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Isolation and molecular characterization of lambda cyhalothrin pesticide degrading organisms

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ABSTRACT

Soil samples contaminated with pesticides were collected from agricultural fields in and around Pudukkottai district, TamilNadu. Samples were inoculated into mineral salts medium with Lambda cyhalothrin as the sole source of carbon and incubated for 12 days. Intermittent addition of pesticide was carried out for enrichment of cultures. Bacteria capable of degrading lambda cyhalothrin were isolated on mineral salts agar medium. From the 6 samples collected, 52 isolates capable of degrading lambda cyhalothrin was obtained, majority of them being gram negative in nature. Out of these, 3 were capable of showing good growth in mineral salts medium. Screening of the isolates for their efficiencies in degrading lambda cyhalothrin was carried out based on enrichment technique, spectrometric analysis and HPLC studies. Strains JJC1, JJC2 and JJC3 showed the highest tolerance to the pesticide. JJC1 and JJC2 were identified as Stenotrophomonas maltophilia and Enterococcus faecalis respectively based on 16S rRNA and phylogenetic tree analysis. JJC3 was identified as Pseudomonas fluorescens based on cell morphology and various biochemical tests. Of the 3 isolates, Stenotrophomonas maltophilia was found to be effective in tolerating and degrading lambda cyhalothrin. Hence it was used in further studies. The biomass and cell growth were determined initially for three consecutive days for Stenotrophomonas maltophilia. The percentage of residual pesticide remaining was determined by HPLC. The residual level of pesticide decreased with the prolonged incubation of microorganisms along with the pesticide and simultaneously the percentage of degradation also increased gradually indicating that the selected microorganisms were capable of degrading the pesticide, lambda cyhalothrin. Due to their high biodegradation activity, the bacteria isolated from this work merit further study as potent biological agents for the remediation of soil or water contaminated with the pesticide Lambda cyhalothrin.

Keywords: Lambda cyhalothrin, Biodegradation, *Stenotrophomonas maltophilia*, *Enterococcus faecalis* and *Pseudomonas fluorescens*

INTRODUCTION

Bioremediation is an environmental clean–up technique that is currently being investigated for use on a wide variety of chemicals. It is the use of naturally occurring microorganisms to enhance biodegradation, or normal biological breakdown. It involves establishing the condition in contaminated environment so that appropriate microorganisms flourish and carry out the metabolic activities to detoxify the contaminants [1].

Pesticides are indispensable to modern agriculture. Currently among the various groups of pesticide that are being used world over, Organophosphates form a major and most widely used group accounting for more than 40% of the total world market. Parathion, Methyl parathion, Malathion, Lambda cyhalothrin, Monocrotophos, Dimethoate and

Phorate are some of the widely used Organophosphorous pesticides. The wide spread use of these pesticides over the years has resulted in problems caused by their interaction with the biological systems in the environment. Considering the toxic effects of pesticides, it is essential to remove these chemo-pollutants from the environment. Biological removals of chemo-pollutants have become the method of choice since microorganisms can use a variety of xenobiotic compounds including pesticides, for their growth and mineralize and detoxify them. Researchers all over the world are engaged in investigations on biodegradation of pesticide [2].

Microorganisms are key players in determining the environmental fate of novel compounds because they can be used as carbon and energy sources by microorganisms [2]. For this reason there is a need to isolate, identify and distinguish the microorganisms that exist in contaminated environment and to determine the genetic determinants of resistance, frequently located on plasmids [3]. The present study describes the isolation of the pesticide resistant bacterial strains which was isolated from the Lambda cyhalothrin contaminated soil.

MATERIALS AND METHODS

Pesticide description

Lambda-cyhalothrin an insecticide registered by USEPA in 1998, it is a man- made pesticide, belonging to pyrethroids group, it is similar to the natural insecticide pyrethin which are naturally occurring insecticide compounds from flowers of chrysanthemums *(Chrysanthemum cinerariaefolium).* Pyrethroids are important arm used in public health management where in they are applied for of controlling cockroaches, mosquitoes, ticks and flies (etc). This group of pesticides has been widely used as pesticides or chemical warfare agents because of their high toxicity towards insects and other animals. Their mechanism of action involves the irreversible inhibition of acetylcholine esterase, a key enzyme of the central nervous system [4]. Lambda-cyhalothrin in soil surfaces and aqueous solution has the pH range of about 5.5 and melting point is 49.2˚C. The molecular weight of lambda-cyhalothrin is 449.9 g/mol, which has a molecular formula of $C_{23}H_{19}CIF_3NO_3$, chemical name *cycloproanecarboxyphenyl* and it is an active ingredient of several brand names called karate, warrior, scimitar, demand, icon and matador. This is used to control the pests of crops like cotton, rice, cereal, vegetables and sugarcane. The recommended pest for lambda-cyhalothrin are bollworms, jassids, stem and fruit borer, leaf folder, green hopper, hispa, sterm borer etc. This is applied on the plant surfaces like leaves, fruits, and vegetables with power sprayer.

Culture medium

A synthetic Mineral salt medium containing $MgSO_4$ – 200 mg, K_2HPO_4-900 mg, KCl-200 mg, $FeSO_4$-2 mg, $MnSO_4$-2 mg, NH_4NO_3-1000 mg/1 litre of medium was used. The pH was adjusted by 7 using 1N HCl and sterilized by autoclaving at 121˚C for 15 min.

Enrichment of the Soil Samples

The aim of this step was to adapt the soil microflora to pesticide, Lambda cyhalothrin. In this step of enrichment procedure the mineral salt medium (MSM) was used. To obtain this effect, collected soil samples (5 g) were mixed with 100 ml of mineral salts medium containing Lambda cyhalothrin at concentration of 5 ml/L before mixing the sample soils and Lambda cyhalothrin, the MSM was autoclaved and pH maintained at 7 ± 0.1. After mixing the soil suspension, the medium was incubated in the dark at 30˚C. After 10-12 days of incubation, the aliquot of contaminated soil suspension was taken for isolation of bacteria [5].

Isolation and screening of Lambda cyhalothrin degrading bacteria

Different discrete colonies on the plates were selected for isolation. Morphologically dissimilar isolated colonies were picked up from the plates with the help of sterile loop and each individual colony was streaked on Lambda cyhalothrin agar plate to obtain pure culture. Totally 3 isolated colonies were picked from the Lambda cyhalothrin agar plates and screened to observe their rapid growth as well as resistance to Lambda cyhalothrin by replica plate method [6] and transferred to Lambda cyhalothrin agar plates containing up to 5 ml/L of MSM

Purification of potential bacterial isolates

A single isolated colony of the pesticide degrading bacteria was picked up with the help of sterilized wire loop and was streaked on LB agar medium. Each isolated strain was streaked at least 3 to 4 times on LB agar plates for purification. After the purified isolates were obtained, they were re-streaked on mineral salt medium containing pesticide (λ cyhalothrin) for confirmation of isolates. The single colony of bacterial strain was inoculated in 100mL

LB broth, incubated at 37ºC then used for further characterization of isolates. The isolated and purified bacterial strains were stored under refridgeration after preparing slants.

Identification of the Isolate

The selected bacterial isolates JJC1, 2 and 3 were identified by cultural, morphological and biochemical tests as explained in Bergey's Manual of Determinative Bacteriology [7]. Colony characteristics of these isolates were observed after growing on nutrient agar plate after 24h at 30ºC. Further conformation of the most tolerant bacterial isolates JJC1, JJC2 was done using 16S rRNA sequencing. Out of the three organisms which were preliminarily screened, the organism JJC1 (*Stenotrophomonas maltophilia*) was more efficient in degrading the pesticide λ cyhalothrin, hence this alone was used in further studies.

DNA sequencing and Phylogenetic analysis

The partial 16S rRNA gene sequences were initially analyzed at Amnion Biotech, Bangalore. The 16S rDNA gene was amplified as per the method of [8] using universal primer and PCR product for the isolate was sequenced in both directions. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16sF and 16sR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and chromatogram plot using Chromas Lite 2.0 version, Applied Biosystems and Amersham MegaBace automated sequencers.The phylogenetic analysis of evolutionary history was infered using the Neighbor-Joining method [9].

Determination of cell growth (Biomass) in liquid culture

The growth of *Stenotrophomonas maltophilia* was performed in 500 ml Erlenmeyer flask containing 250 ml of minimal medium supplemented with various concentrations (1ppm, 2ppm, 3 ppm and 4 ppm) of lambda cyhalothrin as a sole source carbon. A UV- visible spectrophotometer (Systronics - 118) was used to monitor the cell density by measuring the turbidity at 610 nm for 3 days [10].

Determination of growth rate in solid culture

Stenotrophomonas maltophilia was inoculated into minimal medium containing different concentration of lambda cyhalothrin (1, 2, 3 and 4 ppm) as the sole carbon source. Rate of bacterial growth was estimated based on determination of viable cell count per ml (CFU /ml^{-1}) [11].

Determination of residual Lambda Cyhalothrin

Lambda cyhalothrin was extracted from the culture filtrate using Dichloromethane [12]. Residual Lambda cyhalothrin was determined using High performance liquid chromatography [13] with a C18, Phenomenex column (BIORAD, USA) at 60°C using methanol /water (75:25) as an eluent. A flow rate of 1ml /min and a sample volume of 20 µl were maintained. The elute was monitored with UV -visible detector. The peaks were identified and quantified by comparing with retention times of authentic standard (lambda cyhalothrin) at the 254 nm. The degrading capacity of the isolates was determined by the evaluation of the residual remaining in the culture medium.

RESULTS AND DISCUSSION

Totally 52 isolates were isolated from 6 different samples. Three different distinct colonies were selected among the 52. These fast growing 3 bacterial members were designated as JJC1, 2 and 3. By comparing the results of cultural, morphological and various biochemical tests with Bergey's Manual of Determinative Bacteriology [14], Manual for the Identification of Medical Bacteria [15], it was found that, the isolates JJC1,2 and 3 were *Stenotrophomonas* sp. , *Enterococcus* sp. and *Pseudomonas* sp. respectively (Table-1).

DNA sequencing and Phylogenetic analysis

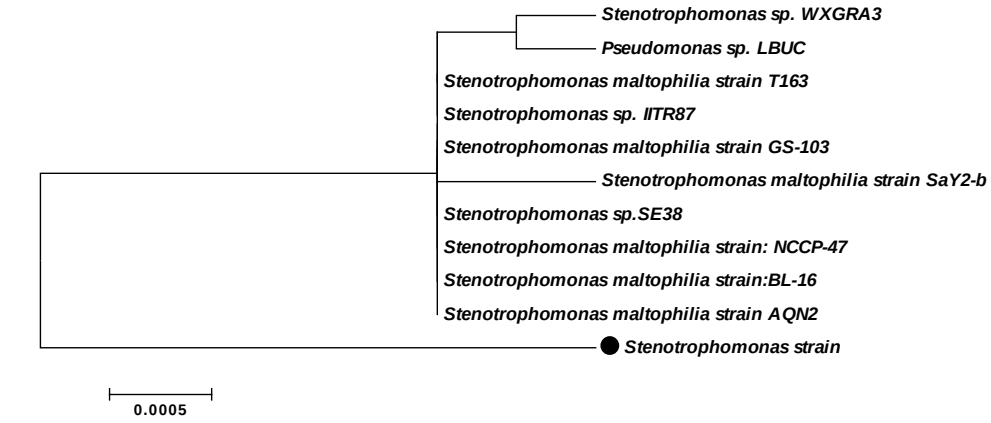

Fig-1 -Sample1- jjc1

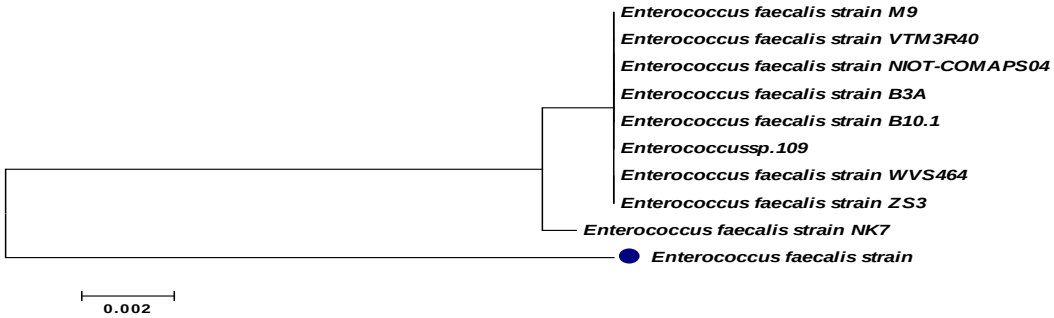

Fig-2 –Sample2- jjc2

DNA sequencing and Phylogenetic analysis

The molecular characterization of the most promising isolates JJC-1 and JJC-2 were done by 16S rRNA sequencing and phylogenetic analysis at Amnion Biotech, Bangalore (Fig-1 and 2) Based on the above tests, the first two bacterial sequences were identified as strains of *Stenotrophomonas maltophilia* and *Enterococcus faecalis*.

Determination of cell growth (Biomass) in liquid culture

In the present study, the screened organisms were evaluated for their ability to degrade the pyrethroid pesticide, lambda cyhalothrin by determining the cell growth in liquid culture, growth rate in solid culture and pesticide residual analysis.The optical density of *Stenotrophomonas maltophilia* was determined on three consecutive days to assess the initial growth of the organism in the presence of the pesticide using UV-Visible Spectrophotometer at 610 nm. On day 1, highest optical density was recorded in 4 ppm (0.82), followed by 3 ppm (0.69), 2 ppm (0.48) and 1 ppm (0.29). The rate of growth was determined to be faster in 4, 3, 2 and 1ppm on three consecutive days. Whereas, the rate of growth in control was found to be comparatively slower. The optical density increased gradually on increasing the incubation and also increasing the pesticide concentration (Table-2). The result of OD was also confirmed with CFU of *Stenotrophomonas maltophilia* isolate. The decrease in CFU on prolonged incubation is related to the nutrient depletion (Table-3). The results of the present finding was in accordance with the work done by Poerlands *et al*.,[16] who isolated a strain of *Pseudomonas cichorii* from soil that was repeatedly treated with the nematicide 1,3-dichloropropene as a sole carbon and energy source. Thus it can be concluded that the isolate *Stenotrophomonas maltophilia* isolate takes up the nutrient source of pesticide for its growth and degrades them by using specific enzymes.

Evaluation of the residual compounds

The lambda cyhalothrin peak was detected at retention time of 6.7 in the HPLC column and its reproducibility was confirmed using, a series of three injections. The peak area of the standard was calculated with 10 ppm standard,

which was then used for further calculations for determining the concentration of the residue in the treated sample. On day 2, the highest lambda cyhalothrin residue was recorded in 4 ppm (3.6 ppm) followed by 3 ppm (3.0 ppm), 2 ppm (1.4 ppm) and 1 ppm (0.6 ppm). On day 12, the highest residue recorded was 4 ppm (2.0 ppm) and 3 ppm (1.5 ppm). No residue was detected in 1 ppm and 2 ppm, on day 12. The percentage of degradation of lambda cyhalothrin was determined using the concentration of the residue obtained. On day 8, 80% degradation of lambda cyhalothrin was recorded in 1ppm, 85% for 2 ppm, 50% for 3ppm and 27.5% for 4ppm and on day 12, 50% was recorded in 3 ppm and 50% for 4 ppm**)** (Table-4).

Pseudomonas sp strain BK8 from soil contaminated with urea herbicide completely degraded diuron herbicide using them as carbon source without accumulating phenolic compounds [17]. Endosulfan degrading bacterial strain KS-2P was isolated from endosulfan polluted soil which utilized endosulfan sulfate as the sole carbon source [18]. Bacteria capable to uptake and degrade various insecticides have been isolated from various sources [19-21]. A widely available insecticide alpha- endosulfan, beta-endosulfan is degraded by single bacteria like *Klebsiella oxytoca*, *Bacillus* sp., *Pandoraea* sp., *Micrococcus* sp. and by mixed bacterial co-culture [22]. *Pseudomonas* sp., *Flavobacterium* sp., *Agrobacterium* sp., *Clostridium* sp., *Ralstonia* sp. etc are reported for degradation of many pesticides like Alachlor, Chlorpropham, DDT, Lindane etc. *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, *Flavimonas oryzihabitans*, and *Morganella morganii* isolated from coffee beans were identified to be efficient in degrading the pesticides, 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) and endosulfan in 50 ppm concentration. The residues were found to be 1,1-dichloro-2,2'-bis (4-chlorophenyl) ethylene (DDE), 1-chloro-2,2-bis (4-chlorophenyl) ethane (DDMU) and 2,2'-bis (p-chlorophenyl) ethanol (DDOH) through GC/MS analysis [23]

Table- 1. Colony characteristics, Morphological and Biochemical characteristics of 3 different Lambda cyhalothrin degrading isolates grown on minimal media at 30 ºC for 24 hours

Biochemical Characteristics	JJC1	JJC2	JJC 3
Catalase	+	+	+
MR	-	-	+
VP	-	+	+
Gas form glucose	-	+	-
H2S production	-	+	+
Indole	-	-	-
Motility	+	-	+
Citrate test	+	-	+
Microbial characteristics			
Gram Reaction	G -	G +	G +
Microscopic view	Short Rod	Short Rod	Rod
Size	1.8 µm	2.25µm	1.75µm
Colony Morphology	Regular pigmented colonies	Punctiform, convex with an entire margin.	Circular, Light yellowish, Flat, Mucoid, Regular

Table-2. Cell growth of the isolate *Stenotrophomonas maltophilia* in liquid culture with different concentration of Lambda cyhalothrin

Lambda cyhalothrin Treatment	**Optical density (610 nm)**		
	Day 1	**Day 2**	**Day 3**
T1 (1ppm)	0.29	0.37	0.41
T2 (2ppm)	0.48	0.51	0.65
T3 (3ppm)	0.69	0.76	0.81
T4 (4ppm)	0.82	0.98	0.86
C (Control) (Without pesticide)	0.11	0.13	0.21

Table -3. Colony Forming Units of the isolate *Stenotrophomonas maltophilia* grown in medium containing Lambda cyhalothrin

Lambda cyhalothrin Treatment	**Colony forming units/ml (10^{-20})**					
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 8**	**Day 12**
T1 (1ppm)	25.7	57.4	115.3	TNTC	TNTC	TNTC
T2 (2ppm)	26.3	60.4	123.5	TNTC	TNTC	TNTC
T3 (3ppm)	24.3	61.7	120.6	TNTC	TNTC	TNTC
T4 (4ppm)	32.5	100.6	TNTC	TNTC	TNTC	TNTC
Control	NIL	NIL	NIL	TLTC	TLTC	TLTC

TNTC: To numerous to count; TLTC: To low to count.

Table-4. Residual level of lambda cyhalothrin and percentage of degradation by *S.maltophilia*

Lambda cyhalothrin Treatment	Concentration in ppm and percentage of degradation									
	Day 0		Day 2		Day 4		Day 8		Day 12	
	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%
T1 (1ppm)	1.0	-	0.6	40	0.3	70	0.2	80	-	100
T2 (2ppm)	2.0	-	1.4	30	0.6	70	0.3	85	-	100
T3 (3ppm)	3.0	-	3.0	-	2.4	20	2.1	30	1.5	50
T4 (4ppm)	4	-	3.6	10	3.2	20	2.9	27.5	2.0	50

CONCLUSION

The choice of the bioremediation strategy should be made on the basis of type of pesticide, environmental matrix and the organisms present in the ecosystem. Among the isolates obtained for degradation of lambda cyhalothrin most of them were gram negative in nature. There are literatures available on efficiencies of gram negative bacteria in degradation of pesticides. The percentage of residual pesticide used was determined by HPLC. The residual level of pesticide decreased with the prolonged incubation of microorganisms and simultaneously the percentage of degradation also increased gradually indicating the capacity of the selected microorganism to degrade the insecticide, lambda cyhalothrin. The percentage of degradation was found to be high in the case of *Stenotrophomonas maltophilia.* This bacterium isolated in the current study was found to be efficient in tolerating lambda cyhalothrin compared to others and can be used for bioremediation of soils contaminated with these pesticides.

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