*Research Article*

**Investigation and application of a novel*****Pseudomonas aeruginosa* (KIBGE-AB9) for the biodegradation of malathion in agriculture soil**

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**Novelty statement**

**Abstract**

Malathion, an organophosphate insecticide despite of its significant benefits in the agriculture productivity, is an important pollutant and food contaminant which affect 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 form 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) was 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 ppm. The isolated strains of *P. aeruginosa* was found to possessorganophosphate hydrolase encoding gene (*ophB*), suggesting that bacterial strain use malathion as carbon source. Bioremediation of malathion by *P. aeruginosa* could be a promising approach against pesticide contamination in agriculture soil.

**Keywords:** Agriculture,malathion, bioremediation, *Pseudomonas aeruginosa*, environmental safety

**1. 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 used annually on crops globally, which is expected to exceed 5.5 million tons in 2021 (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 affects nerve fiber and is neurotoxic in animals and higher-level vertebrates. Several international agencies such as the Food and Drug Administration (FDA) and USEPA have authorized maximum residue malathion concentration on specific food crops. 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 (J *et al.* 2015).

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*. 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 isolate and molecularly characterize *Pseudomonas aeruginosa* from pesticide polluted soil contaminated for potential biodegradation of malathion. The aptness of *Pseudomonas aeruginosa* to grow in the presence of malathion as a source of energy and carbon was estimated, the efficacy of *Pseudomonas aeruginosa* for bioremediation of malathion in soil was evaluated, the effect of different environmental factors (pH, temperature and malathion initial concentration) on the ability of *Pseudomonas aeruginosa* for growth and degradation potential of malathion and finally the presence of *ophB* was also investigated.

**2. Methodology**

**2.1. 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 and transported to the laboratory. 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 last 3-4 years. Soil pH was measured using pH meter, after thoroughly mixing soil with water (1:3 w/v).

**2.2. 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 colony morphologies on nutrient agar and 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.

**2.3. 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 ppm 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).

**2.4. 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 gDNA,10 µL of dream *Taq* green master mix (Thermo Fisher scientific, United States), 0.2 mM 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 minute 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 minutes 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 80V 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.

**2.5. 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.

**2.6. 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 1µL (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 sec at 95 ºC, annealing for 40 sec at 68°C (for *opdA*), 61°C (for *opdE*), 63ºC (for *opdB*) and extension 72ºC for 60 sec. In all cases final extension was set at 72 ºC for 8 min. Amplified products were run on 2% gel agarose gel at 80 V for 1.5 hours and visualized through Gel documentation system.

**2.7. 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 of pH 4, 5, 6, 7, 8, 9 and temperature 25 ºC, 30 ºC, 37 ºC, 40 ºC, 50 ºC and 55 ºC in the presence of malathion at 100 ppm. 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 (25ºC-55ºC). After 48 hours’ 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 ppm. 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).

**2.8. 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 v23, NY, USA). Means were considered significant at *p<0.05.*

**3. Results**

**3.1. 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 (Figure 1). The highest alkaline pH of 7.76 was recorded in *Lactuca sativa* sample (S4).

**3.2. 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 ppm followed by 150 ppm and 200 ppm. Small degradation zone at 200 ppm 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*.

**3.3. 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 (Figure 2a). The reaction was validated using negative (sample without template DNA) and positive control (*Escherichia coli*). The sequencing and matching od amplified product showed similarity (<97%) with *Pseudomonas aeruginosa.* The phylogenetic tree reconstruction (Figure 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*.

**3.4. 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 (Figure 3a), suggesting suitable *in vivo* soil environment from where the test bacterium was isolated. Likewise, at 37°C bacterium showed peak malathion degradation (Figure 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 non-significant effect of 0, 50, 100, 150 and 175 ppm levels in the media. However, at 200 ppm malathion in NB significantly inhibited *P. aeruginosa* (KIBGE AB-9) growth, as indicated by lowed OD (Figure 3c). The presence of organophosphate degradation gene *ophB* was detected in *Pseudomonas aeruginosa* KIBGE-AB9 (Figure 3d) which encodes 31.4KDa 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 *Pseudomonas aeruginosa*. Additionally, the lowest genetic diversity 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). In such case 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 which shows nucleotide variation between species whereas, slight variation within 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. The identified *P. aeruginosa* have the ability to grow well in culture plates in the presence of malathion. 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 higher growth concentration then in absence of malathion. This attribute of increased growth concentration was also seen 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 degrade malathion at 100 ppm concentration and produced zone of degradation.

Several studies on pesticide contaminated soil reported *Pseudomonas* sp. effectively degrade malathion at various concentration (Verma *et al*. 2014; Verma *et al*. 2016; Upadhyay and Dutt, 2017). The study on degradation of malathion revealed 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 involves 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). Presence of organophosphate hydrolase *ophB* gene in *Pseudomonas aeruginosa* indicate degradation pathway involves chemical reaction catalyzed by organophosphate hydrolase enzyme. In recent study *ophB* gene was cloned in *E. coli* DH5α to degrade chlorpyrifos pesticide in nutrient agar plate (Barman *et al.* 2014).

It was observed that soil samples collected from five agricultural fields in Karachi, Pakistan have slightly alkaline pH. It has been reported previously 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 and colleague 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 (Rousk *et al*. 2009). 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 100ppm malathion concentration 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 100ppm 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*. 2019; Swati *et al.* 2020). In the light of the present studies, it provides a remarkable, alternative, and distinct approach to degrade malathion. 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.

**Conclusion**

The *Pseudomonas aeruginosa* (KIBGE-AB9) strain was able to degrade malathion effectively upto 100ppm. 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 use as bioremediation agent against pesticide contamination present in agriculture soil.

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**Author Contributions**

**Basit Khan:**Conceptualization, Investigation, Methodology, Visualization, Formal analysis, writing-original draft. **Ishrat Jamil:** Molecular & Phylogenetic analysis. **Zulifqar Ali Mirani**: Investigation, Methodology.  **Zahoor Ul Hassan:** Manuscript review & editing**. Abid Azhar**: Supervision, Validation. **Saddia Galani**: Conceptualization, Supervision, Writing- Review & Editing.

**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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**Figures and Tables**

Chart, line chart

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**Fig1. pH of the agricultural soil samples**. Symbols (means ± S.D., n = 3) having similar letters are not significantly different from each other (DMR, p <0 .05).

Chart

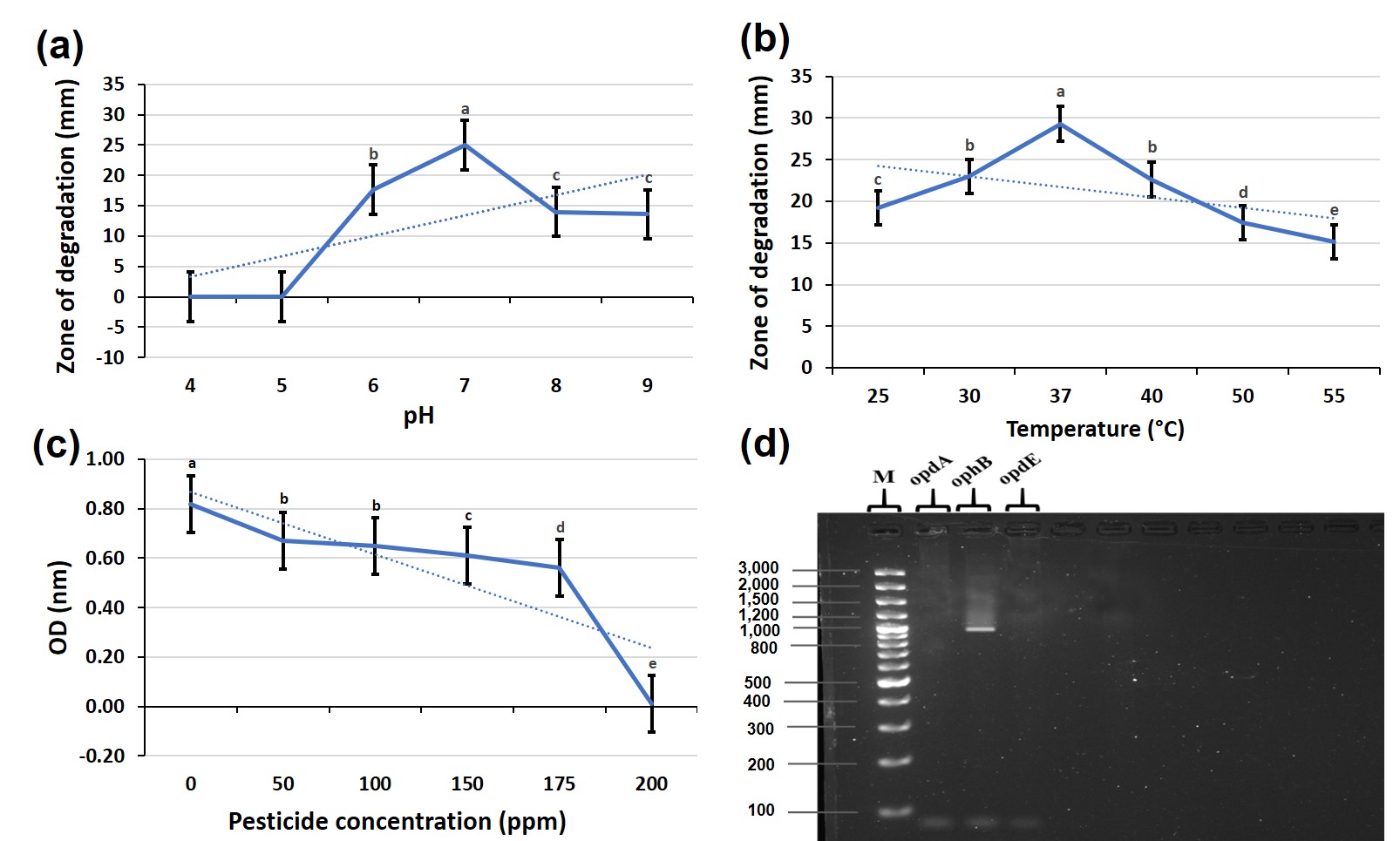
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**Figure 3. Screening of *Pseudomonas aeruginosa* (KIBGE-AB9) for malathion degradation.**

**Fig2. Phylogenetic Analysis of Malathion degrading bacterium.**

(a) PCR amplification of bacterial 16S rRNA gene. Lane M: 100bp marker, Lane 1-2: Amplified PCR product (1542 bp) of bacterial 16S rRNA gene. P.C: Positive control *Escherichia coli*. N.C: Negative control

(b)Evolutionary relationship of malathion-degrading bacteria *Pseudomonas aeruginosa* (KIBGE-AB9) showing consensus of 1,000 bootstrap trees constructed using neighbor-joining distance matrix.



**Fig3. Screening of *Pseudomonas aeruginosa* (KIBGE-AB9) for malathion degradation**. (a) pH (b) temperature optimization for malathion degradation (100ppm). (c) bacterial growth curve showing malathion tolerance by bacterium. (d) Amplification of organophosphate degradation genes (*opdA*, *ophB* and *opdE*). Lane M: 100bp marker. Symbols (means ± S.D., n = 3) having similar letters are not significantly different from each other (DMR, p <0 .05).

**Table 1: Bacterial primers used in the study.**



**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 |

**Table 3: Biochemical characteristics of malathion degrading bacterium**  