, which is tightly regulated and show the host-specific production of OPH on the surface of the cell with a much high degradation rate for parathion in two strains - XL1-Blue and JM105 of *E. coli* (Kaneva *et al*., 1998). Three bacteria *Stenotrophomonas maltophilia* - CPI 15, *Staphylococcus warneri* -CPI 2 and *Pseudomonas putida* -CPI 9 have 4 kb plasmid which can be cured only with sodium azide, when these plasmids are individually transformed into *Escherichia coli* JM109, showed CP degradation in minimal medium. The degradative organophosphorus hydrolase enzyme of ~ 60 KD molecular weight was purified and expressed to 26, 31.85, and 37.74 folds, respectively (John *et al*., 2020).

Thus, it can be concluded that the use of

engineered strains containing such plasmids can be used for bioremediation of OP and with the help of plasmid transformation persistent and continuous competence for the CP degradation can be attained in soil bacteria. *Serratia marcescens* MEW06 have 31.09-kDa MPH which is 54.9% similar to MPH of *Pseudomonas* sp. WBC-3. *mphGM004539* (*mphGM004539*Δ*sp*) without a single peptide was cloned in *Escherichia coli* BL21 (DE3), The expressed enzyme showed 5.26 U/mg enzyme activity at 35 °C and pH 11 (Wang *et al*., 2018). Some intermediates of the OP degradation pathway show an inhibitory effect on the process of degradation and sometimes are toxic to bacterial cells.

Genetically Engineered bacteria can function as 'designer bacteria or biocatalysts,' which have several required enzymes and biodegradation pathways from different microorganisms for the degradation of OP. The strain was engineered for optimization and construction of metabolic pathway for diethyl phosphate (DEP) and paraoxon hydrolysis by introducing another pathway for *p*-nitrophenol (PNP) degradation into the *Pseudomonas putida* so that it can mineralize paraoxon completely (De la Peña Mattozzi *et al*., 2006). Other approaches include the expression of enzymes in a single host for the degradation of multiple pesticides for example - a vector with both CaE B1 and OPH was introduced in *E. coli* bacteria which enabled this strain to degrade both OPs and this is one step ahead toward the target to achieve single biocatalyst which can act on multiple substrates (Lan *et al*., 2006).

Enzyme-based bioremediation

The release of GMOs (genetically modified organisms)is not accepted widely because these organisms can pose an unknown threat to natural biodiversity so researchers are trying to come up with an alternative way for the bioremediation of OP via cell-free enzyme systems (Thakur *et al*., 2019). The rich biodiversity is the need of survival of entire biota (Verma, 2017). The enzyme involved in OP degradation requires high specificity for substrate (Yair *et al*., 2008).

One approach that can be used for attaining good enzyme-substrate interaction is the engineering of an enzyme that specifically has a high affinity for the desired substrate (Broomfield *et al*., 1999). Example- Site-specific mutagenesis at H257L (histidine at 257 place is replaced by leucine) and H254R (histidine at 254 place is substituted by arginine) showed a four to fivefold increase in catalytic activity for P-S bond (Di Sioudi *et al*., 1999). Human paraoxonase 1 (h-PON1) is a 45KDa enzyme. It can hydrolyze various OP's but show low activity but when mutated by random mutagenesis approach it showed more hydrolyzing activity. The mutants showed 10-340 times more hydrolyzing activity for many OP substrates and also had differential arylesterase and lactonase activities. A further detailed study suggested that the mutations have allowed differential binding of OP substrate in the active site of the enzyme (Tripathy *et al*., 2017). Another method used for creating enzymes with high specificity is the shuffling of DNA in a sequential cycle and screening for enhanced activity of OPH for the substrates.

Another screening technique in which surface displacement of enzyme on modified bacterial was used to create enzyme showed improved degradation of methyl parathion. One variant created by this method showed a 25-fold fast degradation rate than the wild type (Cho *et al*., 2002). Conjugation of poly His box to OPH enzyme increased its stability at high temperatures and alkaline pH. This enzyme was more active in cleaving the P-S bond and hydrolyzed methylparathion and parathion with higher efficiency (Efremenko *et al*., 2007). Another problem with the introduction of enzymes at the contaminated site is their short half-life and low thermal stability, therefore it is necessary to develop a good system for proper delivery of the enzyme with an enhancement of self-life (Yair *et al*., 2008). Cross-linking of PTE enzyme on amyloid fibrils made from crystallin and insulin showed improvement in the thermal stability of the enzyme (Raynes *et al*., 2011). PTE conjugated covalently on gold (negatively charged) nanoparticles decreased Km of the

enzyme, increased the value of *Kcat* and *Vmax*, and also made the enzyme more stable at wide temperatures 25-90^{O}C) and pH (2-12) range (Raynes *et al.*, 2011).

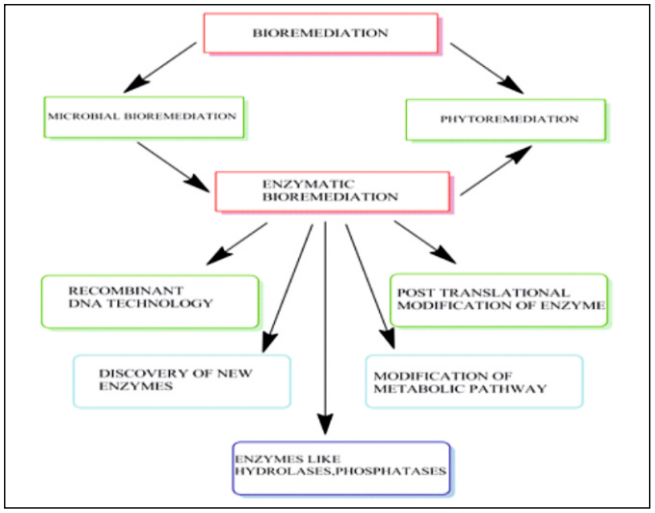

Fig. 2: An overview of bioremediation methodologies.

CONCLUSION

Researchers are using various tools and techniques of protein engineering and molecular biology to modify different OP-degrading bacteria and enzymes to improve the catalytic activity and substrate affinity.Current studies on the role of catabolic genes in OP degradation will enhance the use of recombinant DNA technology (RDT) to develop special bacterial strains which can degrade a large number of OP with high efficiency inturn, these recombinant bacteria can be used for the production of OP- degrading enzymes which are directly applied in the contaminated site, this result in OP degradation in a cell-independent manner. Research for novel microorganisms, genes and enzymes is going on to completely unfold the story of OP degradation by bacteria.

On-field application of various bioremediation techniques at a large scale is yet not achieved due to various regions like the large cost of manufacturing and stabilization of enzymes, instability of microbes on-field, etc. More research needs to be done with collaborations between government, academic institutions, and industries to develop robust bioremediation techniques for commercial use.

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