

Degradation of Profenofos and λ -Cyhalothrin Using Endogenous Bacterial Isolates and Detection of the Responsible Genes

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Abstract

An effective profenofos and λ -cyhalothrin degrading bacterium (strain DB17) was isolated from soil samples collected from Qassim region, Saudi Arabia. Based on the results of phylogenetic similarity of 16SrDNA gene sequences, strain DB17 was identified to be *Pseudomonas putida*. The isolate utilized profenofos and λ -cyhalothrin as the sole source of carbon for its growth. Analytical method was developed and validated for the determination of profenofos and λ -cyhalothrin in bacterial medium to monitor the intended biodegradation of both compounds. The inoculation of isolate DB17 10^9 and 10^{11} cells/ml to mineral salt medium supplemented by 100 mg/liter of profenofos and λ -cyhalothrin resulted in a higher degradation rate than in non-inoculated medium. The genes encoding organophosphorus hydrolase (mpd and opd) and pyrethroid-degrading esterase gene (pytY) were cloned using a PCR cloning strategy. The obtained results highlight the potential of this bacterium to be used in the cleanup of pesticide contaminated sites.

Keywords: Biodegradation; Bacteria; Profenofos; λ - Cyhalothrin; Responsible genes for degradation

Introduction

Crop production of Saudi Arabia is mainly cultivated under greenhouses because of the dominating arid climate of Saudi desert. Plants grown under greenhouse are more prone to infestation and infection by different types of pests. Pesticides usage is a pivotal strategy for pest control. Crop production under greenhouse conditions requires more pesticide usage. The excessive use of pesticides has led to accumulation of these pesticides' residues in the environment, presenting a substantial health hazard to human [1]. Using bacteria for the degradation of pesticides is an efficient approach. Isolation of indigenous bacteria capable of degrading pesticides provides an environmentally friendly way for pesticide detoxification [2].

Degradation by microbes depends not only on the presence of degradative enzymes, but also on a wide range of environmental parameters such as ambient temperature, nutrients status and pH. Also, pesticide concentration is a limiting factor [3]. All the above mentioned factors require investigation to assess its impact on activity and microbial population.

"Catabolic plasmids" are the plasmids of bacteria that carry the responsible genes for pesticide degradation [4]. Depending on the carried genes, the organism is able to use particular compound, hence degrade it [5]. Many catabolic plasmids have been reported in numerous species of *Pseudomonas*, *Arthrobacter*, *Actinobacter*, *Alcaligenes* and *Flavobacterium* [4].

Contaminated environments have participated with time in the evolution of endogenous microbial populations. Therefore, contaminated sites are the most appropriate locations for the isolation of degradative bacterial strains which are able to degrade contaminating compounds and also the related ones [6]. Decontamination of polluted areas is a need of the hour. The conventional means (physio chemical methods) for soil decontamination are rather expensive, labor intensive less efficient and may affect soil properties as well. Thus,

biodegradation is becoming a method of choice for the remediation of polluted sites.

The Objective of this study was investigating the biodegradation of profenofos and λ -cyhalothrin using isolated indigenous bacteria from Qassim region, Saudi Arabia. Also, studying the effect of temperature, incubation time, bacterial inoculum and pesticide concentrations on the efficiency of degradation. Also, a special focus was given to development and validation of the analytical method. In addition, detection of the genes which are responsible for the degradation of tested compounds.

Materials and Methods

Chemicals and reagents

Two insecticides were selected for the current study. Log *P*, chemical group, toxicity and chromatographic parameters of the tested pesticides are summarized in Table 1. Chemical structure of profenofos and λ -cyhalothrin is illustrated in Figure 1. Profenofos and λ -cyhalothrin standards were obtained from Sigma-Aldrich (Germany). The stock solution was prepared in toluene and working solutions were prepared in acetonitrile, HPLC grade, obtained from BDH, UK. MgSO₄ (anhydrous) and NaCl were purchased from Merck, Germany. Mineral salt medium (MSM) components salts (K₂HPO₄, KH₂PO₄, (NH₄)₂SO₄, MgSO₄.7H₂O, CaCO₃, FeCl₂.4H₂O) were all purchased from LOBA Chemie Pvt. Ltd. (India).

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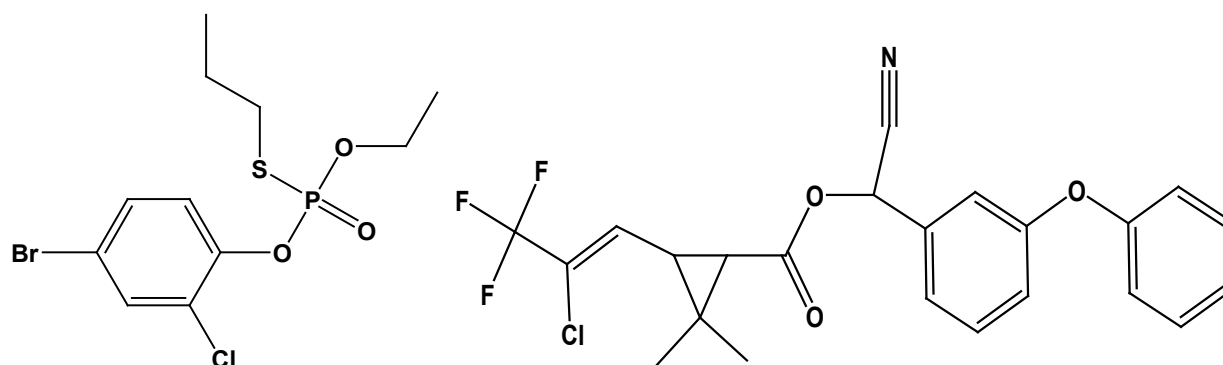


Figure 1: Chemical structure of profenofos (left) and λ -cyhalothrin (right).

| Pesticide | LogP | Chemical group | Toxicity class (WHO) | Retention time (R_t) | Monitored ions (m/z) |
|------------------------|------|------------------|----------------------|--------------------------|--------------------------|
| Profenofos | 4.44 | Organophosphorus | II | 6.16 | 139, 206, 339 |
| λ -Cyhalothrin | 7 | Pyrethroid | II | 8.48 | 181, 197, 208 |

Table 1: Log P, chemical group, toxicity and chromatographic parameters of the tested pesticides.

Soil sample collection for enrichment studies

Five soil samples were utilized in this study for the isolation of profenofos and λ -cyhalothrin degrading microorganisms. Soil samples were collected from different sites with a history of both pesticides applications in Qassim region, Saudi Arabia. The collected soil samples were taken and stored in plastic bags at 4°C until isolation process [7].

Isolation of profenofos and λ -cyhalothrin degrading bacteria

Ten grams of soil sample was placed in a 250 ml conical flask containing 100 ml mineral salt medium MSM ((g L⁻¹ deionized water) K₂HPO₄ 0.225; KH₂PO₄ 0.225; (NH₄)₂SO₄ 0.225; MgSO₄ 7H₂O 0.05; CaCO₃ 0.005; and FeCl₂ 4H₂O 0.005; pH- 7.2) and incubated at 30°C in a rotary shaker at 150 rpm for two days. The flasks were then left for a few hours to allow the soil particles to settle, and the suspension containing microorganisms was then used to inoculate fresh sterilized MSM containing 100 mg L⁻¹ of one of the tested pesticides and incubated for 48 hr. at 28°C. In order to obtain pure cultures of bacteria, 5 ml aliquots of enrichment cultures were centrifuged at 3000 rpm for 5 min and the cell pellets were re-suspended in 2 ml sterile medium. Aliquots of this suspension were spread on MSM profenofos and λ -cyhalothrin agar plates. Inoculated plates were incubated at 28°C and discrete colonies were isolated. Strong resulting colonies in growth were repeatedly sub-cultured using the same procedures above to confirm their utilizing ability. All colonies were transferred into fresh sterile medium to obtain a pure culture [7]. Eighteen isolates of strong growth on the pesticide agar medium were selected for further investigation. Each isolate was cultured in carbon deficient broth medium containing 100 mg L⁻¹ of one of the tested pesticides. Cultures were stirred for 3 days at 30°C before a sample was taken from each treatment. Samples were subjected for pesticide analysis.

Analytical method development and validation

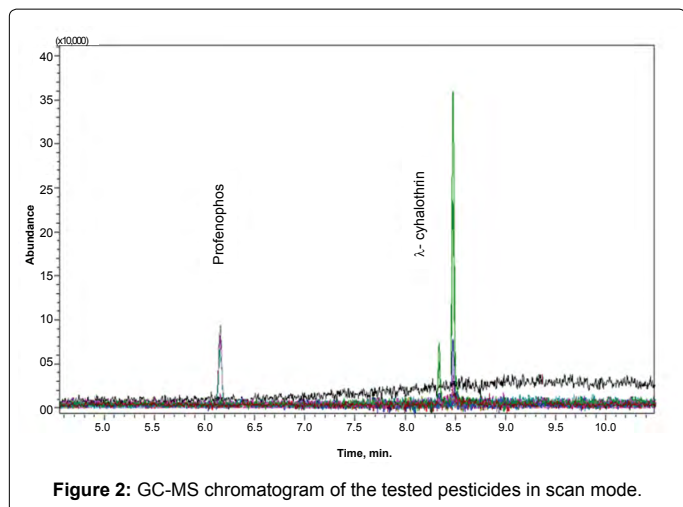
An analytical method was developed using modified QuEChERS method for sample preparation followed by GC/MS for the determination step.

A house made QuEChERS method was followed in sample extraction [8]. Two and half ml sample of bacterial cultures were filtered through a micropore filter 0.2 μ m (Whatman, Inc., USA)

into 15 ml centrifuge tubes. Five ml acetonitrile (1% acetic acid) was added and the tubes were shaken for mixing. Sample tubes were kept in the freezer for 15 min before adding 1 g MgSO₄ and 0.25 g NaCl and tubes were tightly capped and shaken vigorously by hand for 1 min. then tubes were centrifuged (Centra MP4R centrifuge, IEC, MA USA) under cooling for 5 min at 4400 rpm. 1 ml aliquot was taken into 1.5 ml screw capped vial contains 150 mg MgSO₄ and 25 mg PSA (primary secondary amine) and hand shaken for 1 min before centrifugation for 5 min at 4400 rpm. 500 μ l of the extract was transferred to 1.5 ml vial for GC/MS residue analysis. Extracts were diluted when required using blank matrix extracts.

A Shimadzu GC/MS-Q2010 Ultra (Shimadzu Corporation, Japan) was used for determination of pesticide residues. The instrument is equipped with Shimadzu AOC-20i autosampler and split/splitless injector in the split mode at 260°C. RXI-SIL 5 MS fused silica column, 30 m length \times 0.25 mm i.d. \times 0.25 μ m film thickness, sourced from Restek, USA, was used. Oven temperature program started at 200°C with hold time 2 minutes, than raised at 15°C min⁻¹ to 290°C and held at final temperature for 4 minutes. Injected volume was 1 μ l. The MS detector was run in the scan mode (from 50 m/z to 500 m/z). After that SIM mode was utilized for residue determination. Three ions were selected for each pesticide. 139 m/z and 181 m/z were utilized as quantification ions for profenofos and λ -cyhalothrin, respectively. 206 and 339 m/z were used as qualifiers for profenofos; 197 and 208 m/z were use as qualifier ions for λ -cyhalothrin. Injector was in the split mode. Injector temperature was 260°C, transfer line was 285°C and ion source was set at 235°C. Carrier gas, helium, flow was constant at 1 ml/min. GC-MS chromatogram of the tested pesticide is shown in Figure 2.

Validation parameters of the analytical method were investigated to assure the quality of the analytical results. Method accuracy was assessed by extraction and determination of tested compounds concentrations in spiked matrix of bacterial media at different levels i.e., 0.1, 1.0 and 10 mgL⁻¹ of the bacterial media. Method repeatability was calculated as RSD % of the recovery studies. Concerning linearity (R²), 5 concentrations of each compound i.e., 0.05, 0.1, 0.25, 0.5 and 1.0 mg L⁻¹ were prepared. Each concentration was injected three times (n=3) and average area was used for calibration curve development.



Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curve data. In order to compensate for matrix effect, matrix matched standard approach was utilized in pesticide residues quantification.

Biodegradation studies

The bacterial cells of best isolate in degradation process were pre-cultured in LB medium (g L⁻¹ deionized water; NaCl 10.0; Tryptone 10.0; Yeast extract 5.0; pH- 7.2) at 30°C with 150 rpm shaking, harvested by centrifugation at 6000 rpm for 10 min and was washed three times with sterilized mineral salts medium then was quantified by the dilution plate count technique. The obtained bacterial cells were used in biodegradation studies according to the modified method of Ref. [9].

Degradation studies were carried out in liquid mineral salts medium (MSM) supplemented with profenofos and λ - cyhalothrin. These were assayed with batch cultures in MSM by the best isolate which was selected from the first screening of degradation. Effects of incubation temperature, incubation period, pesticide concentration and inoculums density also were studied. Cultures were run in triplicate to ensure accuracy [9]. Degradation of tested pesticides in broth media without inoculation was also investigated (Control treatment). Degradation percent was calculated as the reduction of pesticide concentration after subtraction of the control degradation percent.

Identification of the bacterial isolates by 16S rDNA

The genomic DNA was extracted by phenol-chloroform method according to Ref. [10] and amplified with the 16S rDNA specific primer; P1F (5'-AGA GTT TGA TCC TGG CTC AG-3') and P2R (5'-TGA CTG ACT GAG GCT ACC TG-3') (Macrogen Company, Korea). The thermal cycling conditions were as follows: one cycle of 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 55°C for 1 min. and extension at 72°C for 1 min, followed by final cycle of extension of 72°C for 7 min. The purified PCR product was analyzed for nucleotide sequence determination by using ABI PRISM® 3500 XL DNA Sequencer (Applied Biosystems) at Macrogen Company, Korea. The homology of partial sequences obtained were compared with the sequences from the DNA databases and similarity showing above 95% were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) BLAST server [11].

Detection of organophosphorus hydrolase and pyrethroid esterase genes

The presence of three genes; methyl parathion- degradation gene (mpd) and Organophosphate-degradation gene (opd) as well as pyrethroid-degrading esterase gene (pytY) in the bacterial genome was assessed by polymerase chain reaction (PCR) with specific primers of three genes as shown in Table 2. PCR was performed as following conditions: initial denaturation at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 48 to 55°C for 0.5 min, and extension at 72°C for 1 min, and final chain elongation at 72°C for 7 min. PCR products were analyzed by electrophoresis in a 2% agarose gel with 1 × TBE as the running buffer.

Results

Isolation and identification of profenofos and λ - cyhalothrin degrading bacteria

The selected eighteen bacterial isolates were found to possess ability to use the tested pesticides (profenofos and λ - cyhalothrin) as a carbon source with different efficiencies (Table 3). Strain coded DB17 was the most efficient isolate to degraded both pesticides. DB17 was subjected for further studies. This strain was a rod-shaped, gram negative bacterium. By sequencing the 16SrDNA gene of DB17 and comparing it with previously published 16S rDNA gene sequences, the strain was

| Primer ID | Target | Sequence (5' to 3') | References |
|-----------|-----------|--------------------------------|------------------|
| F | mpd gene | GAATTCATATGCCCTGAAGAAC | Yang et al. [9] |
| R | mpd gene | GAATTCTCGAGCTTGGGGTTGACGACCG | Yang et al. [9] |
| F196 | opd gene | CGCGGTCTATCACAATCTC | Iyer et al. [12] |
| R840 | opd gene | CTTCTAGACCAATCGCACTG | Iyer et al. [12] |
| ZF | pytY gene | CGGGATCCATGACCACTCAAACCTATGAGC | Ruan et al. [17] |
| ZR | pytY gene | CGGAATTCTCAGTATGCGAGAAGCGACTG | Ruan et al. [17] |

Table 2: Sequences of specific primers to detect mpd, opd, pytY genes.

| Bacterial isolate codes | Profenofos | | λ - cyhalothrin | |
|-------------------------|------------|---------------|-------------------------|---------------|
| | Conc. | Degradation % | Conc. | Degradation % |
| Control | 97.21 | 2.79 | 98.45 | 1.55 |
| DB1 | 76.43 | 23.57 | 74.17 | 25.83 |
| DB2 | 84.49 | 15.51 | 73.55 | 26.45 |
| DB3 | 86.43 | 13.57 | 77.02 | 22.98 |
| DB4 | 61.43 | 38.57 | 74.94 | 25.06 |
| DB5 | 77.04 | 22.96 | 80.15 | 19.85 |
| DB6 | 67.65 | 32.35 | 61.64 | 38.36 |
| DB7 | 65.61 | 34.39 | 63.49 | 36.51 |
| DB8 | 56.63 | 43.37 | 64.49 | 35.51 |
| DB9 | 60.82 | 39.18 | 78.64 | 21.36 |
| DB10 | 75.71 | 24.29 | 73.4 | 26.6 |
| DB11 | 59.29 | 40.71 | 68.23 | 31.77 |
| DB12 | 48.06 | 50.92 | 65.88 | 34.12 |
| DB13 | 49.08 | 51.94 | 60.06 | 39.94 |
| DB14 | 71.43 | 28.57 | 64.23 | 35.77 |
| DB15 | 54.29 | 45.71 | 78.18 | 21.82 |
| DB16 | 62.45 | 37.55 | 65.34 | 34.66 |
| DB17 | 32.35 | 67.65 | 54.43 | 45.57 |
| DB18 | 67.76 | 32.24 | 74.79 | 25.21 |

Table 3: Evaluation of the degradation efficiency of the tested bacterial isolates.

classified as a member of the genus *Pseudomonas*. The sequence of the strain displayed the highest identity (98%) with the 16S rDNA gene of a *Pseudomonas putida*.

Validation of the analytical method

Method validation process is the required proof to ensure the ability of an analytical method and the capability of the analyst to provide results of accepted quality. Profenofos and λ - cyhalothrin are widely used insecticides. Polarities of the compounds, expressed as K_{ow} , are 4.44 and 7.0 for profenofos and λ - cyhalothrin, respectively. Analytical method was developed and validated for monitoring of profenofos and λ - cyhalothrin concentrations in liquid bacterial media as affected by the tested bacterial isolates. Validation parameters according to European guidelines i.e., accuracy, repeatability, linearity, limit of detection LOD and limit of quantitation LOQ, were investigated. Chromatographic conditions were optimized to ensure the absence of interferences during the resolution time of the target compound.

Calibration curves of profenofos and λ - cyhalothrin were constructed as a plot of the injected concentrations and the resulting absolute area under peak. Results showed that determination coefficients (R^2) were 0.9997 and 0.9991 for profenofos and λ - cyhalothrin, respectively. LOD and LOQ were calculated from the standard calibration data. LOD was calculated at signal to noise ratio of 3 and LOQ was 3.3 folds of LOD. LODs were 0.02 and 0.04 mg L⁻¹ while LOQs were 0.08 and 0.14 mg L⁻¹ for profenofos and λ - cyhalothrin, respectively. Table 4 shows calibration curve parameters, LOD and LOQ values of both pesticides also recovery and repeatability of the method. The obtained results are within the norms reported by European guidelines to consider a method reliable and accurate.

Biodegradation of profenofos and λ - cyhalothrin in liquid culture media

The degradation of profenofos and λ - cyhalothrin by DB17 isolate (*Pseudomonas putida*) under different conditions of incubation period, inoculum, temperature and pesticide concentration in liquid culture media was analyzed by GC/MS system. Degradation of profenofos and λ - cyhalothrin was monitored for 9 days after incubation. Degradation percentage reached 99.57% and 86.11% for profenofos and λ - cyhalothrin, respectively at the end of the experiment. Rapid degradation rate was observed for first 3 days after incubation. Slower rate was noted during further days after incubation.

The effect of consecutive initial inoculum concentrations of bacterial cells i.e., 10⁵, 10⁷, 10⁹, 10¹¹ on the degradation of the tested pesticides was investigated. Samples were taken after 3 days of incubation. A proportional relation was witnessed in profenofos degradation with inoculum concentration (slope 14.63) in comparison to λ - cyhalothrin where there was a little effect of inoculum concentration on the degradation (slope 3.30). The degradation percentages of profenofos were 5.31% and 95.81% with 10⁵ to 10¹¹ CFU, respectively. While, the degradation percentages of λ - cyhalothrin were 45.82% and 65.22% with 10⁵ to 10¹¹ CFU, respectively. The bacterial strain, DB17 (*Pseudomonas putida*) was more active under 30°C than at 15°C or 45°C as it

| Pesticides | Intercept | Slope | R ² | LOD | LOQ | Recovery | Repeatability |
|-------------------------|-----------|--------|----------------|-----------------------|-----------------------|----------|---------------|
| | | | | (mg L ⁻¹) | (mg L ⁻¹) | (%) | RSD % |
| Profenofos | 4.27 | 470.02 | 0.999 | 0.02 | 0.08 | 97.45 | 7.37 |
| λ - cyhalothrin | 1.26 | 274.17 | 0.999 | 0.04 | 0.14 | 99.95 | 6.86 |

Table 4: Intercept, slope, correlation coefficient (R^2) of the calibration curves and LOD and LOQ of the tested pesticides.

appears from the degradation percent of both pesticides after 3 days of incubation. The ability of the isolate DB17 to tolerate an increasing concentration of the tested pesticides was a concern to investigate. Broth media augmented with successive concentrations of profenofos and λ - cyhalothrin i.e., 100, 200, 300, 400, 500 were cultured with 10⁹ of DB17 bacterial cells as the initial concentration and kept stirred at 30°C for 3 days before a sample was taken. The degradation percentage decreased with pesticide concentration increase. Degradation percent were 74.15% and 25.65% at 100 and 500 ppm of profenofos, respectively while in case of λ - cyhalothrin were 53.74% and 0.53% at 100 and 500 ppm, respectively. Also, it can be inferred that the strain DB17 (*Pseudomonas putida*) was able to degrade profenofos more efficiently than λ - cyhalothrin. Degradation kinetic as affected with different conditions is illustrated in Figure 3.

Detection of the pesticide degrading genes

Detection of pesticide degrading genes (pytY, mpd and opd) in genomic DNA of DB17 isolate was performed according to an established protocol that relies on analytical PCR with their specific primers [12]. The amplified fragments are shown in Figure 4. The sequence BLAST result showed that the mpd gene was 99% similar to mpd gene of *Plesiomonas* sp., whereas the opd gene was 99% similar to opd gene of *Sphingobium fuliginis* and the pytY gene was 99% similar to pytY gene of *Ochrobactrum anthropi* at the nucleotide level. The expected lengths of PCR fragments are 700, 700 and 600 bp, to mpd, opd and pytY genes, respectively as shown in Figure 4.

Discussion

In the present study eighteen profenofos and λ - cyhalothrin degrading bacterial strains were enriched and isolated from soil samples collected from different agricultural sites in Qassim, Saudi Arabia. The isolate DB17 was found more efficient in comparison to other isolates. It was identified to be *Pseudomonas putida* using 16S rDNA gene detection strategy. Ref. [13] mentioned that isolation of bacterial strain through enrichment technique has been extensively used for biodegradation studies of pesticides. Ref. [7] isolated soil bacterial strains able to biodegrade profenofos in soil. They found that *Pseudomonas putida* was efficient to degrade profenofos. Also, Ref. [14] determined the degradation of the pyrethroid insecticide cypermethrin by *Pseudomonas* strains. Their study revealed that *Pseudomonas* strain degraded cypermethrin up to 100% within two days. Many of previous researches mentioned that the use of live biocatalysts for organophosphate degradation was demonstrated with *Pseudomonas putida* [15] and *Pseudomonas* sp [16].

In the present study rapid degradation was found with profenofos compared with λ - cyhalothrin in liquid culture. Also, the suitable conditions of pesticide degradation by *Pseudomonas putida* were 30°C for incubation temperature, 10⁹ to 10¹¹ cells / ml of bacterial inoculum concentrations and 100 to 200 ppm of pesticide concentrations in liquid media. Ref. [7] found that the pesticide profenofos was degraded rapidly in liquid culture of *Pseudomonas putida* where the degradation percentage reached to 27% after 6 hrs and 92.37% after 96 hrs. They used 100 ppm of pesticide concentration and 30°C of incubation temperature in degradation process. In this work some responsible genes for the degradation of organophosphorus and pyrethroid pesticides (mpd, opd and pytY) were detected in the genomic DNA of *Pseudomonas putida* (DB17) isolate. Ref. [9] cloned the gene encoding for organophosphorus hydrolase enzyme using PCR strategy based on the known methyl parathion degrading (mpd) gene of *Plesiomonas* sp. Our results were in agreement with Ref. [9] Sequence BLAST result

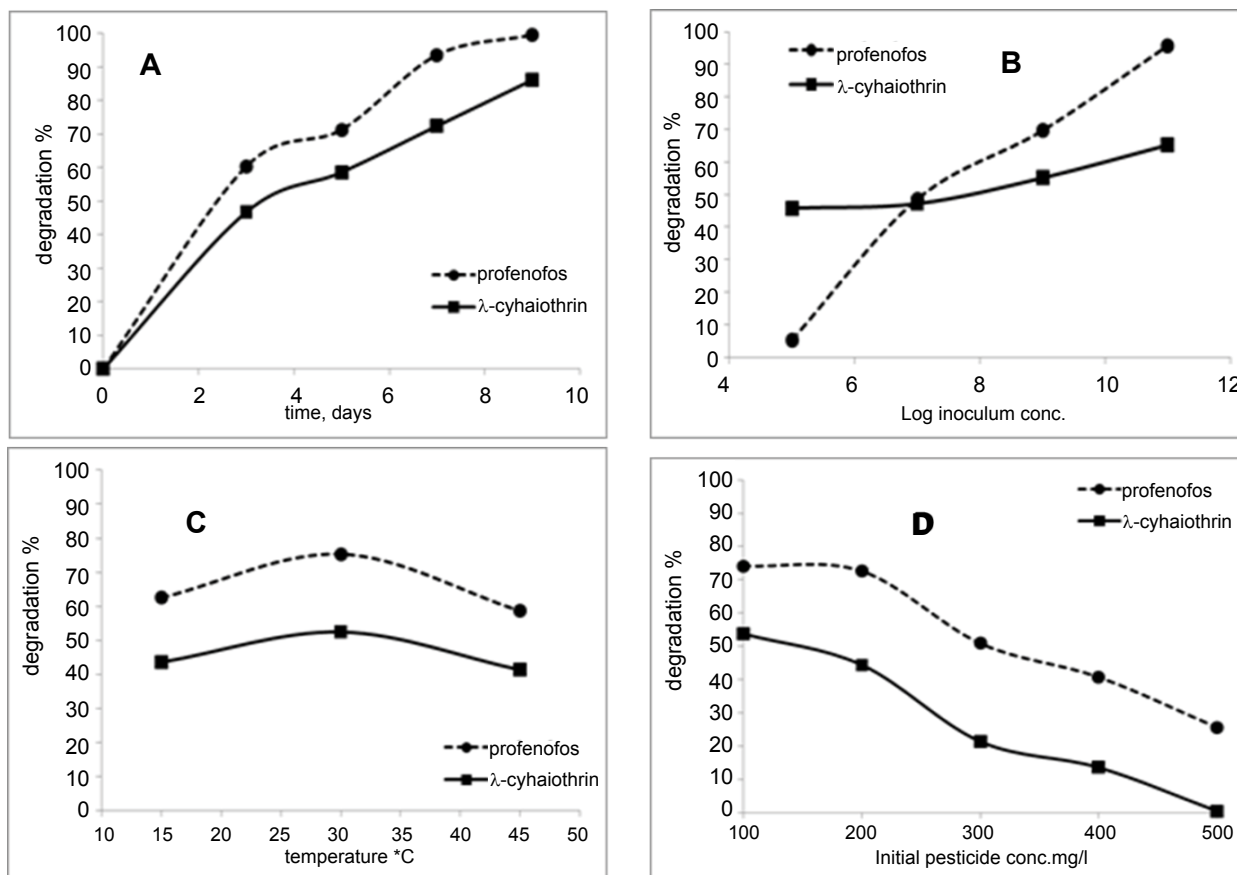


Figure 3: Degradation kinetics of profenofos and λ -cyhalothrin by *Pseudomonas putida* in liquid batch culture media under different conditions.

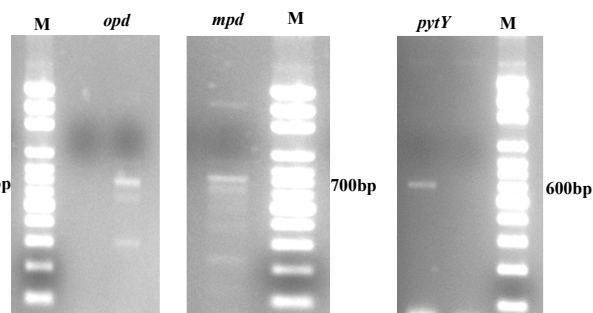


Figure 4: Detection of the *pytY*, *mpd* and *opd* genes in the genomic DNA of the DB17 isolate.

indicated that this gene was 99% similar to *mpd*. Also, they mentioned that the *mpd* and *opd* genes appear to have evolved from different sources. Ref. [17] isolated pyrethroid-degrading gene *pytY* by screening the genomic library of *Ochrobactrum anthropi* YZ-1, and expressed it in *Escherichia coli* BL21 (DE3). Also, they reported *estP*, *pytH*, *pye3*, and *pytZ* as the pyrethroid degrading genes.

In conclusion, several samples of contaminated soils, with a history of profenofos and λ -cyhalothrin insecticides usage, were collected. Eighteen isolates were isolated from the collected soil samples. All the bacterial isolates were subjected to preliminary degradation test to investigate their ability to degrade profenofos and λ -cyhalothrin in liquid broth medium. Isolate DB17 showed the highest efficiency to

degrade both pesticides among other isolates. An analytical method using QuEChERS followed by GC/MS was validated and used for samples' analysis. DB17 isolate was identified to be *Pseudomonas putida*. Genes responsible for organophosphorus and pyrethroid pesticides were detected in DB 17 isolate. The obtained results confirmed that the isolate DB17 *P. putida* can be successfully used for bioremediation of contaminated aquatic environments. Further investigation is required to confirm the ability of DB17 to degrade the tested pesticides in soil.

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