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Research Article

PHYSICOCHEMICAL AND MICROBIOLOGICAL PROPERTIES OF APPLE ORCHARD SOILS AS AFFECTED BY LONG TERM APPLICATION OF CHLOROPYRIFOS

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Abstract- Samples of soil from apple orchards with previous history of continuous and extensive use of chlorpyrifos were collected. Chlorpyrifos residues were determined in soil samples collected from Shimla and Kullu districts of Himachal Pradesh. Residues of Chlorpyrifos were estimated by GC equipped with FID system. A higher percentage recovery of chlorpyrifos was observed (90.80-93.60%) in Kullu district soil samples than Shimla district samples. The pH and electrical conductivity (EC) of the soil samples from apple orchards were recorded and ranged from 6.66-7.23 and 0.27-0.37 dSm^{-1} respectively. The percent organic carbon, nitrogen, phosphorus and potassium content of soil samples ranged from 0.69-1.12%, 312.67-479.67 Kg/ha, 13.37-23.20 Kg/ha and 261.67-365.67 Kg/ha respectively. Mesophilic bacterial population colonizing various apple orchard soil samples were enumerated after 48 hrs of incubation on nutrient agar at 37°C using serial dilution technique and it was observed that bacterial population in the samples ranged between 0.78 x 10^5 to 2.41 x 10^5 cfu/g. A total of seventy two different bacterial strains were further isolated and out of them only fifteen bacterial stains exhibited chlorpyrifos degrading potential. In our study we monitored chlorpyrifos residues along with the physico-chemical analysis of soil samples and also investigated indigenous bacteria inhabiting the apple orchard soils with continuous exposure to chlorpyrifos.

Keywords- Chlorpyrifos, Gas Chromatography (GC), Flame Ionization Detector (FID), Physico-chemical Properties

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Introduction

In Himachal Pradesh, apple is the most ubiquitous among various temperate fruit and Shimla and Kullu are two major apple producing districts of the state. Pesticides are employed to prevent insect pests and obtain higher yields of apples. Chlorpyrifos is a non-systemic, neurotoxic organophosphorus insecticide, with broad spectrum insecticidal activity commonly employed against some of the major pests like San Jose scale (*Quadraspidiotusperniciosus*), wooly apple aphid (*Eriosomalanigerum*), Blossom thrips (*Thripsflavus*), Apple leaf roller (*Archipstermid*), Root borer (*Dorystheneshugelli*), Two-spotted spider mite (*Tetranychusurticae*) etc. Chlorpyrifos is available formulations which include emulsifiable concentrates (EC), granulars (GR) and wettable powder (WP) [1].

The potential damage by chlorpyrifos to non-target organisms is high because acetyl cholinesterase is present in all vertebrates [2]. A diversity of valuable non-target arthropods like bees, ladybird beetles and parasitic wasps are killed by chlorpyrifos usage. Prolonged exposure to chlorpyrifos results in delayed seedling emergence, fruit deformities and abnormal cell division in plants [3]. Chlorpyrifos can remain actively persistence in the soil for periods ranging from few days to even months. Dosage rates, soil type, soil moisture and organic carbon content, pH, temperature and insecticide formulation are the main factors which influence biological persistence [4-6]. This insecticide has been detected in marine, sediments, streams, sumps, sloughs, rivers, urban storm drains, freshwater lakes, groundwater, fog, rain and air [7].

Farmers have adopted various agrochemicals especially fertilizers and pesticides to improve food production and control diseases. These agrochemicals contaminate farmlands and domestic water supply sources and also modify the physicochemical properties and microbial diversity of the affected soil [8, 9]. Microbial populations are well-known to adapt in the presence of agrochemicals by change in species diversity or by adaptation of enzyme systems, so that agrochemical may be more rapidly metabolized and therefore eliminated from the environment [10]. Keeping in view the human health and ecological risks associated with chlorpyrifos and its widespread use in apple orchards of Himachal Pradesh, prompted us to investigate residual chlorpyrifos and its effect on physio-chemical properties and native microbes.

Material & Methods

Soil sample collection

Soil samples from apple orchards topsoil were collected and placed in sterilized polythene bags using sterilized spatula. A total of 30 soil samples from ten sites of the two districts were screened for chlorpyrifos residues and physico-chemical properties.

Extraction and cleanup

Extraction of soil samples was done by means of a soil-packed sintered column. Air dried soil samples were homogenized with 0.5 grams charcoal activated for 4 hrs at 120°C, 1.0 gram florisil activated for 4 hrs 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 cm length and 2.5 cm diameter. For extraction, a mixture of solvent solutions (n-hexane and acetone in ratio 9:1) was used. Eluted material was collected in a 250 ml conical flask and later evaporated on a rotary evaporator (Mac Scientific) to almost dryness. The residue was dissolved in 2.0-5.0 ml n-hexane in small glass vials for GC determination [11].

Residual Chlorpyrifos Determination from extracts

GC-FID analysis of extracts was executed 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. Column pressure of 77 kPa along with 1.0 ml/min column flow and total flow of 12 ml was employed for the analysis in 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 carrier gas used was nitrogen [11]. Percentage recovery of chlorpyrifos was calculated as follows:

$$\% \text{ Recovery of Chlorpyrifos} = \frac{\text{Peak height of sample}}{\text{Peak height of standard}}$$

Soil Physico-Chemical Properties

The physico-chemical properties of experimental soil: pH, Electric conductivity (EC), organic carbon, total nitrogen, available phosphorus and available potassium content, were estimated by combined glass electrode pH meter method, soil water suspension method – using conductivity meter (ESICO-1601), Walkley and Black's rapid titration method, modified macro Kjeldahl method, Olsen's method and Flame photometer method, respectively [12].

Enumeration of Bacterial Population

Ten grams of the chlorpyrifos contaminated soil was suspended in 90 ml of distilled water and tenfold serial dilution of the soil samples from 1:10 to 1:100000 were carried out. 0.1 ml of the 10^{-5} dilution for each soil samples were plated in triplicate on nutrient agar amended with nystatin to suppress the growth of fungi using pour plate methods [13]. The nutrient agar plates were incubated at 37°C for 48 hours. The number of viable micro-organisms in the sample was calculated from the number of colonies formed and the volume of inoculums and the dilution factor expressed in colony forming unit.

Characterization of Isolated Bacterial with Chlorpyrifos Degradation Potential

Enrichment technique using mineral salt medium [14] was employed for isolation of bacterial isolates [15]. Morphological characterization of the isolated bacterial strains was followed by screening for chlorpyrifos degrading potential on eosin methylene blue agar (EMBA) media indicator [16, 17]. Examination was done of the bacterial isolates for their capability of degrading 50 mg/l chlorpyrifos fortified EMBA plates. Isolates were streaked on the surface of each half-strength EMBA indicator medium containing 50mg/l chlorpyrifos and incubated at 37^{0}C for 48hrs. Potential chlorpyrifos degrading ability of the bacterial isolates was observed as the color change of colonies into red. Strains exhibiting chlorpyrifos degrading potential were biochemically characterized and possible genera were determined using Bergey's Manual of Determinative Bacteriology [18]. Secondary screening for presence of Organophosphorus hydrolase (OPH) activity was done using standard method [19]. Isolates exhibiting higher OPH activities were further characterized using 16S *rrna* gene technology, where all the genomic DNA samples were extracted from the isolates and selectively amplified using universal primer 27f (5- AGAGTTTGATCCTGGCTCAG-3, forward) and 1492r (5'-GGTTACCTTGTTACGACTT-3', reverse). Sequencing was carried out with an automated sequencer (Genetic analyzer 31030, Accessories Applied Biosystems). Using the EzTaxon server, phylogenetic neighbors were identified and the pairwise 16S rRNA sequence similarities were calculated [20].

Results and Discussion

Percentage average recovery of chlorpyrifos residues estimated using analytical method was found to be lower in soil samples from Shimla district as compared to Kullu district samples. Highest percentage average recovery 93.60% was observed in soil samples from Manali while lowest recovery of 84.60% was noted in Kotkhai samples of Shimla district [Table-1]. Sharma *et al*. (2016) also reported 90.35% average recovery of chlorpyrifos from soil and water samples from four sites i.e. Bajaura (district Kullu), Mashobra (district Shimla), Kukumseri (district Lahaul & Spiti) and Rekong Peo (district Kinnaur) [21]. Similar observations of soil contamination with chlorpyrifos were recorded by researchers in Northern India [22]. Percentage recovery varied in apple orchard soil samples probably due to different physio-chemicals properties and microbial biota inhabiting apple orchard soils. Low recovery percentage of chlorpyrifos from soil samples of Shimla district sites may be attributed to rapid degradation of the pesticide by microbial flora inhabiting soil having neutral to alkaline soil pH conditions.

Table-1 *Recovery of chlorpyrifos pesticide at different fortification levels from apple orchard soils*

District	Soil sample site	Fortification Levels (mg Kg^{-1})	Recovery (%)
Shimla	Kotkhai	(0.01, 0.05, 0.1)	84.60 (82.30 - 86.90)
	Rohru	(0.01, 0.05, 0.1)	86.26 (80.30 - 92.22)
	Theog	(0.01, 0.05, 0.1)	85.28 (82.34 – 98.22)
	Matiana	(0.01, 0.05, 0.1)	85.70 (84.70 – 86.70)
	Chopal	(0.01, 0.05, 0.1)	87.35 (84.35 – 90.35)
Kullu	Naggar	(0.01, 0.05, 0.1)	91.85 (90.55 – 93.15)
	Manikaran	(0.01, 0.05, 0.1)	90.80 (88.75 – 93.85)
	Lag Valley	(0.01, 0.05, 0.1)	92.25 (91.35 – 93.15)
	Sew Bagh	(0.01, 0.05, 0.1)	91.25 (89.36 – 93.14)
	Manali	(0.01, 0.05, 0.1)	93.60 (91.34 – 95.86)

Physico-chemical properties (pH, electrical conductivity, organic carbon, total nitrogen content, available phosphorus (P_2O_5) and potash (K_2O) contents) of the apple orchard soil samples are presented in [Tables-2]. Apple orchard soil pH for all Shimla district sites was found to be slightly more alkaline then that of Kullu district sites. Previously, also few scientists had reported that soils of dry temperate zone of Himachal Pradesh ranges from neutral to alkaline (pH 6.2-10.3) in reaction [23,21]. The electrical conductivity observed from this study showed that electrical conductivity value of soil samples was between 0.26 - 0.37dsm^{-1} and is acceptable for growth of plants [24].

Table-2 *Mean value*

Variables / Sites	pH	Electrical Conductivity (EC)	Organic carbon (OC%)	Nitrogen (N) (Kg/ha)	Phosphorus (P) (Kg/ha)	Potassium (K) (Kg/ha)
Kotkhai	7.07	0.27	1.07	479.67	22.90	349.33
Rohru	7.12	0.33	1.12	426.00	23.20	365.67
Theog	7.23	0.29	0.85	386.67	17.90	309.00
Matiana	7.08	0.36	0.97	475.33	22.87	350.00
Chopal	6.96	0.37	0.89	387.67	16.73	301.00
Naggar	6.72	0.29	1.08	454.33	18.20	349.33
Manikaran	6.65	0.26	0.77	385.67	14.50	285.33
Lag Valley	6.77	0.30	0.78	370.00	13.37	302.33
Sew Bagh	6.67	0.33	0.69	348.00	14.93	261.67
Manali	6.66	0.30	0.84	312.67	13.47	266.67

For percent organic carbon, maximum mean value of 1.12 % was noted for Rohru, whereas minimum value of 0.69% was found in Sew Bagh sites. For nitrogen content, maximum mean value of 479.67 kg/ha was observed in Kotkhai site followed by Naggar (454.33 kg/ha) and minimum mean value of 312.67 kg/ha was observed for site Manali. For phosphorus content, maximum value of 23.20 kg/ha was observed in Rohru site followed by Kotkhai (22.90 kg/ha) however, the minimum value of 13.37 kg/ha was recorded in Lag Valley site. For potassium content, Rohru gave maximum mean performance of 365.67 kg/ha followed by Matiana (350.00 kg/ha), whereas Sew Bagh site was found to give minimum mean performance of 261.67 kg/ha.

Enumeration Of Bacterial Population In Various Soil Samples

Mesophilic bacterial population colonizing various apple orchard soil samples were enumerated after 48 hrs of incubation on nutrient agar at 37°C using serial dilution technique [Table-3]. Serial dilution technique provides us with information regarding enumeration of culturable bacteria within a particular soil, which further gives indication of soil health. It was found that bacterial population in all soil samples studied ranged from 0.78 x 10^5 - 2.41 x 10^5cfu/g. It was further observed that maximum bacterial population was found in S_2R site of Rohru with value of 2.41 x 10^5cfu/g and minimum bacterial population was recorded at S_9Se site of Sew Bagh with value 0.78 x 10^5cfu/g. Perusal of [Table-3] depicts that bacterial population of 2.39 x 10^5cfu/g at S_1K site of Kotkhai was found to be at par with S_2R site of Rohru.

Table-3 *Enumeration of bacteria from ten apple orchard sites of Himachal Pradesh*

Soil sample Sites	Bacterial population on nutrient agar after 48 hrs of incubation at 37°C(10^5cfu/g)		
	S_1	S_2	S_3
Kotkhai (K)	2.39	2.12	2.18
Rohru (R)	1.96	2.41	2.03
Theog (T)	1.81	2.07	1.97
Matiana (M)	1.48	1.76	1.69
Chopal (C)	1.36	1.54	1.29
Naggar (N)	1.31	1.02	1.27
Manikaran (M)	1.28	1.44	1.33
Lag Valley (L)	0.98	1.21	1.13
Sew Bagh (Se)	0.78	1.10	0.97
Manali (Ma)	1.34	1.16	1.41

S_1, S_2&S_3 sub-sites of ten apple orchard sites of H.P.

Isolation and Characterization

Seventy two different bacteria were isolated by enrichment technique employing mineral salt medium for growth [15]. Selective secondary screening of chlorpyrifos degrading bacteria from 72 isolates was carried out on selective medium i.e. eosin-methylene blue agar (EMBA) containing 50 mg/l chlorpyrifos. Only 15 isolates were able to change the color of colonies from creamish/ yellowish/ whitesh/transparent to red color, which was observed as an indication of chlorpyrifos degradative ability of these bacterial isolates [Fig-1].

Biochemical/metabolic characteristics are also very useful because they are directly related to the nature and activity of microbial enzymes and transport proteins. Important in the identification of a genus and species of bacteria are biochemical tests, including the determination of the kinds of nutrients a cell can use, the products of its metabolism, the response to specific chemicals, and the presence of particular characteristic enzymes. Fifteen chlorpyrifos degrading bacterial isolates were selected on basis of their ability to produce red colonies in the previous experiment were subjected for biochemical characterization and were examined for various biochemical tests *viz.,* catalase, citrate, urease, triple sugar iron agar, indole, methyl red, Voges-Proskauer, oxidase, casein hydrolase, gelatin and fermentation of sugars i.e. lactose, glucose and sucrose [Table-4].

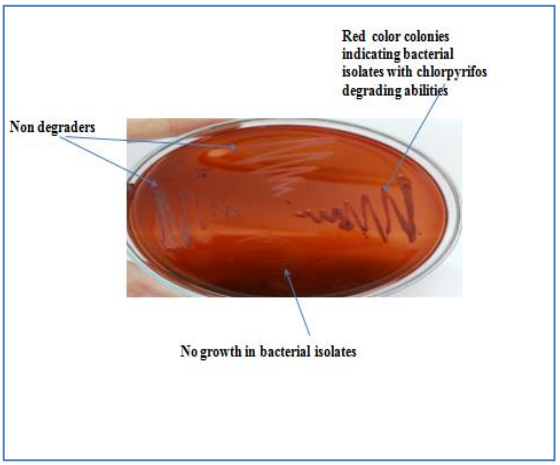


Fig-1 Bacterial isolates with chlorpyrifos degrading potential

Based on comparison of characteristics exhibited by the bacterial isolates with the standard description in the Bergey's Manual of Determinative Bacteriology, the selected isolates were tentatively identified as member species of *Y-Proteobacteria* and *Bacillus* genera. Similarly, Bhagobaty and Malik performed morphological and biochemical tests on the isolated bacteria and found that they belong to the genus *Pseudomonas* [13]. Lu *et al.* also isolated and characterized the chlorpyrifos-degrading bacterium DT-1 as *Cupriavidus* sp. according to the Bergey's Manual of Determinative Bacteriology [18, 25].

Table-4 *Biochemical characterization of selected bacterial isolates with potential chlorpyrifos potential*

Sr. No	Isolate code	Catalase	Citrate utilization	Urease	Triple sugar test	Indole	MR	VP	Oxidase	Casein hydrolase	Gelatin	Lactose	Glucose	Sucrose
1.	$S_1K_{1.1}$	-	-	-	+	-	+	-	+	-	-	+	+	+
2.	$S_1K_{2.1}$	+	+	+	-	-	+	-	-	-	+	-	+	-
3.	$S_1K_{3.2}$	+	+	+	-	+	-	-	+	-	-	-	+	-
4.	$S_2R_{1.1}$	+	+	+	-	-	-	-	+	-	-	-	-	-
5.	$S_2R_{1.2}$	+	+	-	-	-	+	-	-	-	+	-	+	-
6.	$S_3T_{2.1}$	+	+	+	-	-	-	-	+	-	-	-	+	-
7.	$S_3T_{2.2}$	+	+	+	-	-	-	-	+	+	-	-	+	-
8.	$S_3T_{3.2}$	-	+	+	-	-	-	-	+	-	-	-	+	-
9.	$S_3T_{3.3}$	+	+	+	-	-	+	-	-	-	-	+	+	-
10.	$S_4M_{1.1}$	+	+	+	-	-	-	-	+	-	-	-	+	-
11	$S_5C_{1.2}$	+	+	+	+	-	-	+	+	-	-	-	+	+
12.	$S_5C_{2.1}$	+	+	-	-	-	+	-	+	+	+	-	+	-
13.	$S_8L_{1.1}$	+	-	+	+	-	+	-	-	-	-	-	+	-
14.	$S_8L_{3.2}$	+	+	-	-	-	-	+	+	-	-	-	+	-
15.	$S_9Se_{2.1}$	+	+	+	-	-	+	-	-	+	+	-	+	-

S_1K: Isolates from Kotkhai, S_2R: Isolates from Rohru
S_3T: Isolates from Theog S_4M: Isolates from Matiana
S_5C: Isolates from Chopal S_6N: Isolates from Naggar
S_7M: Isolates from Manikaran S_8L: Isolates from Lag Valley S_9Se: Isolates from Sewbagh S_{10}M: Isolates from Manali

Results of all the fifteen bacterial isolates were analysed by SAHN module of NTSYS-Pc (version 2.20) and dendrogram was constructed using UPGMA. The dendrogram constructed based on results of biochemical characters showed that all the bacterial isolates possess 25% similarity. The dendrogram clearly divides into 2 major clusters i.e. A and B with 25% similarity [Fig-2]. Cluster A clearly separates $S_1K_{1.1}$ isolate from rest of the 14 bacterial isolates. Cluster B was further divided in C and D sub-clusters. Sub-clusters C and D were further divided into sub-sub-clusters with further divided into 6 and 7 different groups respectively [Fig-2].

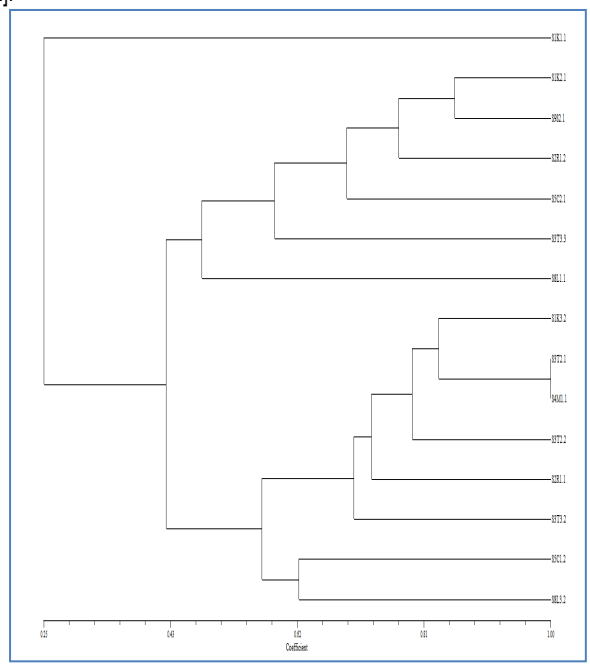


Fig-2 Dendrogram showing relatedness among 15 bacterial isolates with chlorpyrifos degrading potential based on biochemical characters

Screening for Organophosphorus Hydrolase activity

Extracellular and intracellular organophosphate hydrolase enzyme activity of crude enzyme preparation of 15 chlorpyrifos degrading bacterial isolates was determined quantitatively using organophosphate hydrolase enzyme activity assay [19]. Organophosphate hydrolase activity was determined after 24 and 48 hrs of incubation for all the 15 bacterial isolates and only 6 isolates ($S_1K_{3.2}$, $S_2R_{1.1}$, $S_3T_{2.1}$, $S_3T_{2.2}$, $S_3T_{3.2}$ and $S_4M_{1.1}$) were found to produce organophosphate hydrolase enzyme activity.

Chlorpyrifos degrading bacterial isolate $S_1K_{3.2}$ showed maximum extracellular organophosphate hydrolase activity value of 0.165 U/ml after 48hrs of incubation respectively followed by $S_3T_{2.1}$, $S_4M_{1.1}$, $S_2R_{1.1}$ $S_3T_{3.2}$ and $S_3T_{2.2}$ depicting extracellular organophosphate hydrolase activity of 0.157, 0.104, 0.080, 0.076 and 0.052 U/ml respectively after 48hrs of incubation. Extracellular activity is significantly higher in six bacterial isolates as compared to the respective intracellular activity and further these results only six bacterial isolates were selected for further molecular characterization.

Molecular Characterization

Total genomic DNA of the selected six bacterial isolates were extracted successfully using Genomic DNA extraction Mini kit (Real Genomics) and DNA quality was checked using 1.0% agarose gel. After 35 cycles of PCR amplification employing universal primers for 16S *rrna* gene were able to successfully amplify 16S *rrna* gene of only 5 bacterial isolates [Fig-3]. These primers were not able to amplify 16S *rrna* gene of bacterial isolate $S_3T_{2.2}$. Eztaxon-e database was employed to identify these 5 sequences with the most similar 16S *rrna* gene sequences available in this database http://eztaxon-e.ezbiocloud.net/ezt_identify). The 16S *rrna* gene sequence analysis of chlorpyrifos degrading bacterial isolates from apple orchard soil samples of Shimla district - $S_1K_{3.2}$ bacterial isolate from Kotkhai site, showed 98.48% similarity with *Pseudomonas indoloxydans* IPL-1(T) strain, 16S ribosomal RNA, complete sequence - $S_2R_{1.1}$ bacterial isolate from Rohru site, showed 98.54 percent similarity with *Pseudomonas aeruginosa* strain JCM5962(T), 16S ribosomal RNA, complete sequence - $S_3T_{2.1}$ bacterial isolate from Theog site showed 97.70% similarity with *Pseudomonas resinovorans* strain LMG 2274(T), 16S ribosomal RNA, complete sequence - $S_3T_{3.2}$ bacterial isolate from Theog site showed 100% similarity with *Pseudomonas otitidis* strain MCC 10330(T) and 16S ribosomal RNA, complete sequence - $S_4M_{1.1}$ bacterial isolate from Matiana site depicted 98.61% similarity with *Pseudomonas stutzeri* strain ATCC 17588(T) [Table-5].

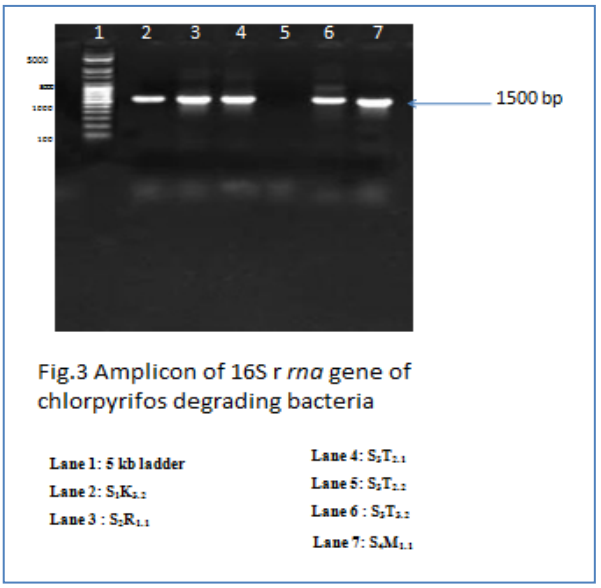
Table-5 *Similarity values of 16S rrna gene sequences of selected five chlorpyrifos degrading bacterial isolates using Eztaxon-e*

Bacterial Isolate	Closest Match	Accession Number	Per cent Similarity	Per cent Completeness
$S_1K_{3.2}$	*Pseudomonas indoloxydans* IPL-1(T)	DQ916277	98.48	74.4
$S_2R_{1.1}$	*Pseudomonas aeruginosa* JCM5962(T)	BAMA01000316	98.54	61.0
$S_3T_{2.1}$	*Pseudomonas resinovorans* LMG 2274(T)	Z76668	97.70	84.0
$S_3T_{3.2}$	*Pseudomonas otitidis* MCC 10330(T)	AY953147	100	63.7
$S_4M_{1.1}$	*Pseudomonas stutzeri* ATCC 17588(T)	CP002881	98.61	74.2

All the five chlorpyrifos degrading bacterial isolates 16S r *rna* gene sequences were submitted to NCBI database and were assigned specific accession number as mentioned in [Table-6]. Our results clearly indicate that *Pseudomonas* species are dominant bacterial population inhabiting chlorpyrifos contaminated apple orchard soils and possess potential for chlorpyrifos degradation.

Table-6 *Chlorpyrifos degrading bacterial isolates with NCBI Accession no.*

S No.	Bacteria code	Strain	Base pairs (bp)	Accession No.
1.	S1K3.2	*Pseudomonas indoloxydans* strain ASK3.2	1141	KP322757
2.	S2R1.1	*Pseudomonas aeruginosa* strain ASR1.1	890	KP322755
3.	S3T2.1	*Pseudomonas resinovorans* strain AST2.2	1231	KP322753
4.	S3T3.2	*Pseudomonas otitidis* strain AST3.2	929	KP322754
5.	S4M1.1	*Pseudomonas stutzeri* strain ASM1.1	1082	KP322756



Conclusion

Lower chlorpyrifos recoveries clearly indicate the higher degradation rate of chlorpyrifos under slightly alkaline soil pH and due to presence of microbial community. Bacterial isolates belonging to *Pseudomonas* genera's are actively involved in chlorpyrifos degradation in apple orchard soils and exhibit high extracellular Organophosphorus hydrolase activity. All the bacterial isolates further can also be used in bioremediation of chlorpyrifos contaminated soils.

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Abbreviations: KPa: Kilopascal, dsm^{-1}: DeciSiemens per meter, kg/ha: Kilograms per hectare, EC: Electrical conductivity, EMBA: Eosin Methylene Blue Agar, Cfu/g: Colony-Forming Unit per gram, NTSYS-Pc: Numerical Taxonomy and Multivariate Analysis System, UPGMA: Un-weighted Pair Group Method with Arithmetic Mean**.,**

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Conflict of Interest: None declared

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