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Research Article

Degradation of Monochrotophos Insecticide using *Bacillus subtilis* Isolated from Gut of *Perionyx sps*

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Abstract

The earthworms are considered as soil engineers, which plays a major role in agriculture. The prolonged application of insecticides in soil alters the fertility of the soil and the population of earthworms. In spite the toxicity of insecticides, the earthworms survive by developing resistance, which might be the results of adaptation of bacterial community in the gut of earthworms. The present work relays on isolation of bacterial strains from earthworm gut and degradation of Monocrotophos insecticide at various concentrations. The bacterial strains were isolated from the gut of the *Perionyx species* and were found to degrade 90% and 75% of 50 ppm and 200 ppm of Monocrotophos. The optimum pH of 9.0 resulted in 95% of degradation and 90% of degradation was observed at 10°C. The metabolites produced on Monocrotophos degradation were analysed using FTIR, HPLC and GC-MS. The molecular characterization of the efficient insecticide-degrading bacterial strains was found to be *Bacillus subtilis* and *Bacillus nakamurai.* This study showed that the bacterial strains in the gut of earthworm were able to degrade a wide concentration of Monocrotophos insecticide.

Keywords: *Perionyx species*; Monocrotophos; *Bacillus subtilis* and *Bacillus nakamurai*; Biodegradation

Introduction

Earthworms comprises the majority of invertebrate soil fauna which plays an important role in maintaining soil fertility by producing humus. The digestive tract of earthworm contains diversity of microorganisms which are responsible for digestion of organic materials and for the survival of the earthworm. The earthworm distribution and the gut microfloral diversity varies according to the pH, moisture and organic content in the soil. The earthworms improve the phyisco-chemical properties of the soil, reduce the salinity and aids in enhancing the soil fertility [1]. In India, the major economy depends on agriculture. There was more than 30% of agricultural loss due to insects and pests. Hence the application of insecticide was used for improving the productivity of crops. The chemically synthesized insecticides have been introduced which resulted in an increase in insecticide pollution [2]. About 500 pesticide compounds have been reported worldwide in which India is the largest consumer among the South Asian countries. The insecticides are lethal to targeted pests and insects and also to non-targeted organisms. The insecticidal residues in soil and food cause drastic health effects in human-beings [3]. Organophosphorous (OP) insecticide are used exclusively in agriculture with 34% sales worldwide which inhibits Acetylcholinesterases Enzymes (AchE) [4]. OP insecticide is widely used in rice, tobacco, maize, sugarcane, cotton and other vegetables [5]. It is also considered as a major groundwater contaminant [6,7].

The toxic effects of the pesticides and insecticides used in the agriculture requires prior attention and detoxification. The effects caused due to insecticide contamination needs effective clean-up process and the technologies. This includes physical treatments, chemical treatments, photodegradation and biological degradation [8]. The physical and chemical methods reduce the toxicity of the pesticides, which also results in the accumulation the less toxic compounds in the soil and water. The effective process for the decontamination of these toxic pesticides were not yet understood clearly. Thus, Bioremediation is an effective process for the degradation or detoxification of the pesticides and its residues in the environment. Degradation of pesticides and insecticides using bacteria has its own advantages as they possess degradative mechanism for conversion of toxic compounds into less toxic compounds [4].

Earthworms are highly resistant to inorganic, organic and chemical contaminants in the soil and studies show that earthworms bioaccumulate these contaminants in their tissues or ingest the contaminated soils which alter the microbial diversity in its gut [9,10]. Earthworms are considered as waste eaters and better decomposers as it contains beneficial bacteria in their gut which acts as a biological stimulator for degradation [11]. The earthworm gut also contains several enzymes which also plays a critical role in degradation [12].

Monocrotophos (MCP) insecticide, has a great public concern due to its toxicity. Bacterial strains such as *Pseudomonas aeruginosa* [4], *Pseudomonas mendocina* [13], *Bacillus megaterium* [14] were reported in the MCP degradation. The microorganisms which can be used as an alternative electron acceptor and degrade synthetic organic compounds by an anaerobic process are still explored [15]. This present study focuses on the degradation of MCP insecticide by the bacterial strains isolated from the gut of the earthworm *Perionyx sps*. The isolated bacteria strains were screened for MCP degradation and the degradation conditions were optimized using various parameters. The degradation of MCP was analyzed using FT-IR, HPLC and GC-MS. The bacterial strains that were effective in MCP degradation were

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molecularly characterized and identified.

Materials and Methods

Isolation of bacteria from the earthworm gut

The worm bed was set in Stella Maris College Campus in a small concrete trough. The worm bed was prepared using soil, dry leaves, vegetable wastes (generated in college canteen), cocopeat etc. All these were layered one after the other, watered and kept undisturbed for about 2 weeks. Later the *Perionyx sps* was purchased from Manidharma Biotech Pvt.Ltd, and introduced into the worm bed with suitable conditions. The moisture level was maintained and the worm bed was turned over every 15 days. The mature earthworms with developed clitellum were selected for the bacterial isolation and degutted for removing the gut content. The earthworms were surface sterilized by swabbing gently with 70% ethanol. The region below the gizzard was dissected and homogenized in 2 ml of sterile distilled water and centrifuged to obtain the supernatant. The supernatant was then serial diluted and (10^{-5}, 10^{-6}, 10^{-7}) dilutions were plated on the nutrient agar. The plates were incubated at 37°C for 24 h [16].

Enrichment of isolated bacterial strains

The pure cultures were inoculated in Mineral Salts Medium (MSM) with agar having the following composition (MSM- g/l): Ammonium nitrate- 1.5, Dipotassium hydrogen phosphate- 1.5, Potassium dihydrogen phosphate- 0.5, Magnesium sulphate- 0.2, Sodium chloride- 0.5, Agar- 12, pH-7. The MSM medium was amended with 100 ppm of Monocrotophos and kept incubated at 37°C for 3 days. Then the pure isolates were inoculated in MSM broth for further degradation studies [14].

Screening of MCP degradation by plate assay

The isolated bacterial strains were preliminarily screened for degradation of MCP by determining the phosphatase and esterase enzyme activity. The enriched bacterial strains were screened for extracellular phosphatase production using Pikovskaya's agar as a screening medium. The pure cultures were streaked at the centre of the sterile PVK plates and the plates were incubated at 37°C for 24 hours. The observation was made to see the phosphate solubilization zone around the colony. The bacterial strains forming zone were used for further studies [17]. For the detection of esterase enzyme, the isolates were spot inoculated on Tributyrin agar plates and the incubated at 37°C for 24 hours [18].

Optimisation of Degradation parameters

The MSM broth containing 24 hours culture of bacterial strains were used for optimising the degradation conditions such as pH, temperature, MCP concentration, Carbon and Nitrogen sources. For pH optimisation, the pH of the MSM was altered to pH 5.0 and 6.0 using 1N Hcl and 1N NaOH was used to increase from 8.0 to 10. 0 pH and incubated at 37°C. The degradation temperature was optimised (10, 25 and 37°C) at neutral pH and the cultures are incubated for 10 days in 150 rpm [19]. The carbon (glucose, sucrose, maltose, fructose, lactose, and cellulose) and nitrogen sources (ammonium sulphate, peptone, urea, yeast extract and meat extract) of 0.3% was added to the MSM and incubated for interval for 10 days to monitor the enhancement of MCP degradation. The tolerance of the bacterial strains to various MCP concentrations were performed (100 ppm, 200 ppm, 300 ppm and 400 ppm) and the results were noted for 10 days with 24 hours interval [20,21].

Characterization of isolated bacterial strains

The MCP degrading efficient bacterial strain were observed under the Scanning Electron Microscope (FEI Quanta 200 F) in IIT Madras, Chennai. The sample preparation for Scanning Electron Microscopy (SEM) was carried out according to the method of Prior and Perkins [22]. The isolated bacterial strains were grown individually on MSM for 24 hours. The bacterial strains in the MSM were centrifuged at 8000 rpm for 10 minutes and the pellets were immediately re-suspended in 2% Glutaraldehyde with 0.05 M Phosphate buffer and 4% sucrose (pH-7.3). Fixation was obtained overnight at 4°C. After 24 hours the pellet was centrifuged at 8000 rpm for 10 minutes, washed 4 times with distilled water and placed on aluminium foil. The samples were then dehydrated with series of gradient ethanol (10%, 20%, 30% till 90%) air dried and finally, the dried flakes were coated with gold and observed.

The bacterial strains resulting in better degradation was molecularly characterized using 16S rRNA sequencing. The DNA was isolated from the MCP degrading bacterial strains using Shrimpex Microbial DNA Kit. The quantity was measured using NanoDrop Spectrophotometer and the quality was determined using 2% agarose gel. A single band of high-molecular-weight DNA has been observed. The microbial 16S ribosomal RNA (16SrRNA gene) region was amplified using universal primers by PCR from the above-isolated DNA. A single discrete PCR amplicon band of 800 bp was observed when resolved on Agarose Gel [23]. The phylogenetic tree was constructed in MEGA 7 using the nucleotide sequences of the bacterial strains.

Results

Screening of MCP degrading bacterial strains

The bacterial strains in the gut of *Perionyx sps,* were isolated in nutrient agar supplemented with 100 ppm of MCP as a sole carbon source. The plates were observed with eight morphologically different bacterial colonies, which was labelled as PKVG1, PKVG2, PKVG3, PKVG4, PKVG5, PKVG6, PKVG7 and PKVG8. The MCP degrading ability of the isolated bacterial strains were determined by screening the presence of Phosphatase and esterase enzyme activity. The bacterial strains PKVG1 and PKVG2 produced zone of inhibition of 1.5 cm and 0.8 cm in Pikovskaya's agar medium. A zone of clearance was observed in the plates containing Tributyrin with diameter 1.2 cm, 1 cm and 0.8 cm. This proves the ability of the bacterial strains PKVG1 and PKVG6 to degrade the MCP.

Optimisation of MCP degradation

The bacterial strain PKVG1 were able to survive and degrade MCP to maximum of pH 10.0. It was observed that pH 9.0 was found to be the optimum condition for the growth and degradation of MCP. The bacterial strains equally survived from pH 5.0 to pH 10.0 with 85%, 75%, 70%, 95% and 80% at 100 ppm of MCPs. At 10°C, the bacterial strain was able to degrade 90 % MCP efficiently. It was observed that the bacterial strains were able to degrade minimally at 25°C and 37°C. The bacterial strain was able to utilize glucose as a carbon source and the MCP was not degraded completely due to its complexity. The bacterial strain equally consumed fructose, sucrose, maltose, lactose and cellulose which aided in the degradation resulting in 90%, 80%,

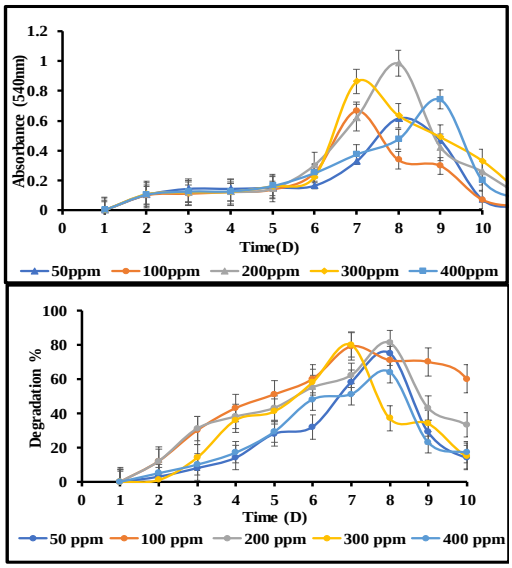

Figure 1: Optimisation of MCP Concentration - PKVG1 a) Growth b) degradation MCP.

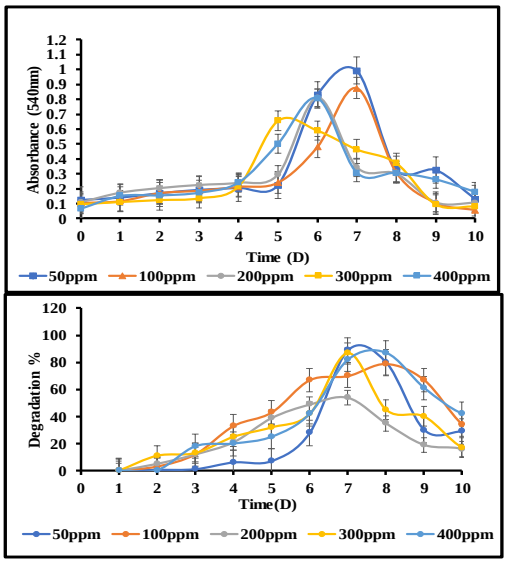

Figure 2: Optimisation of MCP Concentration – PKVG6 a) Growth b) degradation MCP.

80%, 75% and 65% of degradation. The ammonium sulphate was found to be better nitrogen source for the bacterial strain PKVG1 that resulted in 80% of degradation followed by meat extract, yeast extract, urea and peptone showing negligible changes in the degradation percentage.

The bacterial strain PKVG6 showed maximum degradation at pH 9 with 95% degradation of MCP. It resulted that the bacterial strain were able to survive equally at varied pH (pH 5.0 to pH 10.0) with 85%, 78%, 85%, 90% and 50%, which were able to survive and degrade MCP at 10 to 37°C. The optimum temperature was found to be 10°C resulting in 90% of degradation followed by 25°C and 37°C with 70% and 72% showing negligible changes in the percentage of

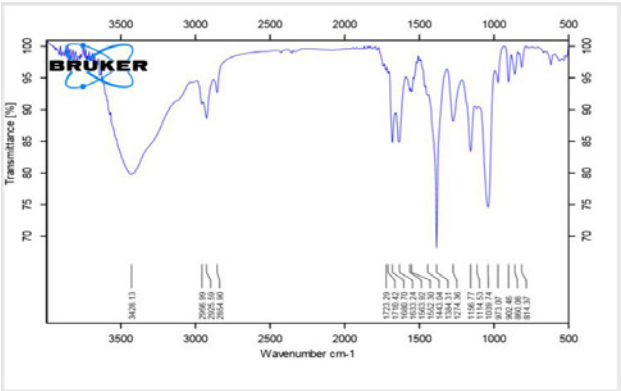

Figure 3: FT-IR Spectrum of MCP – Control.

degradation. The bacterial strain equally consumed sucrose, maltose, glucose and cellulose which aided in the degradation resulting in 85%, 78%, 60% and 80% of degradation. The Urea was found to be a better nitrogen source which resulted in 97% of degradation followed by peptone, yeast extract, meat extract and ammonium sulphate showing negligible changes in the degradation percentage. Thus, it shows that the bacterial strain utilized a minimum amount of urea compared to other nitrogen sources.

The bacterial strain PKVG1 were able to utilize MCP from 50 ppm to 300 ppm effectively but the growth was found to be reduced at 400 ppm. This shows that the growth of bacterial strains showed 200 ppm as optimum concentration resulting in 75% of degradation. This shows that the bacterial strains were able to tolerate and degrade up to 300 ppm (Figure 1a, b).

The bacterial strain PKVG6 utilized MCP at 50 ppm to 400 ppm. The degradation percentage was greater at 50 ppm but (Figure 2a) shows that the bacterial strain PKVG6 were able to utilize and degrade up to 400 ppm. (Figure 2b) explains that the bacterial strain utilized 50 ppm of MCP with 90% degradation. Thus the bacterial strain PKVG6 were able to effectively degrade minimum concentration of MCP but it was also able to survive and degrade at higher MCP ppm.

Analysis of MCP degradation

The FT-IR analysis of the non-degraded MCP showed O-H stretching at 3428 cm^{-1} and C-H stretching was observed at 2926 cm^{-1}. The peak 1681 cm^{-1} and 1633 cm^{-1} was assumed to be C=O stretching and C=C stretching which showed the presence of ketone group in the MCP. The C-H bending was observed at 1384 cm^{-1} and the C-O stretching was observed and it might be indicating the presence of alkyl aryl ether in the compound. The CO-O-CO stretching was observed at 1040 cm^{-1} which was assumed to be the presence of anhydride group as shown in (Figure 3). The FT-IR analysis of MCP degraded by PKVG6 showed O-H stretching at 3436 cm^{-1} and C-H stretching was observed at 2921cm^{-1}. The peak 1635 cm^{-1} and 1048 cm^{-1} showed the presence of alkene and primary alcohol group in the MCP. The C-H stretching was observed at 2921 cm^{-1} and the C-O stretching was observed and it might be indicating the presence of benzene derivative in the compound as shown in (Figure 4a). The FT-IR analysis of the MCP degraded by the bacterial strain PKVG1 showed O-H stretching at 3551 cm^{-1} and N-H stretching was observed

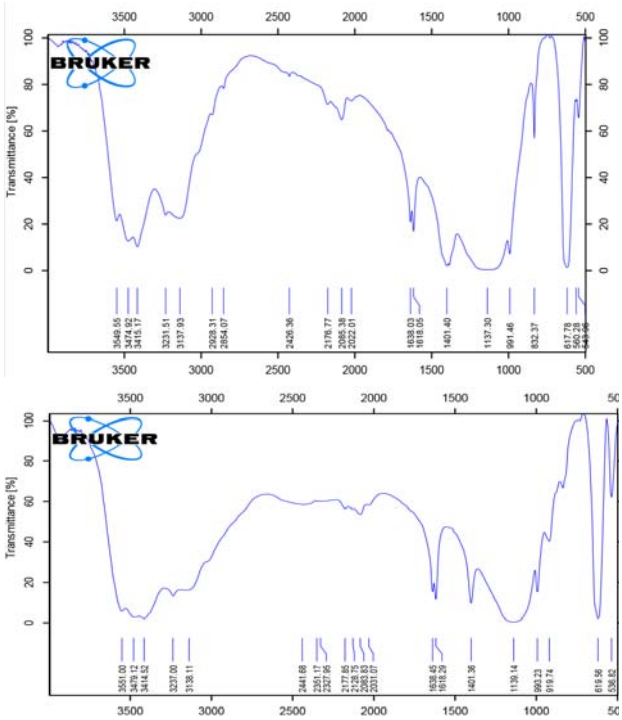

Figure 4: FT-IR Spectrum of degraded metabolites A) PKVG6 B) PKVG1.

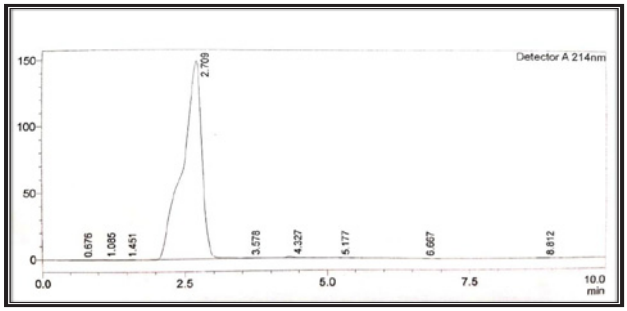

Figure 5: Non-degraded MCP (control).

at 3479 cm^{-1} and 3414 cm^{-1} was assumed to be aliphatic primary amine in the group. The C-H stretching was observed at 3138 cm^{-1} and C=C stretching was was observed at 1638 cm^{-1} and 1618 cm^{-1} which might be indicating the presence of α, β- unsaturated ketone group in the compound and C-F stretching was observed at 1401 cm^{-1} which was assumed to be the presence of fluoro compound and C-Br stretching was observed at 619 cm^{-1} which indicates the presence of halo compound as shown in (Figure 4b).

The HPLC chromatogram of non-degraded MCP showed a peak at a retention time (RT) of 2.709 (Figure 5). The MCP degraded by PKVG6 bacterial strain showed peaks at 3.725, 3.938, 4.837 and 6.307 RT as shown in (Figure 6a). Whereas the MCP degraded by PKVG1 bacterial strain showed peaks at 3.126, 4.353 RT and 3.725, 3.938, 4.837 RT as shown in (Figure 6b). This shows that the compound present in the standard at 2.709 RT was shifted to RT greater than 3.

The GC-MS spectrum (Figure 7) the metabolites formed by MCP degradation. The degradation of MCP by the bacterial strain

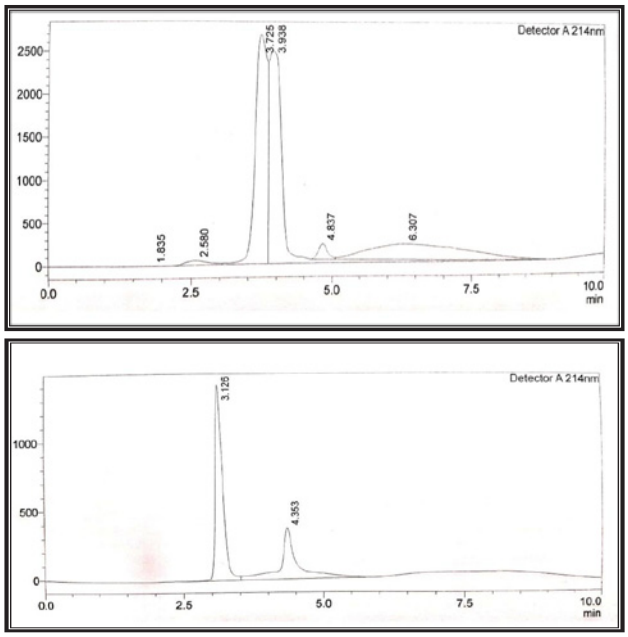

Figure 6: HPLC Chromatogram of metabolites A) PKVG1 B) PKVG6.

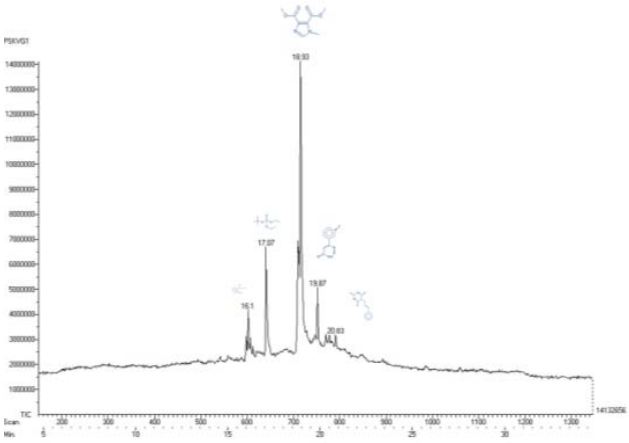

Figure 7: GC-MS Spectrum of metabolites produced by PKVG1.

sowed the formation of five major metabolites which was found to be 1-[2-hydroxyphenyl]-1- Butanone at 16.1 RT, 1, 1-dimethyl ethyl diethyl ester phosphoric acid at 17.1 RT, 1-methyl-dimethyl ester imidazole-4, 5-dicarboxylic acid at 18.93 RT, 6-oxo-4-[3-flurophenyl]-1, 6-dihydro pyrimidine at 19.87 RT and hexahydro-4-[3-phenyl-2-propenyl]-2-thioxo-, pyrimidine-4, 6-dione at RT 20.83.

Characterization of bacterial strains

The bacterial strains were sequenced and the DNA observed in the agarose gel. The bacterial strain PKVG1 was determined to be *Bacillus subtilis* and PKVG6 to be *Bacillus nakamurai*, which showed maximum similarities when blasted using Blastn. The bacterial strain PKVG1 was observed using Scanning Electron Microscope (SEM). The SEM observations correlate with the gram's staining which showed gram-positive *bacilli*. The length of the bacterium ranges from 1.43μm to 1.80μm and width ranges from 490μm to 620μm in 15,000 X and 40,000 X.

Discussion

Organophosphorus insecticides are widely used in India against pests. Excessive levels of organophosphorus insecticides can result in deterioration of soil quality, reduction of crop yield and inferior agricultural products. Herbicides and insecticides cause serious damage to life present in water, lack access to safe drinking water excess use of fertilizers. Among the insecticides, 98% were classified as acutely toxic for fishes and crustaceans. Effect of the contamination may result in the loss of biodiversity and functioning of soil like nutrient cycling, weathering, filtering etc. heavy metals also inhibit microbial activity. Microbial degradation depends not only on the presence of microbes with the appropriate degradative enzymes but also on a wide range of the environmental parameter. Microorganisms have the ability to interact, both chemically and physically, with substances leading to structural changes or complete degradation of the target molecule. The promising technology, which utilizes the ability of microorganisms to remove pollution from the environment eco-friendly, economical and versatile in the environment. The present study focuses on the degradation of Monocrotophos by the bacterial strains isolated from *Perionyx species*. The optimisation of carbon and nitrogen sources were analyzed. The effect of pH, temperature and concentration of MCPs in the degradation process were also studied.

The degradation of organophosphorus insecticides by microorganisms isolated from various sites like cotton fields [21], paddy fields in Tiruvallur district [24] and from many agricultural lands. The present work focuses on the isolation of organophosphorus degrading bacterial strains from earthworm as it is an indirect target of insecticides application. Eight potent bacterial strains were selected for degradation studies. The bacterial metabolism cleaves the complex ring structure of insecticide and is majorly carried by the enzymes produced by the bacteria. The enzymes possessing minimal substrate specificity can degrade a wide range of insecticides [25]. The production of phosphatase and esterase enzyme was screened as it sets the preliminary step for the MCPs degradation for cleaving into smaller compounds. The bacterial strains PKVG1 *(Bacillus subtilis*) and PKVG6 *(Bacillus nakamurai*) were found to be efficient producers of phosphatase and esterase enzymes. The bacterium *Bacillus subtilis* and *Bacillus nakamurai* resulted in 95% and 95% MCPs degradation at pH 10.0 and 9.0. The temperature had a similar effect on the degradation of MCPs by *Bacillus subtilis* and *Bacillus nakamurai* at 10°C which resulted in 90% of MCPs degradation. The tolerability of the bacterium to different MCPs concentration was used to optimise the insecticide concentration. The *Bacillus subtilis* was found to degrade 200 ppm of MCPs (70%) and 50 ppm of MCPs (90%) by *Bacillus nakamurai.*

The degradation of MCP by *Bacillus subtilis* was determined by GC – MS as the metabolites formed are 1-[2-hydroxyphenyl]-1-Butanone, 1,1-dimethyl ethyl diethyl ester phosphoric acid, 1-methyl-dimethyl ester imidazole-4,5-dicarboxylic acid, 6-oxo-4-[3-flurophenyl]-1,6-dihydro pyrimidine and hexahydro-4-[3-phenyl-2-propenyl]-2-thioxo-,pyrimidine-4,6-dione. The degradation of MCPs by *Bacillus subtilis* was efficient compared to *Bacillus nakamurai*. The bacterial strains in earthworm resulted in effective biodegradability of MCPs and this could be correlated with the resistance of earthworms to MCPs. This study can be extrapolated by determining the toxicity of MCPs on *Perionyx* species and its effect in alteration of the microbiome in the earthworm. This perspective will lead to determining the role of bacterial strains in the survival of earthworms under toxic conditions.

Conclusion

The Monocrotophos insecticide is moderately toxic and widely used in agriculture. The continuous usage of MCP makes the soil unfertile as the residues of the monocrotophos are non-degradable. The degradation of MCP by biological means might help which produces metabolites that are less toxic. The MCP applied to the agricultural fields has a great impact on earthworms. The gut of earthworm is a well-known habitat for bacteria as it aids in the breakdown of organic compounds. Prolonged exposure to insecticides makes these bacteria resistant. Thus, the degradation of MCP by bacterial strains isolated from the earthworm gut is relevant to study the degradation. The future studies focuses on the importance of bacteria in the survival of earthworm in the insecticide-contaminated sites which will have a great impact on agriculture.

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