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An environmentally sustainable strategy for the efficient biodegradation of profenofos and plant growth enhancement by indigenous soil bacterium *Bacillus substilis*

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Abstract

In modern agriculture, the widespread use of organophosphorus pesticides results in environmental pollution and pose severe threats to human, flora and fauna. Hence, the efficient removal of these pesticides from polluted environment remains an enduring inspiration. Bioremediation can be used as a proficient detoxifying strategy for polluted ecosystem since it can breakdown or convert toxic pollutants into less hazardous by-products while also being cost-effective, extremely efficient, and environmental sustainable. Further, a detailed analysis of the plant growth-promoting activity of plant–bacteria relationships could be used to promote sustainable agricultural output as well as the remediation of pesticide-polluted soil and water. Hence, a bacterial strain PDB1 was extracted from the soil sample exposed to pesticide for an extended period of time at Karur district, Tamilnadu, India for the bioremediation. Using morphological, biochemical, and 16S rRNA gene analysis, the bacterium PDB1 was characterized as *Bacillus substilis*. The impact of *Bacillus substilis* on several plant - growth indices was studied in the presence and absence of the pesticide profenofos. The capacity of *Bacillus substilis* to degrade profenofos (PF) was examined using gas chromatography-mass spectroscopy (GC-MS). Since this isolated bacterial strain has (PF) degrading capacity as well as other characteristics that promote plant growth, the isolate could be an attractive opportunity for the advancement of bioremediation technique.

Keywords: bioremediation; 4-bromo-2-chlorophenol; degradation; plant growth parameters; profenofos; soil bacteria

Introduction

Modern agriculture has relied on the constant administration of agrochemicals predominantly pesticides to increase crop productivity and food security [1]. Despite the fact that pesticides play a crucial function in contemporary agriculture, their prolonged use causes severe environmental problems [2]. Continuous pesticide exposure causes immunological issues, immunodeficiency syndromes, and cancer-related problems. The excessive use of pesticides pollutes the soil and water. Furthermore, it has a negative impact on the genetic variation of soil microbiota, accompanying in deteriorated soil fertility and plant development, which together jeopardize the long-term productivity of agricultural field. To complicate the issue further, pesticide residues and their by-products frequently penetrate through the surface soil into groundwater, provoking widespread pollution of aquatic environments [3]. Organophosphates are by far the most frequently used insecticides, accounting for 34% of global pesticides consumption. They are known to inhibit cholinesterase neurotransmitters irreversibly, causing disruption of endocrine activities, nervous system failure, and defects in childbirth, infertility, distortion of growth, development, and reproduction in fauna, birds, and mankind [4]. PF [O-(4-Bromo-2-chlorophenyl) O-ethyl S-propyl(S)-phosphorothioate)] is a highly active organophosphate insecticide that is commonly used on crops, vegetables and fruits. The molecular structure of PF comprises phenolic and phosphate ester components (Fig. 1). It is one among the most widely used pesticides because of its easy fabrication, cost-effectiveness and high efficiency. PF has been categorised as moderately dangerous pesticide of toxicity class II by the World Health Organization (WHO), and it has been shown to inhibit acetylcholinesterase. PF is genotoxic to off-target organisms, including terrestrial and aquatic creatures. PF is widely used in agriculture in many countries across the world. It has been widely used to manage and control numerous lepidopterous insects, aphids, whiteflies, spider mites, and hoppers on a variety of crops including tomato, potato, corn, sugarcane, cotton, tobacco, and vegetables. The residual levels of PF have been measured in a variety of plants and food samples. Alarmingly, these organophosphate pesticide residues have been identified in human blood, breast milk, plasma, serum and urine after exposure [5].

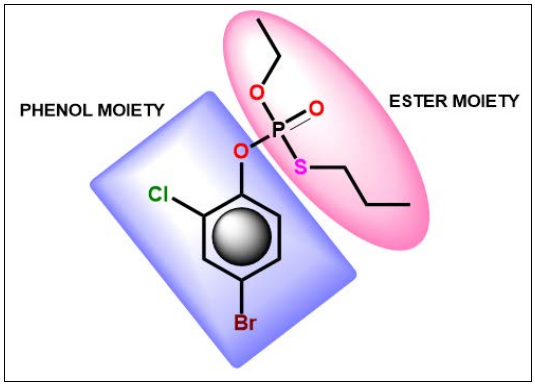

Fig 1: Chemical Structure of Profenofos

To address the ever-increasing demand and over consumption of synthetic pesticides while taking into account their long-term negative impacts on global agro-ecosystems and living organisms, we desperately need to develop, adopt, and promote environmentally sustainable approaches [6]. Traditional methods for removing and/or degrading organophosphorus pesticides, such as chemical treatment, combustion, and landfills, were later shown to be troublesome due to the possibility of secondary exposure. Furthermore, such procedures are prohibitively expensive. As a result, sustainable agriculture is critical in this period since it has the capabilities to fulfil our future agricultural demands [7]. Hence, bio-remediation, bio-mineralization, bio-protection, bio-stimulation have been considered as safe, convenient, environmentally friendly, and economically viable methods for the decontamination of organophosphorus pesticides [8].

Bioremediation is a technique which uses the potential of microbial degradation to provide a cost-effective and dependable way of pesticide diminution. Numerous soil and aqueous atmospheres have effectively recovered by utilising bacteria capable of the degrading the contaminants. PF is removed through hydrolysis, either chemically or through microbial activity, by transforming it to diethylthiophosphoric acid and 4-bromo-2-chlorophenol [5]. Malghani *et al*. described the isolation and characterization of bacterial strains capable of PF decomposition [9]. Salunkhe *et al.* extracted *Bacillus subtilis* from grapevines and examined its ability to degrade PF [10]. Jabeen and colleagues used the surface response technique to analyse the degradation capacity of a bacterial consortium [11]. The research team of Siripattanakul-Ratpukdi explored PF biodegradation by bacterial consortium [12]. Verma and Chatterjee briefly demonstrated a molecular mechanism of PF biodegradation [13].

Plant-assisted bioremediation has a lot of potential for cleaning up of pesticide contaminated soil. Pesticide degrading bacteria may improve plant tolerance to pollutants by detoxifying polluted soils by direct mineralization of contaminants. Furthermore, plant exudates increase the density and efficiency of novel bacteria in the root zone. The ability of microorganisms to detoxify pollutants while also increasing plant development has previously been explored for various organophosphorus insecticides such as malathion, methyl parathion chlorpyrifos and monochrotofos [14]. Despite, the significance of plant–bacteria partnerships towards the remediation of various pollutants has been demonstrated in various studies, research on PF degradation using this strategy is currently limited.

Akbar and Sultan demonstrated that the chlorpyrifos-degrading bacteria *Achromobacter xylosoxidans* and *Ochrobactrum sp*. have the potential to become excellent options for increasing crop productivity in pesticide-contaminated soils [15]. Nivedita and Sundari examined the impact of direct inoculation of specified consortia on plants in the presence of the root disease *Sclerotium rolfsii* and the organophosphate insecticides malathion and methyl parathion [16]. D. M. Dash and J. W. Osborne investigated the bioremediation of monocrotophos by native isolates obtained from cultivable sugarcane soil. The inclusion of *Bacillus aryabhattai* to the rhizosphere of *Liriope muscari* improved plant growth and monocrotophos degradation in soil [17]. Meng Di *et al.* presented the entire genome sequence of *Bacillus amyloliquefaciens* as well as its capacity to breakdown a variety of organophosphorus insecticides [18]. Fiaz Ahmad's research group portrayed the ability of inoculated external bacteria *Bacillus pumilus* to accelerate the cleanup of chlorpyrifos-contaminated soil and reduce levels of harmful pesticide residues in agricultural crops [19]. Govarthanan *et al*. explored the potential for speedy biodegradation of chlopyrifos and plant growth promoting psychrophilic *Shewanella sp*. cultures were obtained from salt water using the enrichment technique [20]. Ghani *et al.* examined the efficacy of a bacterial strain, *Enterobacter cloacae*, competent of promoting plant growth and biodegrading PF [21]. Jaoti examined the potency of *rhizobacteria* towards the biodegradation of PF and analysed the progress of growth of the plants [22]. Vinay Kumar *et al.* assessed the plant development and PF elimination efficacy of *Acinetobacter* and *Comamonas sp.* bacteria as separate strains and in combination and found that the consortium has greater plant growth-promoting properties than the individual bacterium [23].

Inspired by the facts and results, we attempted to isolate and identify a novel bacterial strain from a pesticide-contaminated soil and succeeded. Further, the metabolites derived from PF degradation was identified by using GC-MS and probable mechanism has been proposed. Furthermore, the isolated bacteria was tested for its ability to promote plant development.

Materials and Methods

1. Sample collection

Technical grade profenofos (99 % purity) was obtained from the Scientific Fertilizer Co Pvt. Ltd, Pesticide Division, Gundur, Tiruchirappalli, Tamil Nadu, India. Profenofos-degrading microorganism has been isolated from agricultural soils from Karur district, Tamil Nadu, India.

2. Medium for isolation

Mineral salt media MSM (in g/ L) (KH_2PO_4 – 3; NaCl – 0.5; Na_2SO_4 – 5.8; NH_4Cl – 1; $MgSO_4.7H_2O$ – 0.2) and nutrient broth were purchased from HIMEDIA, India.

3. Isolation of bacterium by Enrichment Method

The bacterial strain was isolated sing an enrichment method as reported earlier [24]. 5 g of soil from the agricultural field is mixed with 100 mL soil enrichment MSM medium supplemented with PF (25 mg/L) concentration. For a week, these enriched cultures have been incubated at 28 ºC with shaking (150 rpm/min). For four cycles, enrichment culture (5 mL) was subcultured into 100 mL fresh enrichment medium comprising PF (25 to 100 mg/L). Pure cultures were developed by proper serial dilutions of the enrichment culture in MSM medium and plating them onto MSM plates comprising 100 mg/L PF. The colonies were inoculated into MSM medium containing 100 mg/ L of PF and pure colony was selected for further investigation. The morphological features of the isolated strain were analysed.

4. Genomic DNA isolation and sequencing of 16SrRNA gene

The genomic DNA of the isolated bacterial strain was extracted using the standard chloroform–isoamyl alcohol extraction method as per our previous report [24]. To identify the strain, partial gene sequencing of 16S rRNA was performed and confirmed using biochemical properties. The

16S rRNA gene was amplified by PCR and sequenced as previously described. BLAST was used to search for sequence similarities in the NCBI Gene Bank. (http://www.ncbi.nlm. nih.gov). Biochemical and molecular characterization of the isolates were performed. For PCR amplification of the 16S rRNA gene, the following primers were used: 27 F(5'- AGAGTTTGATCCTGGCTCAG-3'), and 1492R (5' GGTTACCTTGTTACGACTT-3'). The purified PCR products of 16S rRNA gene were sequenced (Agrigenome labs Pvt Ltd, Kochi).

5. Biodegradation studies

5.1. Plant growth enhancement in *Vigna mungo* by pot culture method

Pot experiments with *Vigna mungo* (L.) Hepper were performed in order to analyse the effects of bacterial inoculation on plant growth parameters and pesticide degradation. Seeds of *V. mungo* were surface sterilized by treatment with 0.1% $HgCl_2$ solution for 5 min followed by washing with sterilized distilled water. Soil samples (1.0 kg) were spiked with PF to a concentration of 100 mg /kg. Samples were then inoculated with microbial suspension to give final concentration of 1.6×10^7cells /g. The test was performed in triplicate and sterilized soil sample was used as control. Sterilized seeds of *V.mungo* were sown in the sample soils and then the soil was moistened with water. Three experimental sets of pots with at least six seedlings were grown under the following conditions: (A) normal growth condition: Sterilized soil as control without bacteria and PF; (B) Under pesticide load: Sterilized soil with PF and without bacteria, (C) Under pesticide with isolated strain: Sterilized soil with isolated strain and profenofos. The pots were carefully monitored to ensure that they have been maintained at ambient environmental conditions. The seed germination process was monitored on a regular basis, and plants were allowed to develop for 3 weeks. The following parameters of plant development were documented: % germination, shoot length (cm), root length (cm), leaf length (cm), shoot fresh weight (g), root fresh weight (g), shoot dry weight (g) and root dry weight.

5.2. PF degradation analysis by GC-MS

PF degradation was determined after 3 weeks. For the GC-MS analysis, 5 g soil sample was collected from the pots B and C. 2 mL of aliquots were extracted after 3 weeks and analysed gas chromatography-mass spectroscopy (GC-MS) analysis. For the detection of intermediate metabolites, GC-MS system (GCMS-QP 2010 Shimadzu) provided with an auto-sampler and a mass spectrometer detector was used. For compound separation, a Phenomenex ZB 5MS column was used. Helium was used as the carrier gas, with a flow rate of 1 mL /min. The temperature system listed below was used: The temperature of the oven was initially held at 60 ºC for 1 minute before ramping from 10 ºC per minute to 290 ºC with a 20 min hold time; total run time was 45 minutes. The injector's temperature was kept at 250 ºC. The ion trap was set to 70 eV and had a scan range of 40 to 800 m/z. In split mode, 1 L of each sample was injected (10:1). Metabolite identification was accomplished by comparing the results to standard compound mass fragmentation patterns as well as instrumental library searches [24].

5.3. Statistical analysis

All the tests were conducted in triplicates and statistical analysis was performed to analyze significant differences. Statistical analysis was performed using a one-way analysis of variance (ANOVA) for % germination, shoot length (cm), root length (cm), leaf length (cm), shoot fresh weight (g), root fresh weight (g), shoot dry weight (g) and root dry weight with multiple comparisons. Statistical analysis for plant growth parameters was performed with one-way ANOVA where significant differences at $p \leq 0.05$ levels with multiple comparisons.

Results and Discussion

1. Isolation, identification and characterization of PDB1

By successive sub-culturing of soil samples, PDB 1 was isolated using an enrichment method. A single bacterial strain PDB 1 was isolated from the soil samples from Karur district of Tamil Nadu, India under prolonged exposure of pesticide applications. The morphological analysis, biochemical characteristics and 16S rRNA gene sequencing analysis of the bacterium PDB1 confirmed a high degree of similarity with *Bacillus substilis* strain [24].

2. Growth experiment of *Vigna mungo* (L.) Hepper

Plant growth studies were used to investigate the effect of bacterial activity on plant growth and PF pesticide breakdown. In pot soil experiments, % germination, shoot length (cm), root length (cm), leaf length (cm), shoot fresh weight (g), root fresh weight (g), shoot dry weight (g) and root dry weight were determined. The results are given in Table 1 and Fig. 2. PF addition to soil (B) affected a reduction in all parameters analysed as compared to the plants grown without PF exposure (A). However, when compared to pot B, the plants in pot C exhibited comparatively higher plant growth characteristics. This may be due to the presence of *Bacillus substilis* which utilised the PF pesticide as only carbon source thus causing the degradation of PF.

Table 1: Measurement of growth parameters of *V. mungo* in pots A, B and C

Growth parameters	Control	PF +*V. mungo*	PFF + *V. mungo* +PDB1
% germination	90.33 ± 0.88^a	66.33±0.66^a	75.00±1.15^a
Shoot length (cm)	4.64±0.15^c	3.13±0.03^c	4.16±0.13^c
Root length (cm)	7.43±0.37^b	5.03±0.21^b	6.06±0.16^b
Leaf length (cm)	3.63±0.12^c	2.5±0.17^c	3.11±0.10^c
Shoot fresh weight (g)	1.83±0.03^d	1.13±0.04^d	1.50±0.04^d
Root fresh weight (g	0.80±0.03^{de}	0.56±0.01^{de}	0.63±0.02^e
Shoot dry weight (g)	0.62±0.02^e	0.25±0.01^e	0.57±0.01^e
Root dry weight (g)	0.25±0.01^e	0.06±0.00^e	0.12±0.02^e

The values indicate the mean ± SD of three replicates.
Different letters in same rows indicate significantly different values.

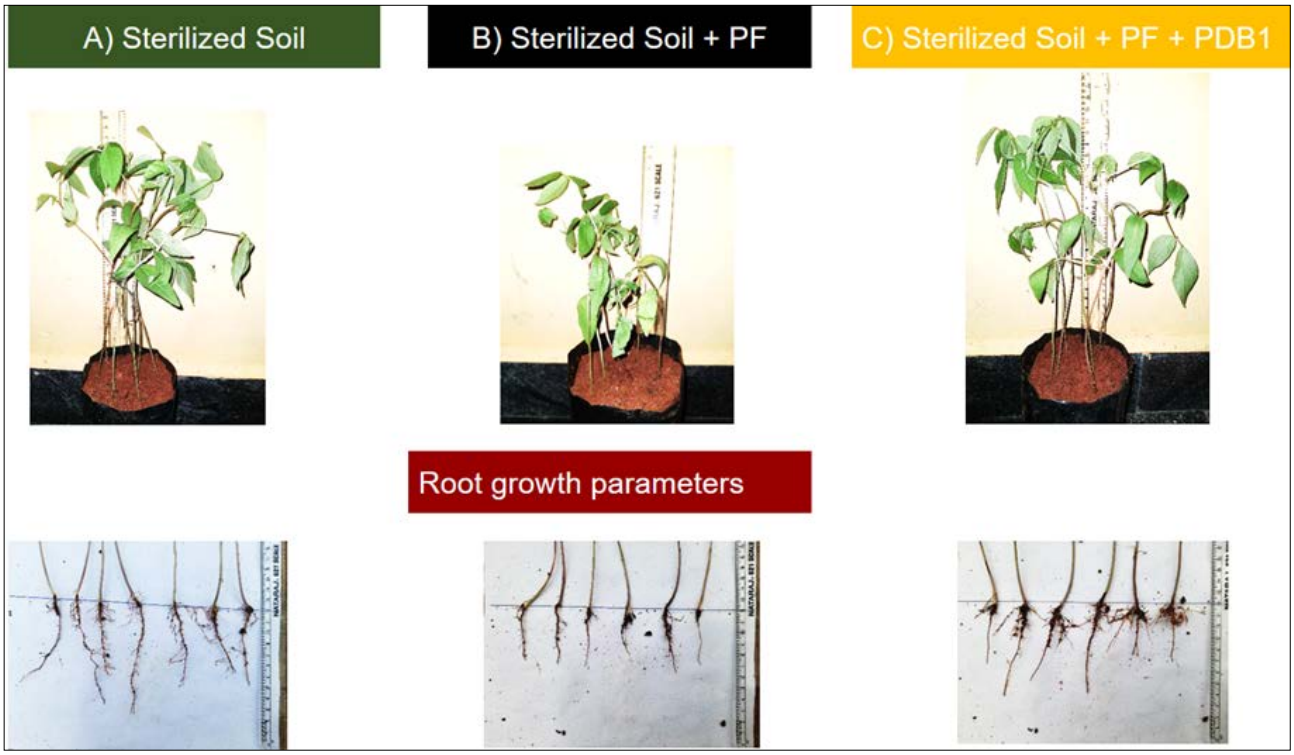

Fig 2: Growth experiment with *V. mungo* in soil supplemented with PF (100 mg /kg).

3. Biodegradation of profenofos

The ability of *Bacillus substilis* to degrade PF in soil environment was studied using GC-MS. The GC-MS was performed for sterilized soil with PF (B) along with sterilized soil with PF and PDB1 (C). The results of GC-MS are given in Fig. 3 and Fig.4. Fig. 3 displayed a single peak of 20.005 at 19.6 min retention time. The peak is attributed to PF pesticide. Fig. 4 exhibits the GC-MS result of pot C which contain sterilized soil with PF and PDB1. In contrast to Fig.3, The spectrum showed multiple peaks due to the biodegradation of PF. Specifically, the emergence of two peaks corresponding to the metabolites of PF namely, 4-bromo-2-cholorophenol (peak of 5.978 at 6.3 min retention time) and diethyl thiophosphoric acid (peak of 13.067 at 13.2 min retention time) confirmed the *Bacillus substilis* mediated biodegradation of PF [5].

Figure 5 depicts a probable mechanistic model of PF biodegradation. Nonetheless, more studies are needed to determine the entire breakdown route and intermediate mineralization.

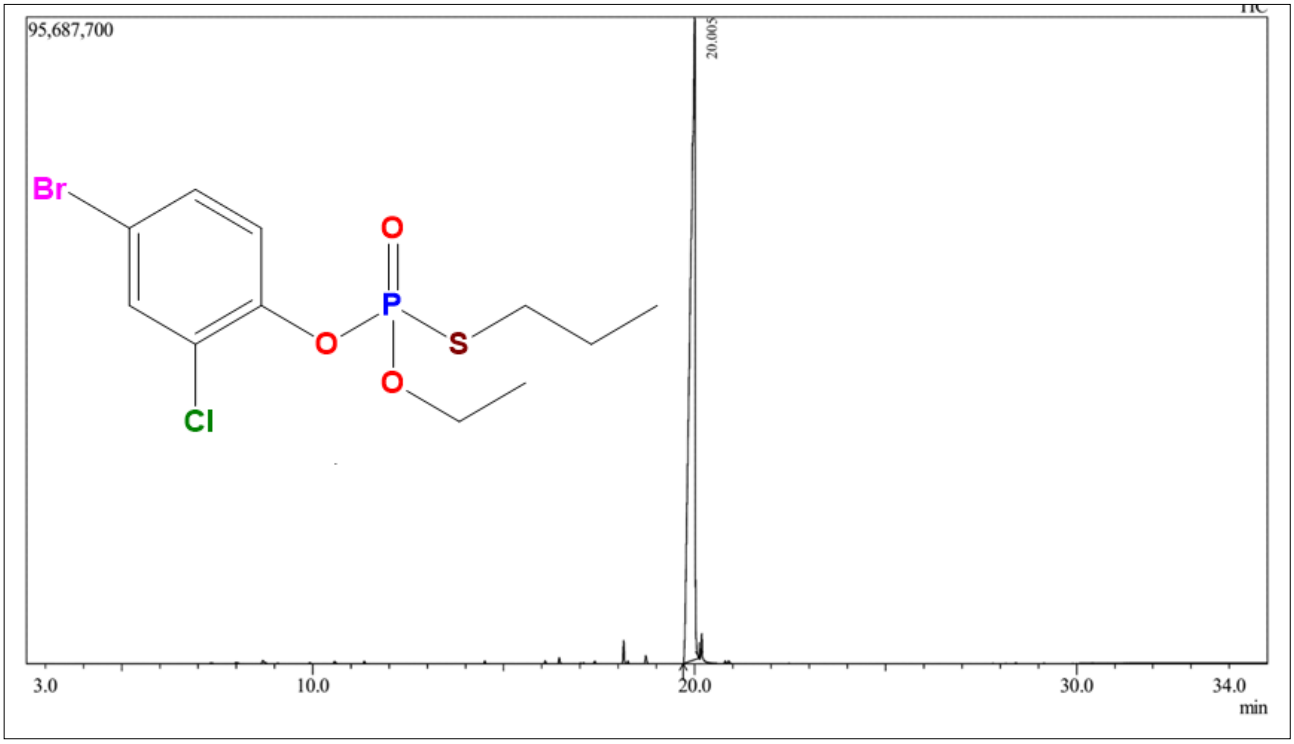

Fig 3: GC-MS result of sterilized soil with PF

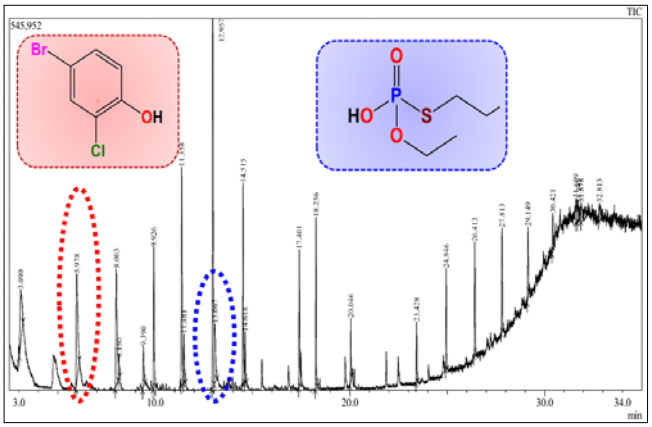

Fig 4: GC-MS result of sterilized soil with PF and PDB1

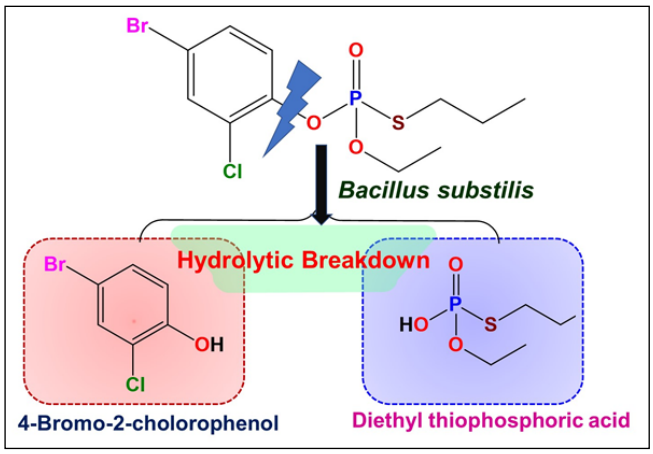

Fig 5: Plausible mechanistic representation of profenofos biodegradation

Conclusion

Plant-bacteria relationships can be used to improve pesticide-contaminated soil remediation. A bacterial strain was isolated using the enrichment methodology for the efficient remediation of PF-contaminated soil. *Bacillus substilis* was identified using morphological, biochemical, and 16S rRNA gene studies on the bacterium PDB1. *Bacillus substilis* was tested in the presence and absence of the pesticide profenofos on many plant, *V. mungo* growth variables, including percent germination, shoot length, root length, leaf length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight. According to the tested criteria, the pot soil reinforced with profenofos and *Bacillus substilis* exhibited comparatively better growth of *V. mungo* than the pot soil containing simply profenofos. The hydrolytic breakdown of PF into 4-bromo-2-chlorophenol and diethylthiophosphoric acid metabolites was demonstrated in mechanistic studies of PF degradation using GC-MS. This isolate, *Bacillus substilis* has the potential to be a key contender for the advancement of bioremediation approach due to its high biodegradation and plant growth stimulating potential.

Conflict of the interest

The authors declare no competing financial interest.

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