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Degradation of The Pesticide: Cypermethrin.

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ABSTRACT

This study extends by using microorganisms to eliminate cypermethrin from contaminated fields for bioremediation purpose at proper condition as an approach to reduce pyrethroid toxicity in the environment. The organisms degrading cypermethrin were isoloated and characterised. The growth and the range of degradation of cypermethrin with different pH, temperature and concentration were determined. The degradation of the pesticide was determined by thin layer chromatography and spectrophotometric method. The plasmids capable of degrading the pesticide were isolated and transformation was carried out.

Keywords: Cypermethrin, Degradation, Leuco crystal violet.

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INTRODUCTION

Pesticides have made a great impact on human health. Pyrethroid is the most important pesticide because even at very low concentration it is more effective Pyrethroids have four major generations among this cypermethrin belong to the fourth generation (Casida,1980).Cypermethrin is more effective against pests including moth pests of cotton, fruits and vegetable crops. Cypermethrin has moderate persistence in soils. Under laboratory conditions, cypermethrin degrades more rapidly in soils (Saraswat and Gaur, 1995) and in aerobic conditions the half-life of cypermethrin is 4 days to 8 weeks (Wauchope et al., 1992)

Cypermethrin is widely used by farmers to control insect pests of vegetables. A simple and sensitive spectrophotometric method is described for the determination of cypermethrin, where cypermethrin is hydrolysed to give cyanide ion, which further reacts with potassium iodide and leuco crystal violet to produce a crystal violet dye in acidic medium. The reagent is selective for cypermethrin, amongst the pyrethroid group. The colour system obeys Beer's law. The method has been applied to the determination of cypermethrin in various samples of water, vegetables, fruits, foliage and biological samples.

CYPERMETHRIN DEGRADING MICROORGANISMS:

Bacteria from liquid cultures of treated soils which were capable of degrading cypermethrin as sole carbon and nitrogen sources. Degradation was more rapid in alkaline soil than in acidic soils. Some of the cypermethrin degrading microorganisms are Pseudomonas, Serratia marcescens, Bacillus, Streptobacillus and Staphylococcus. The present study was conducted with an aim to isolate local strains of insecticide degrading bacteria and evaluate their characteristics for purposes of bioremediation of insecticide contaminated soil, in cypermethrin contaminated fields. These isolates will have the potential to clean up the environment from such persistent pollutants.

MATERIALS AND METHODS

Collection of soil samples:

The soil samples were collected from different cultivated fields. Some of the fields had been sprayed with cypermethrin for past few years. Soil samples were collected at different sites of the field, and samples were transferred to sterile polythene bag and used for analysis.

S. NO.	FIELDS	AREAS
1.	Brinjal	Vellore
2.	Beans	Coimbatore
3.	Wheat	G.K.V.K Bangalore
4.	Sunflower	G.K.V.K Bangalore
5.	Jackfruit	G.K.V.K Bangalore
6.	Cotton	Coimbatore
7.	Mango	Vellore
8.	R.M.S Block	WCC College
9.	Railway station	Nellore
10.	Lawn	Tambaram, Chennai
11.	Cardamom	Kollam, Kerala

Pesticide Collection:

Cypermethrin insecticide was available in 10% EC and 25% EC in liquid form of pesticide. The pesticide was bought from Sri Venkateshwara Hybrid Seeds and Agricultural seed store.

Culture media for bacteria:

Micro organisms were isolated from the pesticide contaminated soils with

- Nutrient Broth
- MM medium
- 10ml of nutrient broth was weighed and mixed well and transferred to conical flask and autoclaved.
- To the above 2 g of soil sample was added and incubated in shaker for 24 hours.
- 250 ml Nutrient agar was prepared and autoclaved, to it 1ml of cypermethrin was added and poured on sterile petri plates.
- Minimal agar was prepared and autoclaved. After autoclave, add cypermethrin of known concentration.
- Take a loop full of the culture and streak on the minimal agar plates and incubate at 37˚C for 24-48 hours.

Screening of pesticide degrading Bacteria:

Samples were enriched by incubation in minimal media containing 1% pesticide at 37˚C. A loop full of the culture was spread plated on to minimal medium with cypermethrin pesticide and kept for incubation at 37˚C for 24-48 hours. Colonies were isolated for further studies.

Isolation and maintenance of bacterial colonies:

The bacterial cultures capable of degrading cypermethrin was isolated from different soils using enrichment technique with different concentrations of cypermethrin in the medium. The soil sample (2-5g) from different agricultural site was inoculated in minimal medium in Erlenmeyer flasks. The flasks were incubated in shaker incubator for 24 hours at room temperature (30-35˚C). A loop full of this enrichment culture from the flasks was streaked on the Minimal Agar plates with cypermethrin and incubated at 35˚C for 24-48 hours. Individual colonies were subcultures into nutrient agar plates containing same concentration of cypermethrin until pure culture was isolated. The isolated strains were maintained at 4˚C.

Enumeration of Cypermethrin utilizing bacteria:

Cypermethrin was used as a carbon source. The microbial strains of cypermethrin resistant bacteria were streaked on the minimal medium plates containing cypermethrin at different concentrations. After incubation the cypermethrin utilizing colonies were isolated.

Identification of bacterial isolates:

The isolates were subjected to morphological, cultural and biochemical studies which included Gram staining, Motility by hanging drop technique, standard Biochemical tests included Indole, Methyl red, Voges-Proskauer , Citrate Test, Starch Test, Coagulase Test and Catalase Test.

Antibiotic sensitivity test by Disc Diffusion method

All the bacterial isolates were tested for their sensitivity to different antibiotics by Disc Diffusion method. The following antibiotics were used: Oflaxacin (OFL), Ceftriaxone (CRO), Clindamycin (DA), Methicilin (ME), Doxycycline hydrochloride (Do), Meropenem (MEM).

Determination of optimal growth and degradation levels of bacteria using Leuco Crystal Violet dye a spectrophotometric method

Concentration, pH and Temperature of pesticides were considered for the optimal growth of the bacterial isolates.

An aliquot of test solution containing different concentrations of cypermethrin was taken (2µl- 500µl). Different test tubes were taken with 10ml of autoclaved MM medium solution. To this different concentrations of cypermethrin was added. 1ml of ethanol was also added to dissolve the cypermethrin in each tube and 1ml of 0.1% of KI was added and kept aside for 10 minutes. 1ml of LCV was added, shaken well

and kept for colour development. Crystal violet dye was produced and absorbance was taken at 595nm with reagent blank.

Effect of Concentration, pH and Temperature of pesticide

Effect of Concentration of pesticides:

Different concentration of cypermethrin (10µl, 50µl, 100µl, 150µl and 200µl) was added to different test tube containing 10 ml of MM medium each, and incubated with different organisms and take at different intervals like (0hour, 24 hours, 48 hours, 11 days and 18 days). Were 1ml of the sample was taken at different time intervals and to it 1ml of ethanol, 1ml 0.1% KI and 1ml of LCV was added and observed for colour change and their absorbance was measured at 595nm .

Effect of Temperature of pesticides:

The degradation was checked using different temperature (4˚C, 16˚C, 37˚C and 45˚C) at different time intervals like (0hour, 24 hours, 96 hours, 11 days) using 10ml of MM medium with a common concentration of cypermethrin (100µl) was added to all and Ethanol, 0.1% KI and LCV was being added to the samples and absorbance was taken at 595nm.

Effect of pH of pesticides:

To every 10 ml of MM medium a known concentration of cypermethrin (100µl) was added, were the media was with different pH like (pH2, pH4, pH6, pH7, pH8 and pH10) and at different time interval like (0hour, 24 hours, 96 hours, 11 days) were 1ml Ethanol, 1ml of 0.1% KI and 1ml of LCV was being added to the samples and absorbance was taken at 595nm.

Thin Layer Chromatography

- The solvent hexane : acetone (3:1) was used as a mobile phase for the compound in the sample to be identified.
- Silica Slurry coated with TLC plate was used as a stationary phase.
- A line was drawn at the bottom of the TLC plate, the sample was placed using the capillary tube over the line marked.
- The TLC plate was placed in a beaker containing the mobile phase and was left undisturbed for the solvent to reach the top of the TLC plate.
- The TLC plate was removed and air dried.
- TLC plate was sprayed with Leuco crystal violet to check for purple spots.
- The pigment was identified by observing under UV trans illuminator.
- The retention factor (R_f) of the compound was calculated using the formula.

R_f = Distance travelled by the compound / Distance travelled by the solvent

Isolation of plasmid DNA and Agarose Gel Electrophoresis

The presence of plasmid DNA was confirmed by isolating plasmids from the pesticide tolerating bacteria pure cultures of all the 7 isolates were grown overnight in 10ml Sterile Luria-Broth(Hi-Media). Plasmids were isolated using Alkaline lysis method. The plasmids extracted were characterized by agarose gel electrophoresis.

Transformation

Isolated plasmids carrying the degraded genes from the 7 isolates were strongly responsible for tolerance to higher concentrations of the pesticides used (Cypermethrin). Therefore, the plasmid DNA was transferred to E.coli DH5α strain by transformation at 37˚C room temperature condition in the laboratory.

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Bioremediation of Cypermethrin using Soil

Soil sample was taken from non pesticide contaminated field. The soil was air dried & autoclaved. Soil was put in different plastic container (200g) of soil, 100ml of Minimal medium was prepared for every 200g of soil, then 0.1ml of cypermethrin was added (100µl) with the desired organisms was added under aseptic conditions. The soil was collected at different time intervals like 0 hour, 11 days, 18 days.

Check for the colour change by using leuco crystal violet. Compare the colour change between the different intervals of soil collection and absorbance is taken at 595nm.

High Performance Liquid Chromatography (HPLC) :

A standard was prepared by dissolving them in acetonitrile to the final concentration. The dilution run at different conditions of pesticide. Cypermethrin was detected at Wave length 254nm and 231 nm at Flow rate 1mL/min using a Mobile phase HPLC grade Acetonitrile70% and water 30%.Before injection samples are filtered through 0.22µm syringe filter. 20µL of sample was injected through auto sampler, decline in the cypermethrin concentration is monitored using the mobile phase of Acetonitrile (70%) and water. This has been done at a wavelength of 254nm at a run time of 5 minutes.

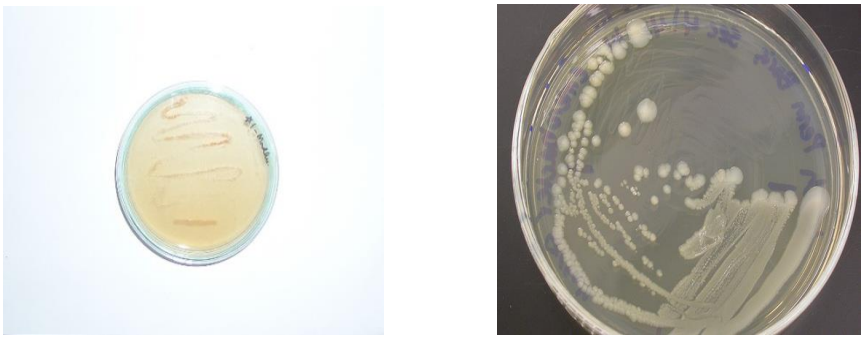
Gas Chromatography- Mass Spectrophotometry (GC-MS) :

Standard was prepared by dissolving them in Hexane to the final concentration. Cypermethrinwas detected at 5% Phenyl Methyl Silox -60°C-325°C, were 1µL of sample injected through auto sampler program at different conditions like 50C -2min, 10C/min 160C-5min, 10c/min 200C-1min, 10C/min 280C-10minutes with a total run time of 41minutes at 290C interface temperature which is done at a flow rate of 1mL/minute.

RESULTS AND DISCUSSION

Isolation of microorganisms from pesticide contaminated soil:

Two organisms were isolated. They were found to carry out successful degradation on cypermethrin pesticide.



Microorganisms isolated from the pesticide contaminated soil (Bacteria).

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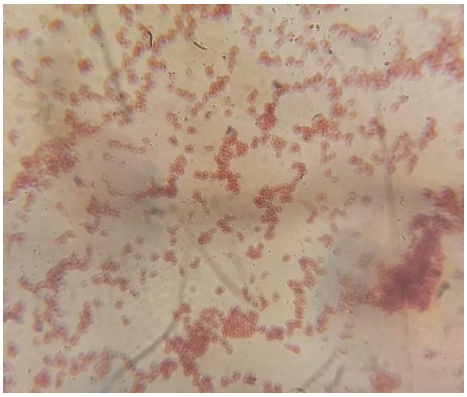
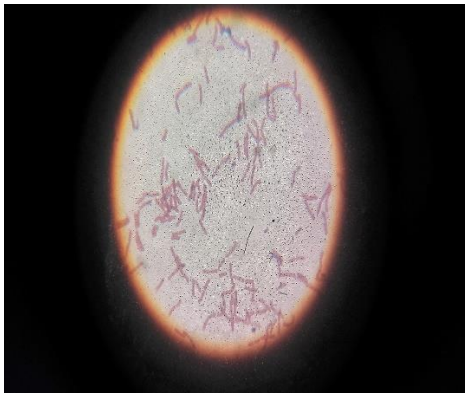
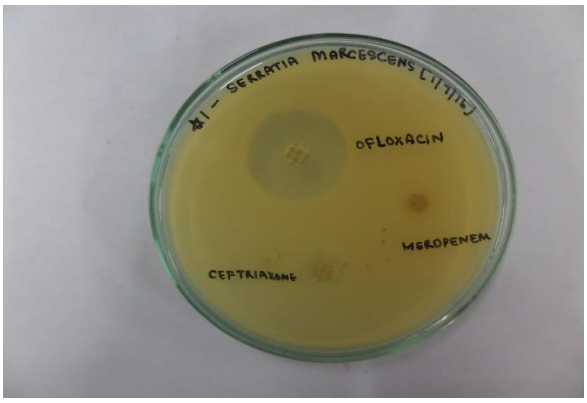
Staining:

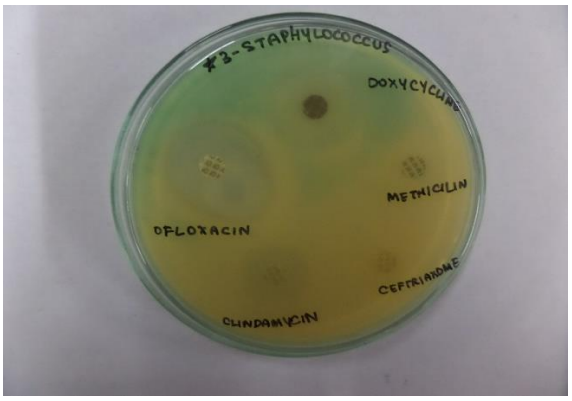




Determination of Antibiotic sensitivity:

Organisms	ofloxacin	Meropenen	clindamycin	doxycycline	cefltriaxone	Methicilin
Serratia marcercens	1.35mm	-	-	-	0.65mm	-
Staphylococcus spp	1.05mm	-	0.6mm	-	-	-





The zone of inhibition was measured in millimetre and the resistance and sensitivity of isolated bacteria towards antibiotics used was determined. It was observed that Chlorpyrifos was easily tolerable pesticide which grows faster in Endosulfan.

TLC –THIN LAYER CHROMATOGRAPHY:

The degradation of the pesticide was found out using TLC which showed positive results. Later calorimetric analysis was done.

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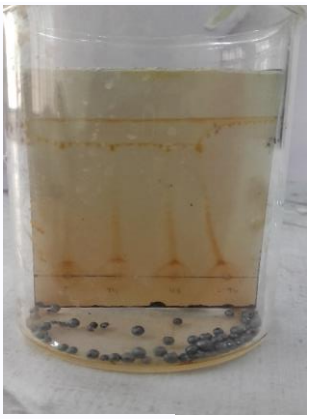
TLC (purple spots)



TLC (brown spots)

Effect of Concentration, pH and Temperature of pesticide:

The effect of temperature , pH and concentration of the pesticide on the growth medium was determined. The optimum degradation level was checked by using the leuco crystal violet , which on production of purple colour indicates that the degradation is less and compared using the OD levels.

Effect of concentration

Serratia marcersens and Staphylococcus spp showed maximum degaradation at 150ul concentration

Effect of pH :

Maximum growth occured at pH 2 for Serratia marcersens and for Staphylococcus spp growth occured at pH 7.

Effect of Temperature:

Serratia marcersens- maximum growth occured at 24 hours at 16c Staphylococcus spp- 24 hours at 4c.

Transformation:

The extracted plasmid DNA from isolates were further used for transforming the competent cells of E.coli DH5α strain. The transformation mixture was plated on LB agar plates containing cypermethrin and culture condition was 37˚C. It was found that competent cells of E.coli DH5α were successfully transformed with the plasmid DNAs isolated from pesticide tolerating bacteria and thus acquired a new extra-chromosomal property of tolerating higher concentration of toxic chemicals The transformation mixture was plated on LB agar plates containing 8,000ppm of Endosulfan, 20,000ppm of Chlorpyrifos and 2000ppm of Cypermethrin. It was found out that competent cells of E. coli DH5α were successfully transformed with the plasmid DNAs isolated from pesticide tolerating bacteria and thus acquired a new extra-chromosomal property of tolerating higher concentrations of toxic chemicals. **(Sayali R. Naphade et.al 2012).**

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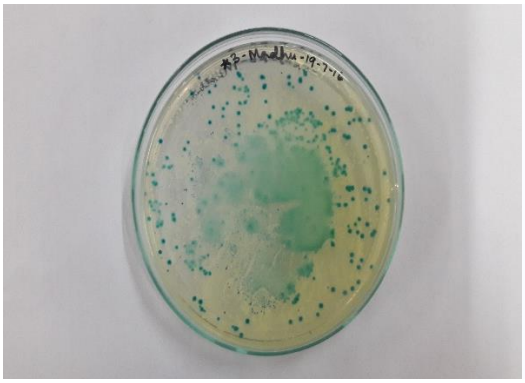

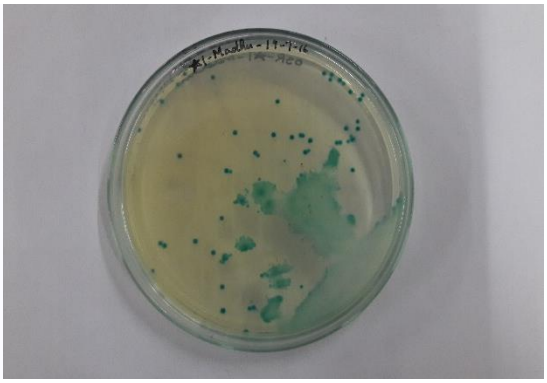

Bioremediation of Cypermethrin using Soil

The addition of cypermethrin to soil resulted in a more rapid degradation of cypermethrin than by indigenous microflora. Degradation of cypermethrin was significant in autoclaved soil after 11 days of incubation studies. In the present work the bacterial system successfully degraded cypermethrin in autoclaved soil indicating that it can survive and compete with local microflora. Degradation of cypermethrin was insignificant in unautoclaved (uninoculated) and autoclaved (uninoculated) soil after 30-days of incubation studies. Degradation of cypermethrinwas significant in unautoclaved(inoculated) and autoclaved (inoculated) soil, whereas 97.5%and 95% of applied cypermethrin degraded respectively in 30-days of incubation studies.

SOIL SAMPLES	0 HOUR	11 DAYS
1	0.46	0.46
3	0.49	0.24

HPLC:

Peaks at retention time(RT) : 26, 27, 28 which cypermethrin, the area of the peak is reduced with time which clearly indicating the degradation of cypermethrin.

GC MS :

The products formed by degradation was analysed by GC-MS and the products formed were:

- Cyclotrisiloxane, hexamethyl ($C_6H_{18}O_3Si_3$)
- Cyclotetrasiloxane, octamethyl ($C_8H_{24}O_4Si_4$)
- Cyclopentasiloxane, decamethyl ($C_{10}H_{30}O_5Si_5$)
- Cyclohexasiloxane, dodecamethyl ($C_{12}H_{36}O_6Si_6$)
- Cycloheptasiloxane, tetradecamethyl ($C_{14}H_{42}O_7Si_7$)

CONCLUSION

This discussion suggests that the isolated organisms Serratia marscensens and Staphylococcus spp can flourish in the cypermethrin pesticide using farms by utilizing them as their source of energy when other sources are limited or unavailable. degradation was also checked by using Leuco crystal violet dye and absorbance was measured. The plasmid from all organisms were isolated and transformed into E.coli DH5αcells and colonies were inoculated to check degradation ability. Using, HPLC the degradation capacity was monitored and the organisms were able to degrade cypermethrin pesticide. using GC-MS it was found that these organisms had higher capability of degrading the pesticide.

REFERENCES

[1] A.G. Murugesan*, T. Jeyasanthi and S. Maheswari Isolation and characterization of cypermethrin utilizing bacteria from Brinjal cultivated soil African Journal of Microbiology Research Vol. 4 (1), pp. 010-013, 4 January 2010Available online ISSN 1996-0808 ©2010 Academic Journals

[2] AlbaseerSaeed S. Development of A Reversed-Phase High Performance Liquid Chromatographic Method for Efficient Diastereomeric Separation and Quantification of Cypermethrin, Resmethrin and PermethrinResearch Journal of Chemical Sciences ISSN 2231-606XVol. 2(10), 26-31, October (2012)

[3] Ali Mohammad Latifi* SamanehKhodi, MortezaMirzaei, Mohsen Miresmaeiliand Hamid Babavalian Isolation and characterization of five chlorpyrifos degrading bacteriaAfrican Journal of Biotechnology Vol. 11(13), pp. 3140-3146, 14 February, 2012 ISSN 1684–5315 © 2012

[4] Amritha G Kulkarni and Kaliwal BB* Bioremediation of Methomyl by Soil Isolate - Pseudomonas aeruginosa , IOSR Journal of Environmental Science, Toxicity and Food Technology, e-ISSN: 2319-2402,P- ISSN: 2319-2399. Volume 8, Dec 2014

[5] C. Ravi*, M. Ananthapriyan and M. Vinoth Kumar Isolation and characterization of endosulfan degrading bacteria from asugarcane field soil at Panaiyur, Madurai district, Tamilnadu.

[6] D Malik, M Singh, P Bhatia. Biodegradation of Cypermethrin by a Pseudomonas Strain Cyp19 and its use in Bioremediation of contaminated soil. The Internet Journal of Microbiology. 2008 Volume 6 Number 2.

[7] E.K. Janghel , J.K Rai, M.K Rai , V.K. Gupta , A new sensitive spectrophotometric determination of cypermethrin insecticide in environmental and biological samples, Chhattisgarh Council of Sience and Technology ,India. May 3, 2007. E-Journal of Chemistry.

[8] Haoyu Zhao, YucongGeng, Long Chen, Ke Tao, and Taiping Hou Biodegradation of cypermethrin by a novel Catellibacteriumsp. StrainCC-5 isolated from contaminated soil Can. J. Microbiol. Vol. 59, 2013

[9] M. Debbab1, S. El Hajjaji2*, Amal H. Aly3, A. Dahchour4, M. El Azzouzi1*, A. Zrineh1 Cypermethrin Residues in Fresh Vegetables: Detection by HPLC and LC-ESIMS and their Effect on Antioxidant Activity Mater. Environ. Sci. 5 (S1) (2014) 2257-2266 Debbab et al. ISSN: 2028-2508

[10] M. Surekha Rani1, K.Vijaya Lakshmi1* P. Suvarnalatha Devi1, R. Jaya Madhuri1, S. Aruna1, K. Jyothi1, G. Narasimha2 and K.Venkateswarlu3 Isolation and characterization of a chlorpyrifosdegradingbacterium from agricultural soil and its growth response African Journal of Microbiology Research Vol. (2) pp. 026-031, February, 2008 ISSN 1996-0808 ©2008 Academic Journals

[11] M.Radhika and M.Kannahi* Bioremediation of pesticide (Cypermethrin) usingbacterial species in contaminated soil International journal of current microbiology and applied sciences ISSN: 2319-7706 Volume 3 Number 7 (2014) pp. 427-435