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Isolation of Pesticide Degrading Bacteria From Paddy Fields and Evaluation of Its Bioremediation Potential Efficiency

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ABSTRACT

Pesticides are chemicals that are widely used in the agricultural sector to control pests in the environment. Lambda cyhalothrin is an insecticide that belongs to a group of pyrethroids. As lambda cyhalothrin is persistent in the soil, there is an urgent need to take remedial measures to control environmental pollution. In this study, the ability of bacteria to degrade lambda cyhalothrin from a paddy field was evaluated. Tolerance of bacterial isolates was tested at different concentrations. Among the ten different genera, isolate *Pseudomonas* sp designated as GMMC1was found to tolerate pesticides up to 500 ppm and was selected for further degradation studies. GMMC1, lambda-cyhalothrin degrading bacterium identified by Sequence BLAST analysis,was isolated from the paddy crop soil and found to be the dominant bacteria which tolerates pesticide. Results of a phylogenetic analysis of GMMC1 found it to be closely related to *Pseudomonas fluorescens*. *Pseudomonas fluorescens* (GMMC1) isolate as appears to be the best short term choice for bioremediation of pesticide-contaminated agricultural fields.

Keywords: Pesticides, lambda cyhalothrin, pyrethroids, bioremediation, environmental pollution.

INTRODUCTION

Pesticides are used in agricultural fields to control all kinds of pests in order to increase crop yield and to control weeds. Pesticides contain different products with several different functions but the designation is formed by combining the names of pest and the suffix. Though they help farmers and all agriculturists on a large scale, they are considered hazardous as they can harm humans and animals and are toxic to the environment.There are various types of pesticides, e.g., organochloride pesticides, organophosphorous pesticides, carbamates, neonicotinoids and pyrethroids. Organochloride pesticides are cumulative in the organisms and pose chronic health effects such as cancer and neurological and teratogenic effects (Alewu and Nosiri 2011). The most widely known

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organochlorine pesticide is dichlorodiphenyltrichloroethane, i.e., the insecticide DDT (Turusov *et al.* 2002; Van den Berg 2009).

Organophosphates, which are promoted as a more ecological alternative to organochlorines, include a great variety of pesticides, the most common of which is glyphosate. They affect the nervous system of insects and humans, in addition to influencing the reproductive system (Colosio *et al*.2009). The carbamates are transformed into various products as a consequence of several processes such as hydrolysis, biodegradation, oxidation, photolysis, biotransformation and metabolic reactions in living organisms (Soriano *et al*.2001). Some synthetic pyrethroids such as fenvalerate, sumithrin and permethrin are considered to be safer. There is evidence of pyrethroids affecting the reproductive behaviour of animals and humans too. A recent study related more than one pyrethroid metabolite to DNA damages in human sperm, raising concerns about possible negative effects on human reproductive health (Jurewicz *et al*.2015).The rapid increase in population has resulted in the accumulation of a variety of chemicals in the environment. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, because they are sprayed or spread across entire agricultural fields. Earlier techniques which were used to eliminate them from the environment were landfills, recycling, pyrolysis etc., but these also have adverse effects on the environment and lead to formation of toxic intermediates (Debarati *et al.* 2005). 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 (Singh, 2008).

MATERIALS AND METHODS

Pesticide Used

Lambda cyhalothrin was purchased from a shop dealing with agricultural products in the local market of Pudukkottai, Tamil Nadu.

Collection of Soil Sample

Soil samples were collected from a paddy field located in Keelasivalpatti, Sivaganagai District, Tamil Nadu, India. These fields had been spread with Lambda cyhalothrin for the past few years. The soil samples were collected in sterile polythene bags for further study.

Isolation of Pesticide Degrading Bacteria

The enrichment method was used to isolate pesticide degrading organisms. Enrichment of pesticide degraders was carried out by using 150ml soyabean casein digest medium. The medium was sterilized by autoclaving at 121°C for 15 min. Ten ppm of lambda cyhalothrin was added after autoclaving as a sole carbon source. The soil sample (1g) collected from paddy field was serially diluted with

normal saline and about 1ml of 10^7 was used to isolate bacterial colonies by pour plate method.

Enrichment of Bacterial Culture and pesticide tolerance

The method described by Chen *et al.* (2011) was used. The tryptic soy brothwas autoclaved at 121°C for 20 min, after which it was aseptically spiked with lambda cyhalothrin at different concentrations (100, 250 and 500ppm) andisolated strains were inoculated at OD 1 at 600 nm. Plates were incubated under 35°C for 48 h.The isolate that tolerated 500 ppm was selected for degradation analysis.

Biodegradation of Lambda cyhalothrin

The degradation studies were performed in 1000-ml Erlenmeyer flasks containing 500 ml of sterile MSM supplemented with 100 ppm of Lambda cyhalothrin. Next, the medium was inoculatedwith 50 ml of 24 h bacterial suspension and medium without bacterial culture as control. All samples were incubated on an arbitrary shaker (120 rpm) in a darkened thermostatic chambermaintained at 30±1°C. Samples of MSM were removed aseptically after 48h and cell free culture filtrate was extracted with ethyl acetate and subjected to Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC).

Thin Layer Chromatography (TLC)

Pre-coated silica gel plates (silica gel 60 F254 0.25mm thicknesses, 20×20 cm, Merck Ltd.) were used for TLC of bifenthrin. The TLC plates were spotted with 5µl sample volume at 1cm apart with micropipette with the same volume of standard bifenthrin in lane 1 for comparison of Rf values. The plates were dried and the chromatogram was developed in a pre-saturated tank with Benzene: Ethyl acetate (6:1 by volume) as the solvent system. After developing the plates, the solvent front was immediately marked and extra solvent was evaporated in a fume hood. The plates were kept under UV at 245 nm for 20 min. The spots were marked and Rf values were calculated.

High Pressure Liquid Chromatography (HPLC)

Samples from the treatment were removed on day 2 for pesticide residual analysis. An amount of 0.5 mL of both treated and control was mixed with 0.5 mL of acetonitrile in 2 mL Eppendorf tubes followed by centrifuging at 12,000 rpm for 5 min. The supernatant was transferred to amber HPLC vials using Pasteur pipettes and kept in a refrigerator. Twenty-five microlitre of each sample was injected into the HPLC. Concentrations of bacteria in suspension were estimated by light absorbance value at300nm.

Identification of Active Isolate

Organisms showing growth in the presence of 500 ppm lambda cyhalothrin on minimal medium were morphologically characterized by colony morphology,

Gram staining, catalase, oxidase and motility. Biochemical methods were used to identify one isolate showing maximum growth.

KOH Solubility Test

The principle behind this test is that lipopolysaccharides present in the bacterial cell wall will dissolve in 3% KOH, forming a mucoid thread. A loopful of bacteria from a well grown colony was mixed in a drop of 3% aqueous KOH solution for not more than 10 s with the help of a toothpick. The tooth pick was raised a few centimeters from the microslide and observed for the formation of a mucoid thread. The Gram-positive bacteria did not produce strands even with repeated strokes of the toothpick.

Molecular Characterization of bacterial isolate

Based on the method of De Medici *et al.* (2003), bacterial isolates which were capable of utilizing cyhalothrinon were identified using 16S ribosomal DNA. DNA was extracted by applying the boiling method with some amendments. The 16S rRNA gene was amplified by using the following primers. Forward: 5′-AGA GTT TGA TCC TGG CTC AG-3′ Reverse: 5′-GGT TAC CTT GTT ACG ACT T-3′.The PCR reaction was performed in a final volume of 50 µL containing 25 µLTaq Master Mix (Vivantis, Malaysia), 1 µL of each primer and 2 µL of bacterial DNA template. The final volume was adjusted to 50 µL using nuclease-free water. DNA amplification was performed in a thermo cycler (BioRad) with the following thermal profile: an initial denaturation step of 94°C for 3 min (1 cycle), followed by 35 cycles of 94°C for 1 min, 41°C for 1 min, and 72°C for 2 min with the final extension step of 75°C for 5 min. The amplified DNA was analyzed by electrophoresis on 1.5% agarose (5 µL aliquot of each PCR product) and stained with ethidium bromide. PCR products were sent to SciGenome, Hyderabad for purification and DNA sequencing. Phylogeny was analysed with MEGA version 6.06 software and distances by neighbor joining method.

RESULTS AND DISCUSSION

Bacterial Diversity

Our study analysed the population of bacterial isolates per gram of soil treated with lambdacyhalothrin while the plate count technique was used for colony forming units(CFUs). Lambdacyhalothrin is one of the most potent pyrethroid insecticides widely used in pest management (Renata Colombo *et al.* 2013; Amweg and Weston 2005).Incubation for 24 h at room temperature resulted in high viable counts per gram of soil plated with and without Lambda cyhalothrin. The plate without Lambda cyhalothrin showed 68×10^7 CFU/g of soil while the soil samples plated with 10 ppm lambdacyhalothrin, the CFU was 18×10^7.Studies on colony morphology such as colour, size, margin, elevation, etc. were recorded and are given in Table 1. Based on colony morphology, ten different colonies were selected and designated as GMMC1 to GMMC10.The majority of colonies in the ten isolates were translucent while a few were opaque. Cell wall nature of isolates

revealed that 50% of isolates were Gram-positive while the rest were Gram-negative. Among the isolates, six were catalasepositive and seven were oxidase-negative. Isolation of Gram negative isolates showed high metabolic adaptability to several toxic pollutants (Pirahuata *et al.* 2006).Predominance of Gram-negative isolates from pesticide impacted soils has been reported by Agarry *et al.*(2013).

TABLE 1
Colony morphology and Gram reaction of isolates

Isolate	Opacity	Margin	Colour	Elevation	Gram	Catalase	Oxidase
GMMC1	Opaque	Entire	White	Flat	Negative	Positive	Negative
GMMC2	Translucent	Circular	Creamy	convex	Negative	Positive	Positive
GMMC3	Translucent	Irregular	White	Flat	Positive	Positive	Negative
GMMC4	Opaque	Round	White	Undulate	Positive	Negative	Negative
GMMC5	Translucent	Punctiform	White	Undulate	Positive	Positive	Positive
GMMC6	Translucent	Spindle shape	Creamy	Raised	Negative	negative	Negative
GMMC7	Opaque	Rhizoid	White	Raised	Positive	Positive	Negative
GMMC8	Translucent	Round	White	Raised	Negative	Positive	Negative
GMMC9	Opaque	Filamentous	White	Flat	Positive	Negative	Positive
GMMC10	Translucent	Round	White	Undulate	Negative	negative	Negative

Pesticide Tolerance

Investigation of microbial degradation is useful for developing ecofriendly bioremediation methods for pesticide toxicity. Bacteria with the ability to degrade pesticides have been widely studied but not in practice (Hong *et al*. 2005).The degradation of lambda cyhalothrin was performed under aerobic conditions by each strain for a period of 72 h in mineral salt medium followed by evaluation of its tolerance capacity. Cell growth recorded at different concentrations is given in Figure 1. The tolerance efficiency of isolates was tested by turbidometry assay of up to 500 ppm and GMMC1 was found to be a potent isolate. The growth rate for control was found to be comparatively slower on minimal medium without Lambda cyhalothrin. Maximum OD 1.72 was recorded in GMMC 1 followed by *Bacillus* sp. 1.54 and 1.52 ± 0.03. It was also observed that the rest of the isolates failed to tolerate lambda-cyhalothrin even at a minimum of 250 ppm level. Moderate growth was observed up to 100 ppm for these isolates. The decrease in CFU at increased concentrations of pesticide is related to the toxic effect of the pesticide. Plate assays revealed that one Gram-negative, *Pseudomonas* sp., and two Gram-positive *Bacillus* sp. and *Micrococcus* sp., had a higher tolerance to Lambda cyhalothrin (250 ppm).

The growth of lambda-cyhalothrin resistant isolates of *Pseudomonas* sp., GMCM1, and *Bacillus* sp. (GMMC3 and 5), were observed in the minimal broth amending lambda-cyhalothrin at 500 ppm. Biochemical test results showed

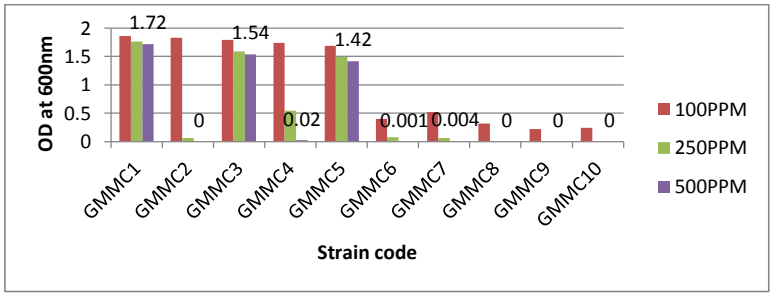
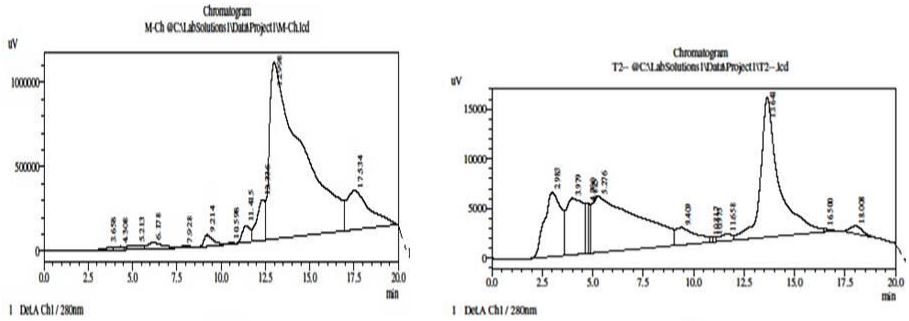


Fig. 1: Effect of Lambda cyhalothrin on growth of bacterial isolates

the isolate GMMC1 is positive on KOH solubility test, catalase test, indole test, oxidase test, and Citrate utilisation test and negative for starch and gelatin hydrolysis. Studies on *Pseudomonas putida* degrading various pesticides were monitored by genetic recombination and a recombinant strain able to completely degrade 50 mg/L of various pesticide compounds was developed by Ting Gong *et al*. (2018). The endosulfan-degrading bacterial strain *Pseudomonas fluorescens* was isolated (Jesitha *et al.* 2015) and degradation of endosulfan by freely suspended and calcium-alginate entrapped bacterial cells was investigated in batch as well as in packed bed column studies. Freely suspended *Pseudomonas fluorescens* cells with biomass maximum OD/OD0 value of 1.68 was found to degrade a number of pesticides. Previous studies had observed that *Pseudomonas* sp. has the ability to degrade pyrethroids (Mariutsz Cycori and Zofia Piotrowska-Seget 2006). Tang *et al.* (2018) reported that pyrethroids are synthetic organic insecticides with mammalian toxicity and are widely used in both rural and urban areas worldwide.

HPLC and TLC Analysis

The composition of the liquid media at the end of degradation was analysed using high performance liquid chromatography (HPLC). TLC analysis found that the cyhalothrin sample treated with *Pseudomonas fluorescens* resulted in the formation of 7 different fractions with Rf values of 0.04, 0.06, 008, 0.14, 0.18 0.22 and 0.28. No fractionation was observed in control. The HPLC chromatogram illustrates (Figure 2a) the separation of soluble metabolites and shows the elution 11 order which depicts the formed intermediate compounds along with the reduction of lambdacyhalothrin with retention times of 3.658, 4.308, 5.213, 6.178, 7.928, 9.214, 10.598, 11.415, 12.336, 12.998, 17.534. Retention times of 6.178 and 7.928 corresponded to the presence of cyhalothrin as reported by Chaaieri Oudou and Bruun Hansen (2002). HPLC analysis of cyhalothrin degradation by strain GMMC1 over time showed 12 different peaks with disappearance of 6.178 and 7.928 retention times (Figure 2b). The retention time peak value suggests that the insecticide residues of lambda-cyhalothrin for *Pseudomonas* sp is 2.9834 along with other peak values of 3.979, 4.700, 4.825, 5.276, 9.409, 10.817,10.975,11.658, 13.641,16.500 and 18.008.



PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.658	708299	20772	0.412	1.157
2	4.308	657053	19757	0.382	1.101
3	5.213	1492847	23787	0.867	1.325
4	6.178	2170805	37045	1.261	2.064
5	7.928	380314	9457	0.221	0.527
6	9.214	2434199	68587	1.414	3.821
7	10.598	241352	5635	0.140	0.314
8	11.415	2985667	97756	1.735	5.446
9	12.336	7605910	239833	4.419	13.360
10	12.998	132352220	1041005	76.896	57.992
11	17.534	21089230	231457	12.253	12.894
Total		172117895	1795091	100.000	100.000

PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.983	377731	6428	13.666	13.860
2	3.979	321879	5694	11.646	12.277
3	4.700	42731	5040	1.546	10.867
4	4.825	42188	4979	1.526	10.736
5	5.276	889582	5649	32.185	12.180
6	9.409	119425	1740	4.321	3.751
7	10.817	5639	564	0.204	1.216
8	10.975	3800	500	0.137	1.078
9	11.658	29800	689	1.078	1.486
10	13.641	892870	14042	32.304	30.277
11	16.500	5829	306	0.211	0.660
12	18.008	32490	748	1.175	1.612
Total		2763964	46378	100.000	100.000

Fig. 2: HPLC spectrum of lambda cyhalothrin. a)control b)degraded

Phylogenetic Analysis

Based on the morphological and biochemical results, the selected isolates were preliminarily identified as *Pseudomonas fluorescens*, and confirmed by their 16S rRNA genes which were amplified from their genomic DNA, and found to be 1500 bp long (Figure 3). Sequence blast showed that the strain GMMC1 had a high similarity (98%) with *Pseudomonas fluorescens* (Accession number KF420847.1) and identified with *Pseudomonas* sp. (Accession number KT377040) at 96%.

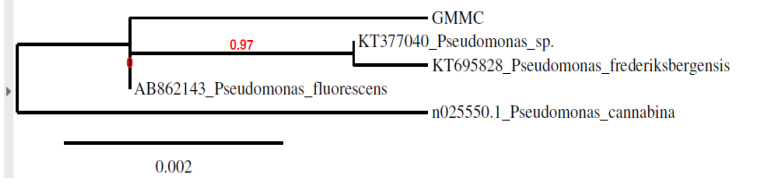


Fig. 3: Phylogenetic relatedness of isolated Pseudomonas sp GMMC1.

CONCLUSION

Lambda-cyhalothrin is broken down in soil through photolysis, chemical hydrolysis and microbial degradation. In laboratory studies, the dissipation of lambda-cyhalothrin in soil was studied by isolation of bacteria. Diverse groups of bacteria were isolated from soil and bacterial strains able to biodegrade cyhalothrin were screened by enrichment of culture with pesticide. GMMC1 was found to

tolerate and efficiently degrade cyhalothrin at 500 ppm concentration level. This is the first report of degradation of cyhalothrin with *Pseudomonas fluorescens.* These findings reveal that increased concentrations of pesticide have a marked effective biodegradation performance of strain *Pseudomonas fluorescens* but do not lead to complete inhibition of cyhalothrin biodegradation.

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