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Investigation and Application of a Novel *Pseudomonas aeruginosa* (KIBGE-AB9) for the Biodegradation of Malathion in Agriculture Soil

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Abstract

Malathion, an organophosphate insecticide despite its significant benefits in agricultural productivity, is an important pollutant and food contaminant which affects the non-target species. This research aimed at finding biological control strategies to degrade malathion in the agriculture soil. For this purpose, soil samples were collected from different agricultural areas known for malathion application continuously from the past five years. The isolated bacterial communities were subjected to identification, characterization and malathion degrading genes. Growth kinetics of bacterial strains at different environmental conditions (pH, temperature and malathion concentration) were performed. An isolate identified as *Pseudomonas aeruginosa*, based on morphological, biochemical and 16S rRNA gene sequencing analysis, showed ability to successfully degrade malathion as assessed by semi-quantitative pesticide hydrolysis test. Optimal degradation activity was observed at pH 7.0, soil temperature 37°C and malathion contents of 100 $mg.kg^{-1}$. The isolated strains of *P. aeruginosa* were found to possess organophosphate hydrolase encoding gene (*ophB*), suggesting that bacterial strain uses malathion as carbon source. Bioremediation of malathion by *P. aeruginosa* could be a promising approach against pesticide contamination in agriculture soil.

Keywords: Agriculture; Bioremediation; Environmental Safety; Malathion; *Pseudomonas aeruginosa*

Introduction

To meet the food requirements of the increasing human population, multidimensional efforts are being made to enhance agriculture productivity. The use of pesticides to protect crops and grains from insect is widely practiced. At present, four million tons of pesticides are being used annually on crops globally and its consumption exceeded to 5.5 million tons in 2021 particularly (Sharma *et al*. 2019). The indiscriminate application of chemical pesticides results in their accumulation in the environment and pose human and animal lives at risk through contaminated drinking water and food (Damalas and Eleftherohorinos 2011).

Malathion, an organophosphate insecticide is widely used in agriculture industry, with more than 13,500 tons applied in the United States annually (Geed *et al*. 2016). It is a highly toxic compound and is reported by the United States Environmental Protection Agency (USEPA) in category 2A. High-level exposure to this chemical affects nerve fiber and is neurotoxic in animals and higher-level vertebrates. In addition, malathion interacts with other toxic compounds in the presence of sunlight in the atmosphere to create malaoxone, a 40 times more toxic compound as compared to other pesticides (Jiang *et al.* 2019).

There are several methods to remediate malathion and other pesticides such as Fenton oxidation, electrochemical oxidation, chemical oxidation, solvent extraction and adsorption (Guivarch *et al.* 2003; Ajiboye *et al*. 2020). These remediation techniques have their own drawbacks such as toxic metabolite formation, sludge production, high cost and limited applicability (Burgos-Castillo *et al*. 2018). Among the others, microbial detoxification of malathion, due to its low-cost operations and low investment is a promising and environmentally friendly approach (Sidhu *et al*. 2019; Kumar and Sachan 2021; Pant *et al*. 2021).

Management and enhancement of bioremediation processes is a dynamic, multi-factor method. Bioremediation success not only depends on the decomposition capacity, but also on the active microorganism's stability under various conditions, such as changes in temperature, pH, pesticide concentration and genes associated with pesticide degradation (Azubuike *et al*.

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2016; Derbalah *et al*. 2020). Therefore, the effects of various environmental factors on the growth potential of tested species must be studied. In this study, we isolated and molecularly characterized *Pseudomonas aeruginosa* from pesticide polluted soil contaminated for potential biodegradation of malathion. The aptness of *P. aeruginosa* to grow in the presence of malathion as a source of energy and carbon was estimated. The efficacy of *P. aeruginosa* for bioremediation of malathion in soil was evaluated by degradation potential of malathion along with effect of different environmental factors (pH, temperature and malathion initial concentration) on the growth of *P. aeruginosa*. Presence of organophosphorus hydrolase *B gene* (*ophB*) in *P. aeruginosa* was also confirmed for degradation of malathion in soil samples.

Materials and Methods

Sample collection and soil characteristics

Soil samples (15 g) from five vegetable fields, S1 (*Spinacia oleracea*), S2 (*Capsicum annuum*), S3 (*Solanum lycopersicum*), S4 (*Lactuca sativa*) and S5 (*Brassica oleracea* var. capitata) were collected in sterile tubes. All the samples were obtained from the top 15 cm of the soil and stored at 4°C. These fields were known for being sprayed with malathion for the last 3–4 years regularly. Soil pH was measured using pH meter from saturation paste, after thoroughly mixing soil with water (1:3 w/v).

Isolation and identification of bacterial communities

Each soil sample (10 g) was stirred in 90 mL of phosphate buffered saline (PBS). Bacteria were isolated through serial dilution method, ten-fold dilutions were prepared and plated on nutrient agar (NA) (consist of sodium chloride (1%), peptone 0.5%, beef extract 0.3%, agar 2%). Plates were incubated overnight at 37°C and individual bacterial colonies were transferred to nutrient broth. Phenotypic identification was performed by visual inspection on nutrient agar and then confirmed by gram staining. Furthermore, biochemical tests such as oxidase, catalase, gelatin hydrolysis, nitrate reduction, citrate utilization, triple sugar iron and cetrimide were performed to elucidate the nature of bacteria.

Pesticide hydrolysis test

Pesticide degradation by bacterial strain was tested by hydrolysis test described by (Trinder *et al*. 2016). For this purpose, 1 μL of overnight bacterial culture maintained in nutrient broth was spotted on NA plates containing 50, 100, 150 and 200 $mg.kg^{-1}$ emulsified malathion. Non-malathion containing NA plates were used as control. All plates were incubated for 48 h at 37°C and degradation zones were measured in mm with scale (Trinder *et al*. 2016).

Molecular characterization of malathion degrading bacteria

Genomic DNA of bacterial strain showing malathion degradation was extracted using CTAB (Cetyl Tri-methyl Ammonium Bromide) method without any modification (Wilson 2001). Extracted DNA was analyzed through gel-electrophoresis and nanodrop for purity and concentration, respectively. Bacterial universal primers, 4F and 1492R (Table 1), were used for the amplification of 16S rRNA gene in 25 μL reaction mixture having 50 ng μL^{-1} gDNA,10 μL of dream *Taq* green master mix (Thermo Fisher scientific, United States), 0.2 m*M* primers and milli-Q water. The conditions of PCR reaction included first hold of temperature at 95°C for 5 min, then denaturation at 95°C for 1 min followed by annealing and elongation at 68°C for 40 s and 72°C for 60 s extension at each cycle, respectively. The entire cycle was repeated 35 times before the final extension for 8 min at 72°C in thermal cycler (Bioneer MyGene 32, Korea). The resulting amplicon were further purified by MQ100 PCR product purification kit (MOLEQULE-ON, New Zealand). Purified product was run on 2% agarose at 80 V for 1.5 h, which was qualitatively analyzed on Gel documentation system (MOLEQULE-ON, New Zealand). The quantity of purified product was measured by Nanophotometer (IMPLEN, Germany) and sent to Bioneer (Korea) for sequencing.

Bioinformatics analysis

Sequencing data on 16S rRNA gene was searched for homology against reference sequences in basic local alignment (BLASTn) tool (Altschul *et al*. 1990). Sequences was subjected to multiple sequence alignment (MSA) by utilizing online tool CLUSTAL W. Query sequence and homologous sequences were added in Molecular Evolutionary Genetics Analysis (MEGA 7.0) software for the reconstruction of phylogenetic tree (Kumar *et al*. 2018) using Neighbor-joining method. The percentage from each branch point was given to the bootstrap of each placement.

Molecular screening of the isolate for malathion degradation potential

Malathion degrading bacteria degrade malathion by an enzyme organophosphate hydrolase, which is encoded by *opdA, opdE,* and *ophB* genes (Horne *et al.* 2002). The presence of these genes in the isolated bacterial strains was explored by applying specific PCR primers listed in Table 1. In each case, PCR mix were prepared by adding forward and reverse primers 0.2 μL (0.2 μM) each, template DNA 1 μL (50 ng/μL), mater mix (10 μL) and deionized water (13.5 μL). Thermal cycler conditions were set at initial denaturation of 95ºC for 5 min, followed by 35 cycles of denaturation for 60 s at 95°C, annealing for 40 sec at 68°C (for *opdA*), 61°C (for *opdE*), 63°C (for *opdB*) and extension

Table 1: Primers and their sequences used for the amplification of bacterial genes

Primer Name	Primer Sequences	Product Size
16S rRNA-F	AGAGTTTGATCCTGGCTCAG	1542 bp
16S rRNA-R	GGTTACCTTGTTACGACTT	
opdA-F	GATCGTCTGCAGCCAATCGGTACAGGCGATCTG	956 bp
opdA-R	GATCGTAAGCTTTCATCGTTCGGTATCTTGACGGGGAAT	
ophB-F	CGTCGTCGGCTGGGCAGGGT	914 bp
ophB-R	GCGTGCGGCCTACCTCGTTG	
opdE-F	TTTACCCGCTCACGCGGTCGTCC	1092 bp
opdE-R	GCTCGAGATCATTGCCGCACCAGAATAC	

72°C for 60 s. In all cases final extension was set at 72°C for 8 min. Amplified products were run on 2% gel agarose gel at 80 V for 1.5 h and visualized through Gel documentation system.

Effect of pH, temperature, malathion contents on the activity of *P. aeruginosa*

In order to study the suitability of bacterial strain at different environmental conditions, test bacterium was allowed to grow at range of pH from 4, 5, 6, 7, 8 and 9 with varying temperatures such as. 25, 30, 37, 40, 50 and 55°C in the presence of malathion at 100 $mg.kg^{-1}$. The pH of the NA was adjusted by adding either HCl or NaOH before pouring the media into Petri dishes. At the center of plates, 10 μL of bacterial cell suspension (prepared in NA broth) was spotted and plates were incubated at different temperatures (25–55°C). After 48 h, zone of pesticide degradation zone was measured around bacterial colony as described by (Jiang *et al.* 2019). To test pesticide tolerance by bacteria, *P. aeruginosa* was inoculated in nutrient broth contaminated with malathion at 50, 100, 150, 175 and 200 $mg.kg^{-1}$. Tubes were incubated for 24 h at 37°C in shaking incubator set at 180 rpm (Trinder *et al.* 2016). Bacterial growth was analyzed by measuring the optical density at 600 nm on UV-1600 Spectrophotometer (TOMOS Life Science Group, China).

Statistical Analysis

Data from five fields for soil pH, zone of concentration and zone of degradation was analyzed using one-way analysis of variance (ANOVA) and post hoc analysis by Duncan's multiple range test through SPSS software (IBM SPSS v. 23, NY, USA). Means were considered significant at $P < 0.05$.

Results

Soil pH analysis

Soil pH, along with many other factors play important role in the microbial communities and thus plants growth and productivity. In the present study, pH value of all the five soil samples was slightly alkaline, indicating fertile soil with high microbial growth (Fig. 1). The highest alkaline pH of 7.76 was recorded in *L. sativa* sample (S4).

Identification of bacterial isolates and their malathion degrading potential

Ten bacterial isolates showing morphological variations on NA were purified by sub-culturing on fresh media and were tested for their malathion potential by using pesticide hydrolysis test. All the bacterial isolates except 3 showed negative reaction to Gram's staining (Table 2).

Among ten bacterial isolates, S2.1 isolated from *Capsicum annuum* filed showed zone of degradation on malathion containing nutrient agar plates. Optimum degradation was observed at 100 $mg.kg^{-1}$ followed by 150 $mg.kg^{-1}$ and 200 $mg.kg^{-1}$. Small degradation zone at 200 $mg.kg^{-1}$ indicated that increased malathion concentration negatively impacted bacterial growth.

Malathion degrading bacterium (S2.1) was further characterized by biochemical tests (Table 3). The biochemical analysis coupled with morphological characteristics suggested that the malathion degrading bacterium (S2.1) might belong to genus *Pseudomonas.*

Genotypic and phylogenetic analysis of malathion degrading bacterium

Amplification of 16S rRNA gene (~1542 bp) from malathion degrading bacteria (S2.1) was achieved in PCR reaction (Fig. 2a). The reaction was validated using negative (sample without template DNA) and positive control (DNA of *Escherichia coli*). After sequencing of amplified product, online BLAST software was used for sequence alignment and analysis showed similarity (< 97%) of amplified sequence with *P. aeruginosa*, confirming its status. The phylogenetic tree reconstruction (Fig. 2b) indicated that malathion degrading bacteria (KIBGE-AB9) was found in a single clade and displayed close relationship with previously identified species of genus *Pseudomonas*, which is *Pseudomonas aeruginosa*, with a bootstrap value of 98%. Keeping *Streptococcus pneumoniae* as an outgroup, majority of the other bacteria in the tree belonged to genus *Pseudomonas*. After that this sequence of strain was

Table 2: Morphological characteristics and Gram's reaction of bacterial isolates from agricultural soil samples

Bacterial Isolate	Source	Morphology		
		Colonial Characteristics	Size (mm)	Gram's Reaction
S1.1	*Spinacia oleracea*	Circular, White, Convex, Entire, Moist, Opaque	3	Gram Negative
S1.2	*Spinacia oleracea*	Circular, White, Convex, Entire, Moist, Opaque	3	Gram Negative
S2.1	*Capsicum annuum*	Irregular, White, Raised, Undulate, Moist, Opaque	2	Gram Negative
S2.2	*Capsicum annuum*	Circular, White, Convex, Entire, Moist, Opaque	3	Gram Negative
S2.3	*Capsicum annuum*	Circular, White, Convex, Entire, Moist, Translucent	2	Gram Negative
S3.1	*Solanum lycopersicum*	Hairy, White, Flat, Filiform, Moist, Opaque	5	Gram Negative
S3.2	*Solanum lycopersicum*	Irregular, White, Raised, Undulate, Moist, Opaque	2	Gram Negative
S4.1	*Lactuca sativa*	Circular, Yellowish green, Flat, Undulate, Moist, Opaque	3	Gram Positive
S4.2	*Lactuca sativa*	Circular, Creamish, Raised, Entire, Moist, Opaque	2	Gram Positive
S5.1	*Brassica oleracea* var. capitata	Circular, Yellowish orange, Convex, Entire, Dry, Translucent	2	Gram Positive

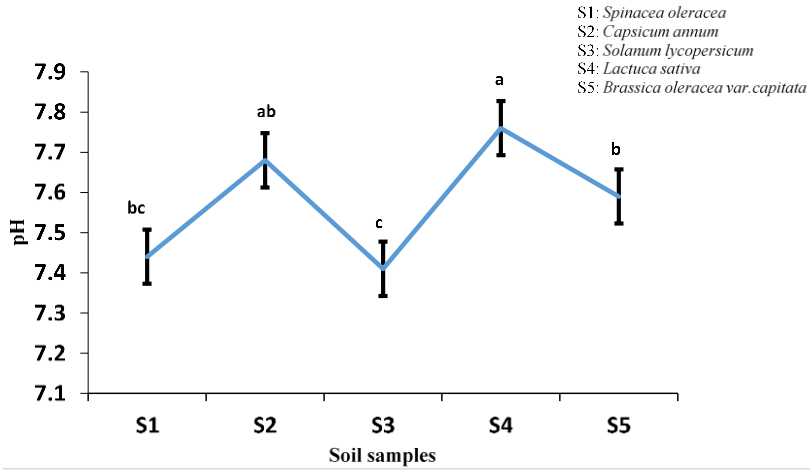

Fig. 1: pH of the agricultural soil samples
Symbols (means ± S.D, n = 3) having similar letters are not significantly different from each other (DMR, $P \leq 0.05$)

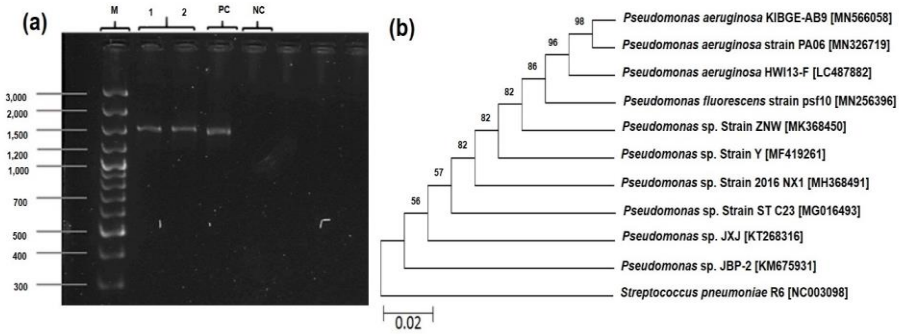

Fig. 2: Amplification of 16S rRNA gene of malathion degrading bacterium and phylogenetic analysis
(**a**) PCR amplification of bacterial 16S rRNA gene: Lane M: 100 bp marker, Lane 1-2: amplified PCR product of bacterial 16S rRNA gene. (product size 1542 bp) P.C: Positive control *Escherichia coli*. N.C: Negative control
(**b**) Evolutionary relationship of malathion-degrading bacteria *Pseudomonas aeruginosa* (KIBGE-AB9) with GenBank Accession Number MN566058 showing consensus

submitted to GenBank with GenBank Accession Number MN566058.

Screening and optimization of indigenously isolated *P. aeruginosa* for bioremediation

Along with other factors, soil pH and ambient temperature are critical physiochemical parameters that influence the microbial growth in agriculture field. In the present study, optimal *in vitro* malathion degradation was achieved at pH 7.0 (Fig. 3a), suggesting suitable *in vivo* soil environment from where the test bacterium was isolated. Likewise, at 37°C bacterium showed peak malathion degradation (Fig. 3b) was optimal for KIBGE-AB9 growth and activity. Testing the growth and survival of bacterium in the presence of increasing malathions levels in the nutrient broth showed a

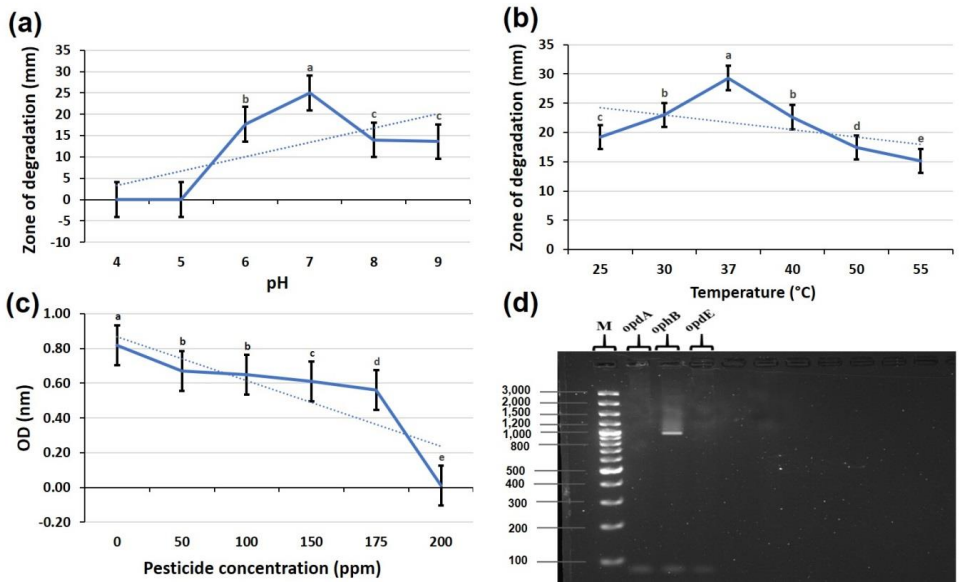

Fig. 3: Screening of *Pseudomonas aeruginosa* (KIBGE-AB9) for malathion degradation. (**a**) pH (**b**) temperature optimization for malathion degradation (100 mg.kg^{-1}). (**c**) bacterial growth curve showing malathion tolerance by bacterium. (**d**) Amplification of organophosphate degradation genes (*opdA, ophB* and *opdE*). Lane M: 100 bp marker. Symbols (means ± S.D., n = 3) having similar letters are not significantly different from each other (DMR, $P \leq 0.05$)

Table 3: Biochemical characteristics of malathion degrading bacterium

Sr. No.	Biochemical Test	Results
1	Oxidase	+ve
2	Catalase	+ve
3	Citrate	+ve
4	Nitrate Reduction	+ve
5	Hydrogen Sulfide (H_2S)	-ve
6	Sugar Fermentation*	-ve
7	Gelatin Hydrolysis	+ve
8	Cetrimide	+ve

non-significant effect of 0, 50, 100, 150 and 175 mg.kg^{-1} levels in the media. However, at 200 mg.kg^{-1} malathion in NB significantly inhibited *P. aeruginosa* (KIBGE AB-9) growth, as indicated by lowed OD (Fig. 3c). The presence of organophosphate degradation gene *ophB* was detected in *P. aeruginosa* KIBGE-AB9 (Fig. 3d) which encodes 31.4 kDa organophosphate hydrolase enzyme responsible for the formation of malathion zone of degradation on nutrient agar plates in pesticide hydrolysis test.

Discussion

Microbial communities in the agriculture soil frequently sprayed with pesticides may play an important role in the remediation process. In this study, morphological and biochemical analysis of the bacterium revealed as belonging to *Pseudomonas*. Molecular characterization was conducted using 16S rRNA gene sequencing which classified this isolate as *P. aeruginosa*. Additionally, the highest similarity between tested strain KIBGE-AB9 and *P. aeruginosa* verified the identification of tested isolate as *P. aeruginosa*. Bacterial identification using conventional microbiological techniques often provides non-specific results due to intraspecies similarities and differences between each other (Franco-Duarte *et al*. 2019). Moreover, *Pseudomonas* shows complex taxonomic characters with similar morphological and biochemical attributes with other bacteria due to intra species similarities (Al-Dhabaan 2019). Whereas, molecular techniques based on 16S rRNA gene sequencing give reliable identification of bacteria and considered as gold standard for phylogenetic analysis as it consists of various conserved and hypervariable regions exhibiting nucleotide variations between species (Janda and Abbott 2007; James 2010).

Analyzing microbial growth rates is substantial for extrapolation of promising colonization capacity in soil, as it gives adequate speed of a microbe's ability for colonization and substrate breakdown. Growth rates also indicate the species dominance over a particular substrate. Species which are fast-growing might have an advantage for utilization of resources at a higher speed, than their competitors. In this study, the identified *P. aeruginosa* have the ability to grow well in culture plates in the presence of malathion. As, it is collected from the soil; thus, it is able to grow well in harsh environmental conditions (polluted areas). Furthermore, the indigenously isolated *P. aeruginosa* from soil contaminated with pesticides showed high growth than in absence of malathion. This attribute of increased growth was also evident in other malathion degrading microbe *i.e., Aspergillus flavus*, isolated from water contaminated with malathion (Derbalah *et al.* 2020). Thus,

distinctive microbial detach are proficient for malathion biodegradation and it proclaims that the secluded organism employed malathion as abundant source of carbon for its growth. Therefore, *P. aeruginosa* having the ability to degrade malathion biologically, affirms that it utilizes malathion as a source of carbon for its development.

In this study, 50 bacteria were initially isolated from malathion contaminated soil samples by serial dilution and spread plate method on nutrient agar (NA). The top 10 bacterial isolates with increased malathion degradation activity were shortlisted for further analysis (Table 2). Among 10 isolates only S2.1, *Pseudomonas aeruginosa*, optimally degraded malathion at 100 $mg.kg^{-1}$ concentration and produced zone of degradation.

Several studies on pesticide contaminated soil reported *Pseudomonas* spp. effectively degrade malathion at various concentration (Verma *et al*. 2014; Verma *et al*. 2016; Upadhyay and Dutt 2017). This degradation is mainly attributed due to the presence of different enzymes such as acid organophosphorus anhydrolase (OPAA), methyl parathion hydrolase (MPH), phosphotriesterase (PTE) and organophosphorus hydrolase (OPH) (Bai *et al.* 2017). The key enzyme involved in detoxification technique is OPH which hydrolyze various organophosphorus compounds. The isolation of OPH was first studied from *P. diminuta* which was involved in hydrolysis of wide range of O-phenylenediamine (OP) compounds (Caldwell *et al.* 1991). In another study *ophB* gene was cloned in *E. coli* DH5α to degrade chlorpyrifos pesticide in nutrient agar plate (Barman *et al.* 2014). In this study, presence of organophosphate hydrolase *ophB* gene in *P. aeruginosa* indicated degradation pathway involves chemical reaction catalyzed by organophosphate hydrolase enzyme.

It was observed that soil samples collected from five agricultural fields in Karachi, Pakistan had slightly alkaline pH. It previously known that microbial community composition has significant ($P < 0.05$) correlations with soil pH and salt content. In fact, bacterial phylogenetic diversity and phylotype richness were also increased in soils with middle-level salt rates and were significantly ($P < 0.05$) correlated with pH and salt content (Zhao *et al.* 2018). Rousk *et al*. (2009) studies radioactively labeled soil with thymidine (TdR) and leucine (Leu) to evaluate the bacterial growth at pH gradient ranges from 8.3–4.0 and observed fivefold decrease in bacterial growth at acidic pH. Additionally, optimum degradation of malathion was observed at pH 6.0 which is near the pH of agricultural soil from where the bacteria were isolated and above 100 ppm malathion showed negative impact on bacterial growth. This might be because malathion applied in agricultural fields was present in diluted form that's why bacteria evolved mechanism to degrade malathion in low concentration. If gradually increase the dose of malathion in experimental fields for longer period of time it might be possible that bacteria evolve mechanism to degrade malathion at above 100 ppm concentration (Khorasani *et al.* 2013).

It is necessary to recognize and isolate suitable microbial strains and maintain their survival and operation after their transfer into the target environment for effective bioremediation. Previously, numerous studies reported variety of microorganism were isolated from contaminated sites to degrade contaminants such as oil, heavy metals, hydrocarbons, and pesticides (Abatenh *et al.* 2017; Liu *et al*. 2020; Wanwari *et al.* 2020).

Conclusion

It is concluded that malathion degrading bacterial strain, isolated from soil is identified as *P. aeruginosa* (KIBGE-AB9) strain, able to degrade malathion effectively up to 100 $mg.kg^{-1}$. The strain gave better performance at 37°C and pH 7.0. This strain was found to possess organophosphate hydrolase encoding gene (*ophB*) that has potential to degrade the malathion. Therefore, it may be used as bioremediation agent in an eco-friendly manner against pesticide contamination present in agriculture soil. However, by cloning a gene fragment which degrades malathion, it is quite difficult to create a highly resistant engineered strain. It is yet to decipher, under complex environmental conditions, how engineered strains execute their functions. Hence, further in-depth analysis requires to understand degradation of organophosphate compounds through bioremediation of genetically engineered strains.

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Author Contributions

BK and SG planned, executed the experiments and wrote the original draft. IJ conducted Phylogenetic analysis. ZAM interpreted results ZH and AA reviewed manuscript.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

The datasets generated and/or analyzed during the current study are available in the NCBI repository, https://www.ncbi.nlm.nih.gov/.

Ethical Approval

Not applicable to this paper.

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