



REVIEW ON BIOREMEDIATION OF METHYL PARATHION CONTAMINATED AGRICULTURAL SOIL BY MICROORGANISMS

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Abstract

Organophosphate pesticides are widely used for crop protection against pests and also to increase crop productivity. Methyl parathion (O, O-dimethyl O-p- nitrophenyl phosphorothioate, MP) is one such organophosphate pesticide. The extensive use of it has led to bioaccumulation in the environment. Thus causing harmful health hazards like inhibition of acetylcholinesterase (an important enzyme in nervous system) activity. Bioremediation, a technique can be applied to overcome bioaccumulation caused by pesticide. The present review is focused on the bioremediation of methyl parathion by degradation through potential soil bacteria and culturing them by immobilization. Also the new perspectives for the bioremediation would enhance in effective degradation of methyl parathion.

Keywords: *Bioremediation, Methyl parathion, Methyl parathion hydrolase gene, Organophosphate pesticide, p-Nitrophenol*

I. INTRODUCTION

India is primarily an agriculture-based country with more than 60-70% of its population dependant on agriculture. A huge portion of arable land already under cultivation is being rapidly depleted by industries and urban encroachments. India's fast growing population is projected to cross 1.3 billion by 2020. To feed this population various strategies are being applied to attain agricultural production [1]. Better harvests require intensive cultivation, irrigation, fertilizers, and the use of chemicals to protect plants from pests and plant diseases. In India, 15–20% of all produce is destroyed by pests [2]. This emphasizes the paramount importance of pesticides in India in preventing agricultural loss and enhancing production. The extensive use of pesticides causes serious environmental concerns, as only 5% or less from the applied pesticides reach the target organisms which resulted in contamination of soil and water bodies (major environmental problem of current age). The periodic use of pesticides makes the situation particularly perturbing. This repetition in the long term necessarily leads to an accumulation of pesticides and their residues in environment; endangering the entire population by their multifaceted toxicity [3]. There is a direct relationship between the contamination of pesticides and their residual detection [4]. In addition to causing toxic effects to humans, there is a high risk of contamination in ecosystem [5]. An enduring threat of volatilization of sprayed pesticides is present that usually hit (directly) non-target vegetation. This leads towards contamination of air, soil, and non-target plants [6]. There are chronic threats to human life, caused by long term, low dose exposure to pesticides. It can cause hormonal disruption, diminished intelligence, and reproductive abnormalities [7]. The constant mobility of applied pesticides through leaching, sorption, and volatilization results in contamination of different levels in the environment [8], [9].

1.1. Microbial flora in soil

Soils are the naturally occurring physical covering of the earth's surface, and represent the interface of three material states: solids (geological and dead biological materials), liquids (water),

and gases (air in soil pores). Each soil is a unique product of the combination of geological parent material, glacial and geomorphological history, the presence and activity of biota and the history of land use and disturbance regimes. Soils are the foundation of all terrestrial ecosystems and are home to a vast diversity of bacteria, archaea, fungi, insects, annelids, and other invertebrates as well as plants and algae. These soil dwellers provide food or nutrients that support organisms that live above and below ground. Soils also play critical roles in buffering and filtering freshwater ecosystems. Consequently, soils are extremely important to human societies. We depend on soils for the basis on which we and our buildings stand and for the production of food, building materials, and other resources; indeed, soils influence most ecosystem services on which we depend [10]. Soil microbes, bacteria, archaea, and fungi play diverse and often critical roles in these ecosystem services. The vast metabolic diversity of soil microbes means their activities drive or contribute to the cycling of all major elements (e.g. C, N, P), and this cycling affects the structure and the functions of soil ecosystems as well as the ability of soils to provide services to people [11].

1.1.1. Soil bacterial phyla

Molecular tools have been used to investigate in situ soil bacterial community composition. These investigations have revealed that although bacteria have been subdivided into more than 100 phyla, fewer than 10 are abundant in soil [12]. The estimated relative abundance of the major phyla varies between different soils (or samples); members of the phyla Proteobacteria, Acidobacteria, and Actinobacteria are widespread and often abundant, whereas members of the Verrucomicrobia, Bacteroidetes, Firmicutes, Chloroflexi, Planctomycetes, and Gemmatimonadetes are generally less prevalent [13].

1.1.2. Soil fungal phyla

Fungi are ancient. Fungal-like organisms appeared in the fossil record at least 1400 million years ago and all modern classes of fungi had appeared by the Late Carboniferous, approximately 300 million years ago. Fungi are thought to have colonised land during the Cambrian period, well in advance of plants. Not surprisingly, given their ancient origins, fungi have evolved to occupy nearly every ecological niche on earth. It is estimated that there are 1.5 million to 5 million species of fungi [14].

With the advent of next-generation sequencing technologies and the analysis of multiple genetic marker datasets, fungal taxonomy has changed substantially in recent years. Seven fungal phyla are currently recognised: Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Ascomycota, Basidiomycota, and the relatively recently evolved lineage of parasitic endobionts, the Microsporidia, which are sometimes considered to be a sister-group of the fungi [15].

II. USE OF PESTICIDE IN CROP PROTECTION

Pesticides are the chemical substances that kill pests. In the context of soil, pests are fungi, bacteria insects, worms, and nematodes etc. that cause damage to field crops. Thus, in broad sense pesticides are insecticides, fungicides, bactericides, herbicides and nematicides that are used to control or inhibit plant diseases and insect pests. Although wide-scale application of pesticides is an essential part of augmenting crop yields; excessive use of these chemicals leads to the microbial imbalance, environmental pollution and health hazards. An ideal pesticide should have the ability to destroy target pest quickly and should be able to degrade non-toxic substances as quickly as possible [16].

III. ORGANOPHOSPHATES

They are esters derived from phosphoric acid. In humans, they act on the central nervous system by inhibiting acetyl cholinesterase, an enzyme that modulates the amount and levels of the neurotransmitter acetylcholine, disrupting the nerve impulse by serine phosphorylation of the hydroxyl group in the active site of the enzyme. The symptoms are causing loss of reflexes,

headache, dizziness, nausea, convulsions, coma and even death. Also described with alkylating properties, which from the point of view of mutagenesis is paramount because they act directly on the deoxyribonucleic acid (DNA) adding alkyl groups, methyl and ethyl mainly to the nitrogenous bases with nucleophilic groups capable of reacting with electrophiles. Organophosphorus compounds are most commonly used in agriculture, most are insecticides and miticides, their way of joining these organizations is by ingestion and contact. They are used in vegetable crops, fruit trees, grains, cotton, and sugarcane, among many others [17].

3.1. Methyl parathion

Methyl parathion (*O*, *O*-dimethyl *O*-*p*- nitrophenyl phosphorothioate, MP) is an organophosphate pesticides used extremely for agriculture crop protection. It has been banned in many countries because of its higher toxicity for mammals [18]. It is highly toxic pesticide with trade name Dimethyl parathion, Mepaton, Mepatox, Methyl E-605 and Methylthophos displaying insecticidal activity against a wide range of insect and arthropod pests [19],[20]. Methyl parathion may also be found in compounds with other insecticides such as acephate, camphechlor, carbaryl, carbophenothion, cypermethrin, dicofol, ethylparathion, ethion, lindane, methoxychlor, monocrotophos, phosal, propargite, petroleum oils, and tetradifon.

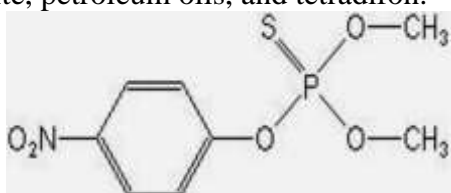


Figure 1. Chemical structure of Methyl Parathion

Methyl parathion is formulated as a number of different commercial products. The most commonly available formulations include a wettable powder (WP), emulsifiable concentrate (EC), dustable powder, and Ultra-low volume liquid and microencapsulated product. It is used for control of insects and mites having chewing and sucking type of mouth parts, including thrips, weevils, aphids and leafhopper in a very wide range of crops, including cereals, fruit, nuts, vines, vegetables, ornamentals, cotton, and field crops[19],[21]. It kills insects and mites by contact stomach and respiratory action. Methyl parathion is highly toxic for warm blooded animals the mammals and birds. It is potent inhibitors of acetylcholinesterase activity, an important enzyme in the nervous system. It is applied to agricultural crops by aerial or ground spraying equipment. Methyl parathion has been detected in surface waters and sediments, rainwater, aquatic organisms, cereals and pulses [22].

3.1.1. Properties of methyl parathion

Table 1. Physical and Chemical Properties of Methyl Parathion [23]

S.No.	Properties	Methyl Parathion (MP)
1.	Molecular Formula	C ₈ H ₁₀ NO ₅ SP
2.	IUPAC Name	<i>O</i> , <i>O</i> - dimethyl <i>O</i> -4-nitrophenyl phosphorothioate
3.	Common Name	Methyl Parathion, Parathion Methyl, Metaphos
4.	Melting Point	35-38°C
5.	Boiling Point	143°C
6.	Vapour Pressure	1.3m Pa at 20°C
7.	Water Solubility	55-60 mg/litre at 25°C
8.	Freezing Point	About 29°C
9.	Non-aqueous solubility	Soluble in ethanol, Chloroform, aliphatic solvents and slightly soluble in light petroleum.
10.	Odour	Like rotten eggs or garlic

3.1.2. Fate of methyl parathion in environment

The environment consists of a series of compartments like soil, water, air and other living macro and microorganisms. Once a pesticide enters the environment, its continuous transformation from one component to another occurs. These processes, in turn, are mediated by several significant biological activities which can be determined by the quantity of residual pesticides that persists in any one compartment for a certain period of time. The distribution of MP in air, water, soil and organism in the environment is influenced by several physical, chemical and biological factors. When dispersed in the air, water and soil it becomes pollutant. Pollution caused by both excessive and continuous use of pesticide. These pesticides enter the environment by diverse modes such as accidental spills, direct applications residuals due to facility cleaning of containers etc [24].

MP takes several months to degrade when applied in soil but when it is applied in higher concentration in soil, as in an accidental spill, its degradation takes several years. The half life of MP in natural water is about one month, which is long enough to be toxic to many living organisms in the environments [25]. Methyl parathion is relatively more mobile in soil. It degraded rapidly in flooded non aerobic soil where its half life period is 7 days but in aerobic flooded soil in half life period has been estimated as 64 days. The half life of MP in water is 24-28 days [26].

3.1.3. Toxicity and hazards caused

Methyl parathion exhibits the high oral and moderate dermal toxicity with a half life of 14 - 21days. The toxicologically relevant mode of action is the inhibition of choline esterase activities and also various clinical effects occur due to OP poisoning in human. MP is lipid soluble and it can penetrate in skin also. It also enters the body through the respiratory and gastrointestinal tracts and when absorbed through alimentary canal, it is stored in adipose tissue. Its primary mechanism for toxicity is its slow release into the bloodstream and subsequently to the nervous system [27].

General health effects

Classical symptoms of methyl parathion poisoning are: headaches, nausea, night-waking (sleeplessness), diarrhea, restlessness, difficulty breathing, dizziness, abdominal cramps, excessive sweating, in-coordination excessive salivation and mental confusion [28].

Neurotoxic effect

Methyl parathion has the ability to bind to acetylcholinesterase and to prevent the hydrolysis of acetylcholine. Neurotoxicity as it is related to high-level exposure, low-level exposure, and neuro degenerative diseases has been studied. There is an association of pesticide exposure and Parkinson's disease with a 1.5 – 7 –fold increase in risk [29].

Respiratory, cardiovascular, hepatic and renal effects

Following effects have been reported due to inhalation of methyl parathion: pulmonary edema, cardiovascular lesions, acute nephrosis of the kidney, and liver lesions. Congestion of the esophageal mucosa and petechial hemorrhages has been associated with a lethal dosage of methyl parathion taken for suicide [30].

Genotoxic effects

MP was observed as genotoxic in in vitro and in vivo causing gene mutations in bacteria, chromosomal aberrations in mammalian cells, sister chromatid exchange (SCE); and was positive on the sex-linked recessive lethal assay in *Drosophila*. In vitro, MP showed binding directly to the cellular DNA[27].

IV. BIOREMEDIATION OF METHYL PARATHION

The degradation of persistent pesticides is very essential for decontaminating soil and water bodies [31].The pesticides degradation processes are of different modes, involved in decontamination of various systems in variable efficiency. The rate of degradation of pesticides is influenced by several factors which include chemical structure of pollutants, pH of soil, concentration of hydrogen peroxide, and concentration of iron. The rate of degradation differs as the

pathway of this process changes. Acceleration of degradation processes results in decontamination in short span of time. Thus, photocatalytic degradation, biodegradation, ozonation, and photo-Fenton reactions are commonly evaluated for pesticides removal studies [32]. Microorganisms are present on earth as an uncountable number of species. These microbes are very vital for the bioremediation of pesticides. The phenomenon of biotransformation is very common and sometimes very essential for the survival of microorganisms, responsible for biodegradation of applied pesticides. There is a natural balance in between microbial evolution and bioremediation [33]. Biodegradation can be approached via microbes and also augmenting this process by artificial means. This approach to environmental decontamination possesses a number of benefits; for example, there is minimum chance of environmental disruption, economical and fewer chances of secondary exposure alongside not causing damage to ecosystem [34, 35].

Biodegradation methodology is widely used for the treatment of xenobiotics such as pesticides in soil. It is employed in many countries due to its low cost and being ecofriendly [36]. Conventional approaches like land filling, recycling, and incineration are not very efficient and cost-effective. Different types of toxic intermediates are also formed during these processes [37]. Studies in pure cultures with the isolated microorganisms revealed that

There are four major reactions involved in OPs metabolism: hydrolysis, oxidation, alkylation and dealkylation. Hydrolysis of the phosphoesteric P–O–C or phosphothiesteric P–S–C bonds present in the OP molecules is considered the initial step in their metabolism.

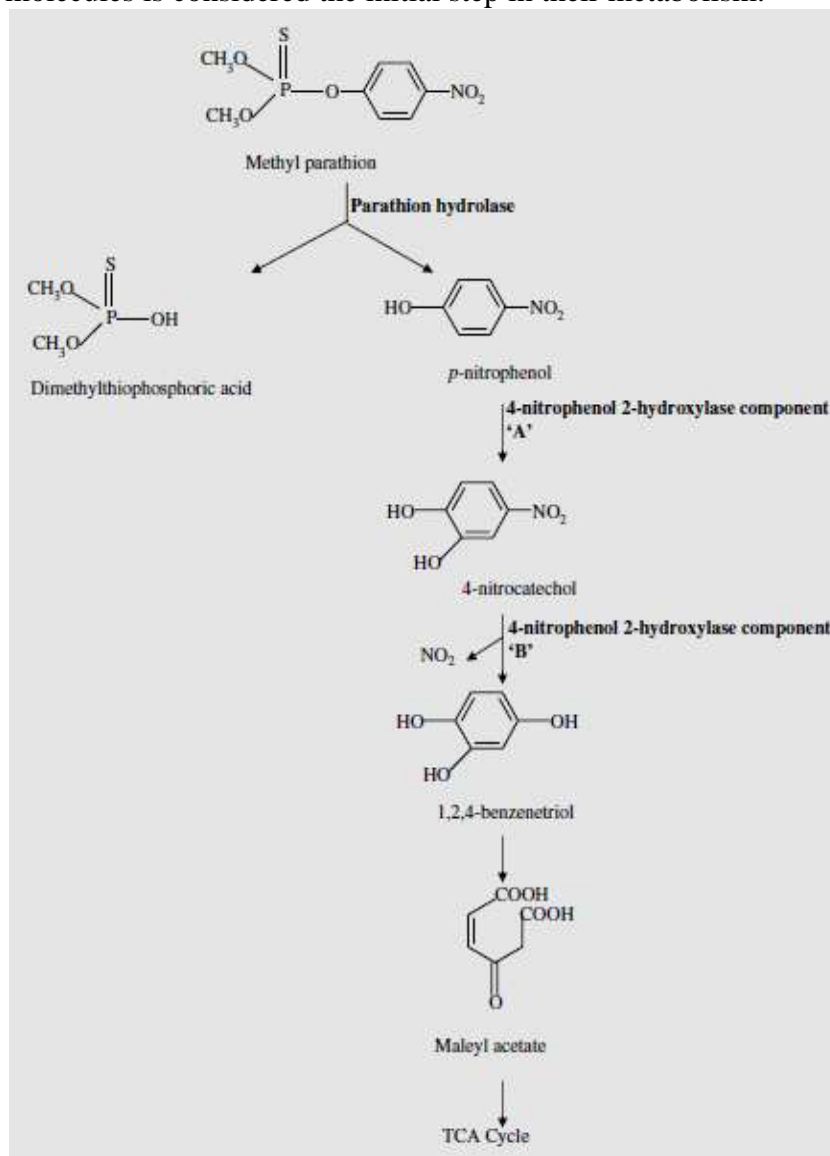


Figure 2. Degradation of methyl parathion by microorganism [38]

Pseudomonas sp. hydrolyses the pesticide to p-nitrophenol but required glucose or another carbon source for growth, unlike *Flavobacterium sp.*, which metabolizes p-nitrophenol by releasing nitrite and was used by the bacterium as a nitrogen source. *Bacillus sp.* hydrolyse methyl parathion in the presence of different concentrations of yeast extract. *Burkholderia cepacia* rapidly degrade methyl parathion and p-nitrophenol and utilize them as sole sources of carbon [39].

4.1. Enzyme involved in methyl parathion mineralization

Methyl parathion degrading bacteria possess a novel triesterase often referred to as methyl parathion hydrolase, encoded by the highly conserved organophosphate degradation (opd) gene, localized either on dissimilar indigenous plasmids or on the chromosome [40]. Methyl parathion hydrolase hydrolyses the characteristic triester bond found in methyl parathion [41]. P-Nitrophenol (PNP) is one of the major hydrolytic products generated when methyl parathion is subjected to microbial degradation [42].

4.2. Gene and Genetic Engineering

Methyl parathion hydrolase gene, *mpd*, which is responsible for hydrolyzing methyl parathion to p-nitrophenol and dimethyl phosphorothioate. The *mpd* gene encoding an organophosphate degrading protein was isolated from a methyl parathion (MP) degrading *Plesiomonas sp.* A stable, genetically engineered strain, JQL4-5-*mpd*, capable of simultaneously degrading methyl parathion was constructed by random insertion of the methyl parathion hydrolase gene (*mpd*) into the chromosome of strain JQL4-5. Soil treatment results indicated that JQL4-5-*mpd* is a promising GEM in the bioremediation of multiple pesticide contaminated environments [43].

The methyl parathion hydrolase gene (*mpd*) and enhanced green fluorescent protein gene (*egfp*) was successfully coexpressed using pETDuet vector in *Escherichia coli* BL21 (DE3). The coexpression of methyl parathion hydrolase (MPH) and enhanced green fluorescent protein (EGFP) were confirmed by determining MPH activity and fluorescence intensity. The recombinant protein MPH showed high enzymatic degradative activity of several widely used OP residues on vegetables. Subsequently, a dual-species consortium comprising engineered *E. coli* and a natural p-nitrophenol (PNP) degrader *Ochrobactrum sp.* strain LL-1 for complete mineralization of dimethyl OPs was studied. The dual-species consortium possesses the enormous potential to be utilized for complete mineralization of PNP substituted OPs in a laboratory-scale bioreactor. These studies demonstrated that MP could be degraded via the MP → PNP → hydroquinone → Krebs cycle by the dual species consortium. The data confirm that the mineralization process of MP is initiated by hydrolysis leading to the generation of PNP and dimethylthiophosphoric acid, and PNP degradation, then, proceeds through the formation of hydroquinone. The accumulation of PNP in suspended culture was prevented [44]. A bacterial consortium of seven (out of 64) Enterobacteriaceae isolates including *Citrobacter*, *Enterobacter* and *Proteus vulgaris* capable of degrading methyl parathion.

4.3. Biodegradation studies using immobilized bacterial cells

The immobilization of bacterial consortium was done in sol-gel by using two types of materials: TEOS (tetraethyl orthosilicate) and sodium silicate. Cell survival and preservation of methyl parathion hydrolase activity was studied. In addition, *E. coli* DH5 α cells transformed with pGFPuv (Clontech) were also immobilised in sol-gel and their ability to express GFP after immobilization was observed by fluorescence microscopy (Leica DM4000 M) as an indicator of preservation of biological activity [45].

The efficiency of removal of methyl parathion from agricultural soil was evaluated using mineral salt medium supplemented with glucose, and the bacterial consortium was cultivated as free cells and immobilized on *Luffa cylindrica* fibers. To improve the structure of the fibrous network and to achieve greater retention of microorganisms, removal was also tested prior to fibers treatment with sodium hydroxide (NaOH). The results indicate that the microorganisms used had better growth as free cells. A removal of 54.88 % for MP was observed using the free cells; but when the cells were immobilized on loofa sponge fibers, the removal was increased to 98 to 100 % of the pesticides. This

pesticide removal was the result of a combined effect among the activity of the microorganisms, the adhesion to the bacterial cells and the adsorption on the support material [46].

4.4. Degradation of methyl parathion: Case study

Experimental Setup

A completely randomized block was designed. Pots measuring 40 cm in length, top and bottom diameters of 40 cm and 15.2 cm, respectively, were filled with 3 kg of soil to which MP was added exogenously. For this, air-dried soil was sieved (2 mm) and aqueous solution of MP was added to the soil to form thick slurry. The final concentration of MP was 60 ppm. Cells of test strain were inoculated in the soil to obtain 1×10^5 cfu/g of soil. Simultaneously, the controls without the microbe were also maintained. The nitrogen and phosphorous sources were applied 1 week after the treatment of MP. The slurry was air dried at 40-45°C for 4 days, and the dried soil was pulverized before being used in the pots. These pots were then placed randomly in natural environmental condition. The moisture level in all the pots (control + experimental) was maintained throughout the period of an experiment by sprinkling tap water periodically whenever necessary. The pots were maintained at room temperature for 30 days [47].

Extraction

1 g soil was withdrawn at various time intervals (0, 5, 10, 15, 20, 25, 30 days). 1 g soil was suspended in 10 ml of 5% NaOH, vortexed for 10 min and centrifuged at 1500 rpm for 10 min. Supernatant was acidified to pH 2.0 with HCl and extracted with double the volume of ethyl acetate. The aqueous phase was extracted again with ethyl acetate; both the extracts were pooled together and passed through anhydrous sodium sulfate to remove the traces of water. The filtrate was dried at room temperature and the residue was finally dissolved in 1 ml of acetone and analyzed through GC-MS [47].

Results

A total 10 strains were isolated from the soil samples supplemented with MP. Out of these, six strains, namely, C1, C2, C3, C6, C8, and C10 which appeared in higher concentration were further screened for their ability to tolerate maximum concentration of MP. Thus, C1 (*Achromobacter xylosoxidans*) was most potent strain, finally selected for further evaluation. Different factors were optimized for maximum degradation of MP by the test strain (C1), such as inoculum concentration, temperature, pH, different carbon sources, different nitrogen sources, and incubation period. It was recorded that maximum degradation was recorded when cells of bacterium C1 were applied at 1×10^5 cfu/ml, at temperature 35°C, at 6.5 pH. It was recorded that utilization of MP was maximum when glucose was used as a carbon source and when NH_4Cl was used as a nitrogen source. It was seen that better degradation of MP was reported after 27 days of incubation. When pot studies were conducted it was reported that, the test bacterium was able to degrade the target pesticide up to 94.44% after 30 days of incubation [48].

4.6. New perspectives involving other approaches for bioremediation

Bioaugmentation of Soil Contaminated with methyl parathion

Bioaugmentation is the compelling method of engineered bioremediation, based on the inoculation of given environments (*e.g.* soil, activated sludge, sediments, water, *etc.*) with microorganisms characterised with desired catalytic capabilities. It is mainly recommended for sites where the number of autochthonous microorganisms that enable contaminants to be degraded is insufficient and/or those in which native populations do not have the catabolic pathways necessary to metabolise pollutants [49]. A successful bioaugmentation of soil contaminated with methyl parathion (MP) with *Pseudomonas* sp. strain WBC-3 was reported [50]. The disappearance of MP was almost complete in inoculated and non-inoculated soil (0.536 mg/g of soil); however, the degradation proceeded faster (13 days) in the bioaugmented soil than in the non-bioaugmented soil (20 days). In comparison, at the end of the experiment only 37.2% of MP was removed from sterile inoculated soil, thus indicating the significant role of indigenous microorganisms in the degradation of MP. Since the introduction of microorganisms into soil may affect the structure of microbial communities, they also assessed the response of autochthonous microorganisms to WBC-3

inoculation. They observed a dynamic shift (determined by DGGE) in the genetic biodiversity of the microbial community in the bioaugmented soil as compared to the control [51].

By improving the efficiency of methyl parathion hydrolase

A methyl parathion hydrolase (MPH) gene, *bjmpd*, was cloned from a newly isolated MP-degrading bacterial strain, *Burkholderia jiangsuensis* MP-1T and heterologously expressed in *Escherichia coli* BL21 (DE3) [52]. Although the amino acid sequence of the *bjmpd*-encoded enzyme, named *BjMPH*, differed from that of MPH from *Pseudomonas* sp. WBC-3 (*PsMPH*) in only three residues, Ser132, Val247 and Ala267, a significantly higher specific activity towards MP was exhibited by *BjMPH* than *PsMPH*. Among them, Ala267 was identified as a key site affecting the catalytic efficiency, and it was rather conservative (Ala or Ser) in homologous proteins, suggesting that a simple substitution of the residue in conservative site with another conservative residue based on the consensus sequence approach might possibly enhance the catalytic efficiency of the MP-degrading enzyme. Inspired by such an observation, a new mutant was identified, *BjMPH*_{T64N}, exhibiting 3.78-fold higher catalytic efficiency (kcat/KM) towards MP than its wild-type, reaching $4.20 \times 10^6 \text{M}^{-1}\text{s}^{-1}$. Homology-modelling analysis indicates that enhanced polar contacts of the 64th residue in this mutant may contribute to stabilizing the structure of the enzyme and promote the interactions between enzyme and substrate. This study generated an efficient MP-degrading enzyme, and provides useful information for enhancing the catalytic efficiency of MPHs via conservative residue substitution based on the consensus approach [53].

Biodegradation by Biosorption

A new isolate of genus *Scytonema* distinct from its closest relative *Scytonema hofmanni* was found efficient in removal and degradation of organophosphorus pesticide methyl parathion (MP). The cyanobacterial isolate was also capable to utilize the phosphorous present in the methyl parathion following its degradation which was evident from the increase in growth (chlorophyll content), biomass, protein content and total phosphorous in comparison to cyanobacterium grown in phosphate deficient cultures. The rapid removal of MP by the cyanobacterium during initial 6 hours of incubation was defined by the pseudo second order of biosorption kinetics model, which has indicated the involvement of chemisorption in initial removal of pesticide. Further degradation of MP was also confirmed by the appearance of p-nitrophenol in the medium after 24 hour of incubation. Thus the cyanobacterial isolate of *Scytonema* sp. BHUS-5 seems to be a potential bioremediation agent for the removal of organophosphorus pesticide methyl parathion from the habitat [54].

V. CONCLUSION

The indiscriminate use of pesticides, especially methyl parathion is generating problems related to environment and health. The microorganisms play an important role in the degradation of pesticide by utilizing them as a carbon source. Thus, bioremediation is emerging as beneficial tool to combat with pesticides. Other approaches for biodegradation of methyl parathion by microorganisms will play a significant role in its efficient degradation like applying molecular approach by increasing the efficiency of methyl parathion hydrolase gene. But still microbial degradation is limited to the lab-scale, and remains to be applied in the field practice. Therefore, there needs more research which is to be done for the degradation of pesticides practically in real field.

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