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High Profile Chlorpyrifos Degrading *Pseudomonas putida* MAS-1 from Indigenous Soil: Gas Chromatographic Analysis and Molecular Characterization

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Abstract – Chlorpyrifos is an organophosphorous insecticide applied to soil to control pests in agricultural fields. It is well known important insecticide used for crop protection in Pakistan and its massively spread contamination is of logical concern. A number of water and land ecosystems may get polluted with organophosphorus compounds. These compounds have a significant level of toxicity for mammals including humans. It is thus essential to exclude these toxicants from the ecosystem as quickly as possible. The feasible approaches to achieve such goals include biodegradation and biomineralization (utilization). The study was designed to determine the potential of *Ps. putida* MAS-1 to degrade / utilize chlorpyrifos. Gas chromatography studies revealed that *Ps. putida* MAS-1 degraded chlorpyrifos rapidly. For the final identification of *Ps. putida* MAS-1 PCR amplification of V4 variable regions of 16S rDNA was done which showed band size of 876 bp. The research findings presented in this manuscript indicate that *Pseudomonas putida* MAS-1 can be exploited for the biodegradation of chlorpyrifos polluted environment.
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Keywords – Biodegradation, Organophosphorous pesticides, Chlorpyrifos

1. Introduction

Organophosphorus compounds are used globally as agri-domestic pesticides. The first ever organophosphorus pesticide (tetraethyl pyrophosphate) was used in 1937 [1]. These compounds constitute 38% of overall pesticides use [2]. Chlorpyrifos (*O*, *O*-diethyl *O*-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate) belongs to organophosphates has the distinction as one of the most extensively and widely used pesticides which is effective against a very wide variety of economically viable crop pests (insects) [3]. Intensive and persistent use of organophosphorus chemicals has been an important source of pollution of a number of environmental systems globally [4] that's why biodegradation of these chemicals has gained considerable attention [5]. Biodegradation is an eco-friendly and highly efficient process and can be used as an alternative to chemical and physical methods [6]. The application of microbial diversity for the sake of biodegradation requires the knowledge based on microbiological, physiological, biochemical, and environmental considerations relevant to contaminant pesticides biotransformation [7]. Microorganisms can utilize pesticides as source of energy. In fact, soil bacteria constitute the principal biological agents that are responsible for accelerated biodegradation [8].

Recently, nine morphologically different bacterial strains were isolated from the chlorpyrifos contaminated soil. Among them four bacterial strains which were more efficient in the biodegradation of chlorpyrifos were developed as consortium [9]**.** In another study, it has been reported that 4 strains of *Pseudomonas* (from a water waste irrigated agri-soil in India) were able to utilize chlorpyrifos as an exclusive carbon source [10**]**. The environmental fate of chlorpyrifos has been studied extensively and its degradation may involve a combination of chemical hydrolysis, photolysis, and microbial degradation [11]. The presence and degradation profile of chlorpyrifos in the ecosystem, food samples and different crops is determined by using methods e.g., Gas Chromatography (GC), Gas Chromatography - Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC) and High Performance Liquid Chromatography - Mass Spectrometry (HPLC–MS). A number of pesticides happen to be thermostable with low polarity and are volatile compounds, thus GC is rated as better technique for pesticide analysis. Flame Photometry based estimation, because of its good selectivity and precision based response for sulfur and phosphorus can be used for the estimation of sulfur or phosphorus based chemicals [12]. Keeping in view, the importance of chlorpyrifos as a hazardous and toxic pesticide and an environmental pollutant, the present study was focused on the biodegradation potential (using gas chromatography) and PCR based 16S rDNA identification of indigenous soil isolate *Pseudomonas putida* MAS-1.

2. Materials and Methods

2.1. Media and Chemicals

Nutrient broth (BioM Laboratories, USA) and minimal salt medium were used for chlorpyrifos degradation studies. Chlorpyrifos (Pakistan Agro Chemical Pvt.) and HPLC grade hexane (Merck, Germany) were used.

2.2. Bacterial Strain

An indigenous cotton soil chlorpyrifos degrading bacterial isolate *Pseudomonas putida* MAS-1 was used in this study. The isolate could resist chlorpyrifos upto 20 mg/mL.

2.3. Study of the Degradation Profile of Chlorpyrifos by Pseudomonas putida MAS-1

2.3.1. Inoculum Preparation for Degradation Studies

Pseudomonas putida MAS-1 was grown in conical flasks containing nutrient broth and minimal medium (20 mL) with 2mg/mL concentration of chlorpyrifos. Flasks were incubated at 37˚C in shakobator for 24 hours at 100 rpm. The inoculated flask contents were centrifuged and washed three times with sterilized distilled water. For gas chromatography experiments $3x10^8$ cells/mL were used [13].

2.3.2. Inoculation and Fortification of Media

The bacterial culture suspension (100 µL) containing $3x10^8$ cells/mL was inoculated into the flasks containing nutrient broth and minimal medium, 20 mL each with 2mg/mL concentration of chlorpyrifos. Uninoculated flasks with nutrient broth and minimal medium (having 2mg/mL chlorpyrifos) served as controls. The flasks were incubated at 37˚C for different time intervals (0 hour, 24 hours, 48 hours, 72 hours and 96 hours) [5].

2.4. Extraction and Partitioning

Using the liquid-liquid extraction method, extraction of chlorpyrifos was performed. Chlorpyrifos containing culture media were transferred to a separating funnel and 20 mL n-hexane was added to each medium. The mixture was shaken vigorously for 4 to 5 minutes and then left undisturbed until separation of two liquids took place. The sample was extracted three times and the n-hexane layer was collected in 250 mL conical flask. The extract was evaporated on rotary evaporator at 50°C to almost dryness under pressure with the help of vacuum pump and residue was redissolved in 2 to 5 mL n-hexane in sterile glass vials for GC determination [14].

2.5. Soil Treatment

The bacterial culture suspension (100 µL) containing $3x10^8$ cells/mL was inoculated into tubes containing 20 grams of autoclaved soil and 2mg / g concentration of chlorpyrifos [15]. Uninoculated soil was kept as control. The tubes were incubated at an ambient temperature for different time intervals (0 hour, 24 hours, 48 hours, 72 hours and 96 hours).

2.6. Extraction of Chlorpyrifos

For extraction purpose, the soil samples were air dried and homogenized with 0.5 grams charcoal activated for 4 hours at 120°C, 1.0 gram florisil activated for 4 hours at 650°C and 5 drops of 25% ammonium hydroxide solution and then placed over a 2.5 cm layer of anhydrous sodium sulphate in a glass column with 34 cms length and 2.5 cms diameter. Extraction was done by using the solution of distilled n-hexane and acetone (9:1). Eluted material was collected in a 250 mL conical flask and later evaporated on a rotary evaporator to almost dryness. The residue was dissolved in 2-5 mL n-hexane in small glass vials for GC determination [15].

2.7. Residual Chlorpyrifos Determination (extraction from soil and nutrient broth)

The chlorpyrifos containing extracts were analyzed on GC (Varian-3600), USA equipped with Flame Ionization Detector (FID). The column size was 2 meters x 1/4" x 2mm internal diameter packed with 1.5% OV-17 +1.75% OV-210 Chrom W, HP 80/100 mesh. Hydrogen and nitrogen flow rate 4.5 mL/min and 175 mL/min. respectively. The column temperature was 250°C, injector temperature 250°C and detector temperature 300°C. Detection limit 1µg and retention time 4.032 minute [16].

2.8. Residual Chlorpyrifos Determination (extraction from minimal medium)

GC-FID analyses of extracts were performed on a Shimadzu GC-17A gas chromatograph. The column size was 30 meters x 0.25 mm internal diameter with Optima-5 (Macherey-Nagel) capillary column. The composition of the capillary column was 5% phenyl-95% methylpolysiloxane. The analyses were performed in the split mode with 1:10 ratio. The column and injector temperatures were maintained at 300°C and 400°C respectively and detector temperature was maintained at 450°C. The column pressure was 77 kPa with 0.905689 mL/min column flow and the total flow was 12 mL. The carrier gas was nitrogen.

$$\% \text{ Recovery of Chlorpyrifos} = \frac{peak\ hight\ of\ sample}{peak\ hight\ of\ standard}$$

2.9. Polymerase Chain Reaction Based Identification of Pseudomonas putida MAS-1

Bacterial DNA for PCR was extracted using the PUREGENE, genomic DNA purification kit. The presence of Ps. putida MAS-1 DNA was confirmed by PCR amplification of V4 variable regions of 16S rDNA sequences with the primers listed below by the method of [17]. S-D-Bact-1390-a-A-20 (5'-AGGCCCGGGAACGTATTCAC-3') 1293-1318 S-S-P.put-0597-a-A-20 (5'-TTGCCAGTTTTGGATGCAGT-3') 1587-1563. The DNA primers targeting the specific V4 variable region of the 16S rDNA region were detected by polymerase chain reaction (PCR). Amplification yielded PCR product of 876 bp DNA fragment, detectable by agarose gel electrophoresis.Positive and negative controls were included in the PCR analysis to make it more reliable and authentic.

3. Results and Discussion

Insect pests pose a potential threat to the world food supply, human-animal health, and the livelihoods of the farmer communities. Scientific development must effectively adapt to provide sustainable tools to manage the insect pests. Insecticides such as chlorpyrifos have played, and will continue to play an important role in a sustainable insect management strategy.

Results of the present study were based on the biodegradation potential and molecular characterizations of *Pseudomonas putida* MAS-1 from cotton grown field of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. This site was selected as a number of pesticides including chlorpyrifos have been in use for many years. Chlorpyrifos degrading Providencia stuartii was isolated from agricultural soil with a 10 year history of chlorpyrifos application [18]. Since it is one of the most commonly used commercial insecticides, it is thus logical to isolate bacteria from contaminated soils [19].

The gas chromatography studies were undertaken, under three different conditions. The results are shown in table 1

and Figures 1 and 2, indicating the percentage recovery of the residual chlorpyrifos. It is clearly evident that with the passage of time, the recovery of the residual chlorpyrifos was gradually reduced.

Table 1. Percentage Recovery of Residual Chlorpyrifos from Nutrient Broth, Soil and Minimal Medium Inoculated with *Pseudomonas putida* MAS-1

Time (hours)	% Recovery		
	Nutrient Broth	Soil	Minimal Medium
0	85.50	85.50	99.79
24	81.57	82.02	9.59
48	66.52	60.86	6.19
72	48.78	42.60	5.27
96	28.60	24.00	2.71

It was revealed that *Pseudomonas putida* MAS-1 degraded chlorpyrifos rapidly. Accordingly, with the passage of time, the recovery of the residual chlorpyrifos was gradually reduced (24% and 28.6% recovery from soil and nutrient broth respectively after 96 hours). Degradation of chlorpyrifos in soil by *Sphingomonas* sp. strain Dsp-2, isolated from the polluted water of an industry, manufacturing chlorpyrifos was also studied [13]. These researchers have shown similar pattern of decrease as of ours, in residual concentration of chlorpyrifos. Recently, chlorpyrifos degradation was studied in soil by *Bacillus cereus* (isolated from a Chinese soil) [20]. The researchers investigated the ability of *B. cereus* to degrade chlorpyrifos under different cultural conditions.

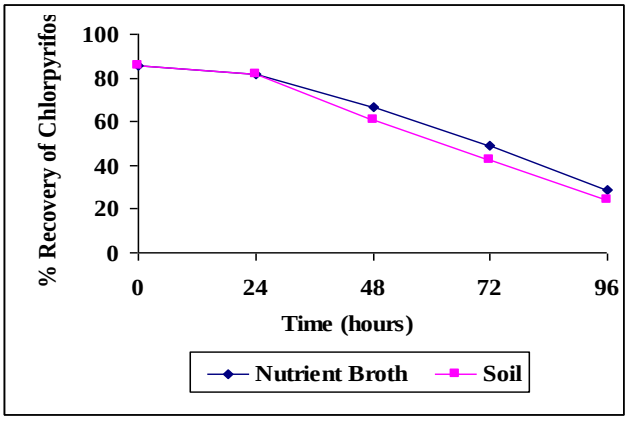

Figure 1. Percentage Recovery of Residual Chlorpyrifos from Nutrient Broth and Soil Inoculated with *Pseudomonas putida* MAS-1.

Pseudomonas putida MAS-1 utilized almost 90% chlorpyrifos during the first 24 hours when grown in minimal salt medium (table 1) containing 2 mg/mL chlorpyrifos (without glucose). Accordingly, that having no other option for the bacterium except chlorpyrifos as carbon and energy source (in the minimal salt medium without glucose), it instantly started degrading / utilizing the pesticide. Earlier, *Enterobacter* strain B-14 was reported that could degrade all chlorpyrifos within 2 days in mineral salt medium with nitrogen (MSMN) without any other source of carbon [5].

In the light of the findings / report of [11], our isolate *Pseudomonas putida* MAS-1 may be equated with *Paracoccus* sp. strain TRP as regards the splendid potential to biodegrade chlorpyrifos. This strain was able to utilize chlorpyrifos and 3,5,6-trichloro-2-pyridinol, methyl parathion etc., when made available as energy and carbon source. Biodegradation of chlorpyrifos by TRP strain was followed by GC-MS and HPLC without observing any intermediate compounds. The left outs of different pesticides using GC-FID by *Alcaligenes faecalis* strain DSP3 were also reported [21]. This strain was able to utilize / degrade chlorpyrifos and 3,5,6-trichloro-2-pyridinol within 10 days (in a mineral salt medium). Similar to our findings, *Alcaligenes faecalis* strain DSP3 rapidly degraded chlorpyrifos and 3,5,6-trichloro-2-pyridinol during the initial 48 hours.

It is worth explaining that the isolate *Pseudomonas putida* MAS-1 did not start utilizing chlorpyrifos (in both conditions / approaches) in their earlier growth phases after inoculation into chlorpyrifos containing nutrient broth and soil. However, the isolate started degrading / utilizing chlorpyrifos after 24 hours (as shown in Figure 2). One of the possible reasons for this non-utilization of chlorpyrifos seems to be the availability of the alternate source of nutrients present in nutrient broth, thus the isolate did not bother to utilize the pesticide.

However, in case of utilization profile of chlorpyrifos present in minimal salt medium without glucose, *Pseudomonas putida* MAS-1 started utilizing chlorpyrifos soon after its inoculation into this medium. It is because of having no option of nutrients availability, thus, it had to rely upon the chlorpyrifos to fulfill their carbon and energy requirements. Consequently, *Pseudomonas putida* MAS-1 started vigorously utilizing chlorpyrifos within 24 hours of inoculation (as shown in Figure 2).

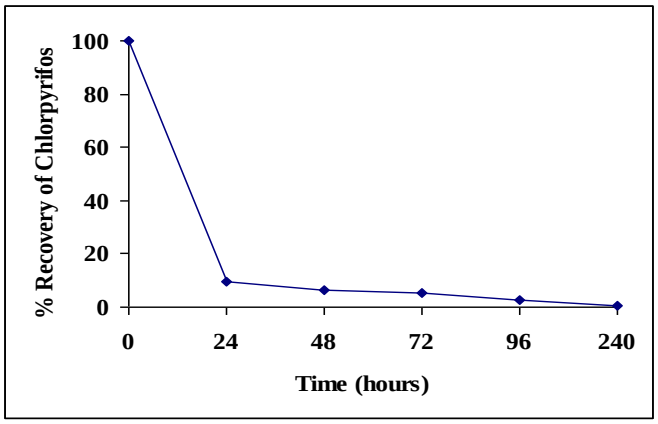

Figure 2. Percentage Recovery of Residual Chlorpyrifos from Minimal Medium Inoculated with *Pseudomonas putida* MAS-1.

It appears that *Pseudomonas putida* MAS-1 has a preference for utilizing chlorpyrifos as a substrate, which served as sole source of carbon and energy. This proposition is supported by the biodegradation profile of *Paracoccus* sp. strain TRP and *Sphingomonas* sp. strain Dsp-2 [11, 13].

PCR based identification is the best method / approach (in addition to other classical methods including morpho-cultural and biochemical tests) for the final identification (of *Pseudomonas putida* MAS-1). The sensitivity, specificity and precision of PCR based diagnosis were established for not only as a confirmatory tool but also strains identification. The identification of *Pseudomonas putida* MAS-1 was done by PCR amplification of V4 variable regions of 16S rDNA showing band size of 876 bp (Fig.3). The identification of *Pseudomonas putida* and *Burkholderia* sp. strain JS 150 and *Bacillus subtilis* ATCC 7003 (hydrocarbon degrading

bacteria), based on V4 variable segment of 16S rRNA was also reported [17].

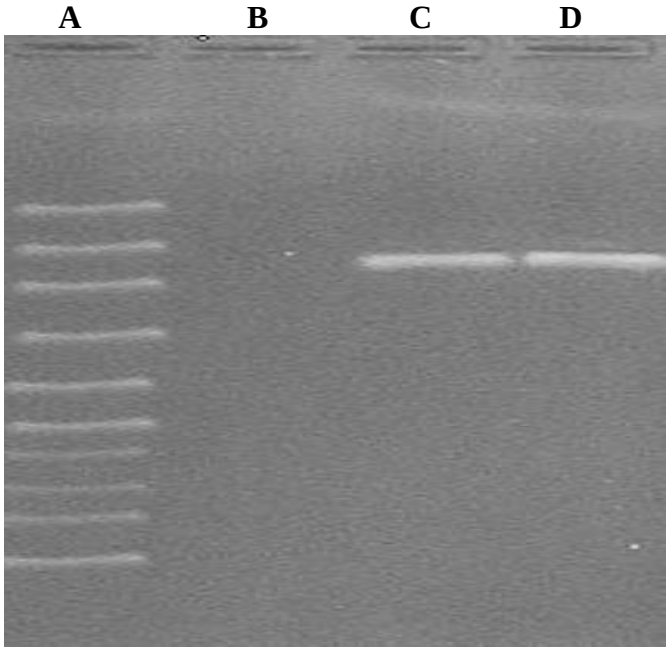

Figure 3. Identification of *Ps. putida* MAS-1 by PCR Amplification of V4 Variable Regions of 16S rDNA Showing Band Size of 876 bp.

Lane A 100 bp ladder (DNA marker)
Lane B Negative amplification control
Lane C 876 bp amplified positive sample of Ps. putida MAS-1
Lane D Positive control of Ps. putida

4. Conclusion

Studies based on selective isolation and characterization of pesticide degrading microorganisms are crucial for understanding the variety of mechanisms and biodegradative metabolic pathways related to the accelerated degradation in the ecosystem(s). Chlorpyrifos, which was previously thought to be immune to the enhanced biodegradation, has now been shown to be efficiently biodegraded by bacterial and fungal systems. Bioremediation technologies are in the process of development for this toxic compound and the associated nerve gas agents using organophosphorus hydrolase enzyme system. Future studies may be focused on the genes responsible for the enhanced biodegradation in order to elucidate the exact degradative pathways involved in microbial biodegradation of chlorpyrifos.

The research findings presented in this communication indicate that *Psuedomonas putida* MAS-1 (an indigenous isolate from cotton fields of Pakistan) can be exploited for the biodegradation of chlorpyrifos polluted environment (including the agricultural and waste water laden soil fields). This isolate seems to be a superb and swift chlorpyrifos biodegrading indigenous bacterium identified as *Pseudomonas putida* MAS-1.

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