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Degradation and Bioremediation of The Pesticide Cypermethrin" by *Pseudomonas spp and Bacillus spp*

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Abstract: In this present study, we have used two species of bacteria namely *Pseudomonas and Bacillus* to degrade cypermethrin from the contaminated crop fields for the purpose of bioremediation and focussing on the reduction of pyrethroid toxicity in the surrounding environment. The above stated two microorganisms were isoloated and characterised. The various underlying factors like different pH, temperature and concentration corresponding to the growth and the degree of degradation of the pesticide cypermethrin were determined. Thin layer chromatography and spectrophotometric method were done in support of the cypermethrin degradation. The genetic transformation of the plasmids which aided in the degrading of the pesticide were carried out in a suitable vector.

Keywords:pesticides**,** Cypermethrin, pyrethroid,

INTRODUCTION

The excessive use of pesticides leads to an accumulation of a huge amount of pesticide residues in the food chain and drinking water environment that further leads to a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds.Pyrethroid is the most important pesticide because even at very low concentration it is more effective. They are very effective against flies, mosquitoes, stored grain insects, aphides. Pyrethroids have four major generations among this cypermethrin belong to the fourth generation [1]. Cypermethrin is a synthetic pyrethroid used as an

insecticide in large- scale commercial agricultural applications as well as in consumer products for domestic purposes.Extensive or improper use of the chemicals leads to greater health risk to plants, animals and humans. Excessive exposure can cause nausea, headache, muscle weakness, salivation, and shortness of breath, seizures and neuro toxic effects to insects[2, 3].

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. Address: shop no. 399/2, opp. KSSC, NH-7, B.B Road, Bayanna Layout, Hebbal, Bengaluru, Karnataka 560024. Phone: 098443435. The cypermethrin pesticide was handled carefully.

Culture media for bacteria: Microorganisms were isolated from the pesticide contaminated soils with

- Nutrient Broth
- MM medium

Nutrient media was initially used as culture media for the growth of microbes present in the pesticide contaminated soil.

- To every 10ml of nutrient broth which 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 incubator 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.
- Observe for isolated colonies and further subculture and store at 16˚C.

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: In the enumeration of cypermethrin utilizing bacteria in minimal medium. The MM consisted of Na_2HPO_4, KH_2PO_4, $MgSO_4.7H2O$, $(NH_4)_2SO_4$, $Ca(NO_3)_2.4H_2O$, $Fe(SO_4)_3$, pH7.5 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. Cypermethrin stock solution of 1mg/mlwas prepared. Working standard solutions were prepared with appropriate dilutuions of stock standard solutions and water. Now to the above add 0.1% KI aqueous solution. Leuco crystal violet (LCV) was prepared in 100ml volumetric flask by adding 0.5g of LCV, 0.3ml of 85% orthophosporic acid and make up volume with water. Gently shake till dye dissolves[4].

Procedure: 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 add different concentrations of cypermethrin, add 1ml of ethanol to dissole the cypermethrin to each tube, add 1ml of 0.1% of KI and keep 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.

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

Procedure 2: The residues were 19nalysed for metabolites by thin layer chromatography on Silica gel plates using the following solvent systems. Chloroform-acetic acid (95:5 v/v). The metabolites were visualized under UV light at 254 nm or by exposure to iodine vapours and also by spraying with 1% $FeCl_3$-$K_3Fe(CN)_6$ solution in water

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.

Bioremediation 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.

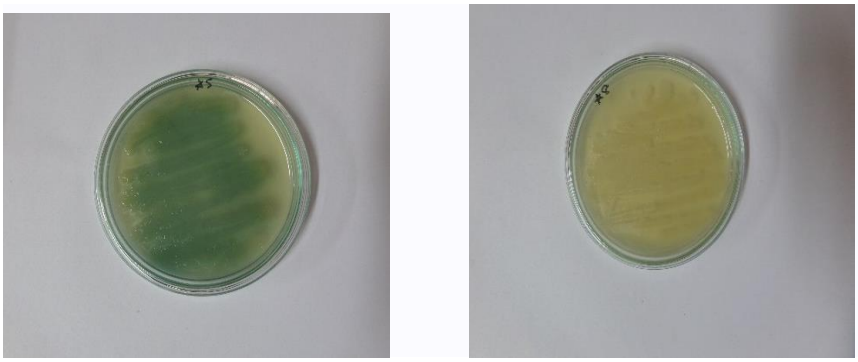
High Performance Liquid Chromatography (HPLC): A standard was prepared by dissolving them in acetonitrile to the final concentration. The dilution run at different conditions of pesticide. Cypermethrin was detected at Wave length 254nm and 231 nm at Flow rate 1mL/min using a Mobile phase HPLC grade Acetonitrile70% 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[5] at a flow rate of 1mL/minute.

RESULT AND DISCUSSION

Isolation of microorganisms from pesticide contaminated soil:

Bacteria: The results indicate from the pesticide contaminated soil 2 organisms were isolated. They were found to carry out successful degradation on cypermethrin pesticide used in this work. For the isolation of organisms , MM medium was used and which showed very high growth of organisms and effective compared to nutrient medium. MM medium suplemented with cypermethrin and resulted that growth was observed within 24-48 hours. But according to research article it says thatthe bacterial culture capable of degrading cypermethrin was isolated from agricultural soil using enrichment technique supplemented with cypermethrin (0.1 - 1%) and incubated at 35°C for 24 - 48 hIndividualcolonies were sub cultured into nutrient agar plates containing same concentration of cypermethrin until pure culture was isolated[6].



Pseudomonas spp *Bacillus spp*

Fig: 1: Microorganisms isolated from the pesticide contaminated soil (Bacteria).

Staining: The stainig techniques was done to know the morphology of the microorganism (bacteria and fungi) which were isolated from esticide contaminated soil.

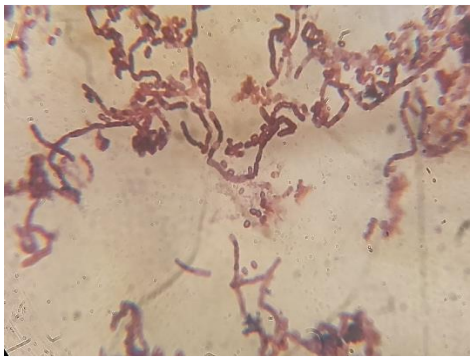


Fig.2: *Bacillus spp*

Biochemical charaterization of isolates: The following biochemical tests were done to identify the isolates founf from the pesticide contaminated soil.

Table 1: Biochemical charaterization of isolates

Content	*Bacillus spp*	*Pseudomonas spp*
Indole	-	-
Methyl Red	+	-
Voges Prokauer test	-	-
Citrate test	+	+
Oxidase test	+	+
Startch test	+	-
Catalase test	+	+
Coagulase test	-	+

Determination of Antibiotic sensitivity: The bacterial isolates were spread on LB agar paltes separately. Then sensitivity discs ofloxacin, meropenen, clindamycin, doxycycline, cefltriaxone and methicilin were placed on the plates and these paltes were incubated overnight. The appearance of clear area around antibiotic discs showed the sensitivity of isolates to those antibiotics, while the growth of the isolates around the discs showed their resistance against those antibiotics. The zone of inhibition was also measured for each disc.The antibiotic resistance / susceptibi;ity profiling, the disc diffusion method was used. The zone of inhibition was measured in millimeter and the resistance and sensitivity of isolated towards antibiotic used was determined. It was found that all the isolates was resistant to ofloxacin. For antibiotic resistance/ susceptibility profiling, the disc diffusion method was used.

Table 2: Antibiotic sensitivity

Antibiotics	*Pseudomonas spp*	*Bacillus spp*
Ofloxacin	1.5mm	0.85mm
Meropenen	-	-
Clindamycin	-	-
Doxycycline	0.65mm	-
Cefltriaxone	-	0.6mm
Methicilin	-	-

The zone of inhibition was measured in millimetre and the resistance and sensitivity of isolated bacteria towards antibiotics used was determined, and according to their study it was found that all five isolates being Gram negative in nature were found to be resistant to penicillin and Ciprofloxacin. The percentage of resistance towards the 15 antibiotics used was found to be lowest in EC1(26.66%) and highest in EC5 (66.66%) whereas the remaining three isolates showed moderate resistance (40%) by observing the growth on the plates. It was observed that Chlorpyrifos was easily tolerable pesticide were growing faster in Endosulfan. Amongst all five isolates, EC5 was capable of tolerating highest concentrations of the pesticides when compared with the remaining four isolates[7].

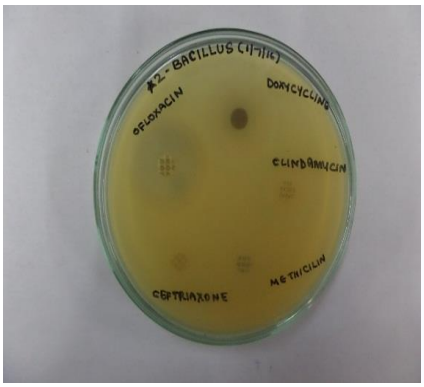


Fig.3: (A) *Bacillus spp*

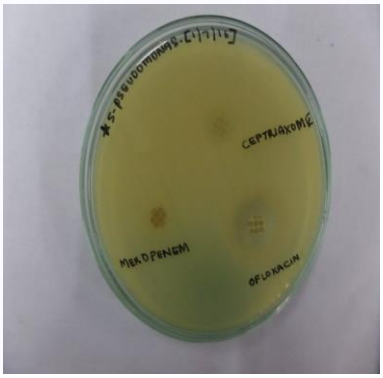


Fig.3: (B) *Pseudomonas spp*

TLC –thin layer chromatography: To find out the degradation using TLC which was found out that it was positive, later we went in for calorimetric analysis.

Fig.4: TLC (purple spots)

Isolation of plasmid DNA and Agarose Gel Electrophoresis: Among the seven isolates, presence of plasmid DNA was detected in all the seven isolates on performing the plasmid DNA extraction, and almost all the isolates had the tolerance for high concentration of heavy metal salts, pesticides and antibiotics. On treatment with the restriction of endonucleases- EcoRI and HindIII showed band indicating sequences. But according to their studyamongst the five isolates, presence of plasmid DNA was detected in EC3, EC4 and EC5 on performing Miniprepmethod of plasmid DNA extraction. The other two isolates EC1 and EC2 were lacking plasmid DNA indicating that the property of these isolates to tolerate high concentrations of heavy metal salts, pesticides and antibiotics was not plasmid borne. On treatment with restriction endonucleases - EcoRI and HindIII, it was seen that the plasmids from EC3, EC4 and EC5 showed single band indicating no recognition sequence (restriction site) of these two enzymes. On comparing with the λ /*Mul*molecular weight marker, molecular weight of EC3, EC4 and EC5 plasmids was found to be approximately[7] between 9,824D and 26,282 D[3]**.**

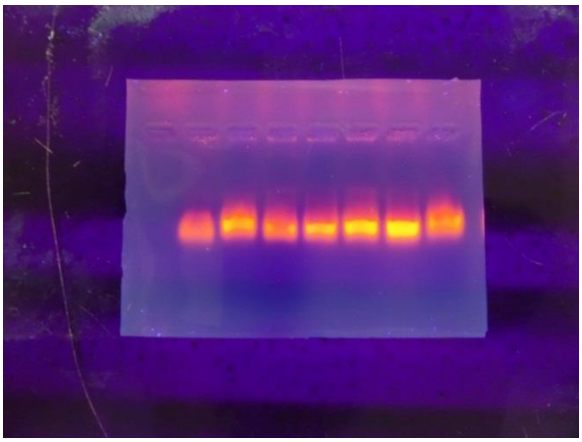


Fig.5: Isolation of plasmid DNA

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. But according to their study it was found to be that the extracted plasmid DNA from EC3, EC4 and EC5 were further used for transforming the competent cells of *E.coli* DH5α strain. 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[7].

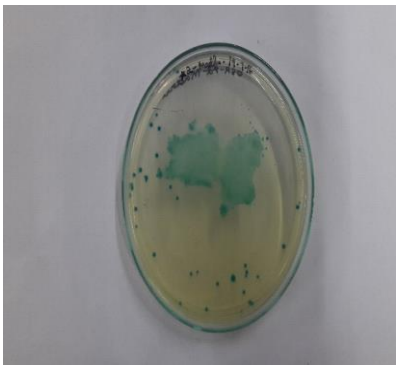


Fig.6:*Bacillus spp*

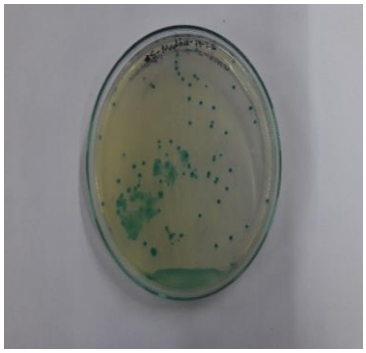
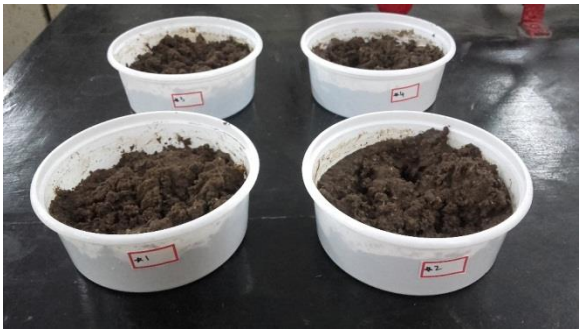


Fig.7:*Pseudomonas spp*

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. But according to their study it was found to be that the addition of strain Cyp19 to soil resulted in a more rapiddegradation of cypermethrin than by indigenous microflora [8,9].

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. Microbial population of strain Cyp19 reduced from 10 6 cells g $^{-1}$ of soil to 10 4 cells g $^{-1}$ ofsoil in 30 days of incubation study. In the present work the bacterial system successfully degraded cypermethrin in autoclaved and unautoclaved soils indicating that it can survive and compete with the local microflora[10].



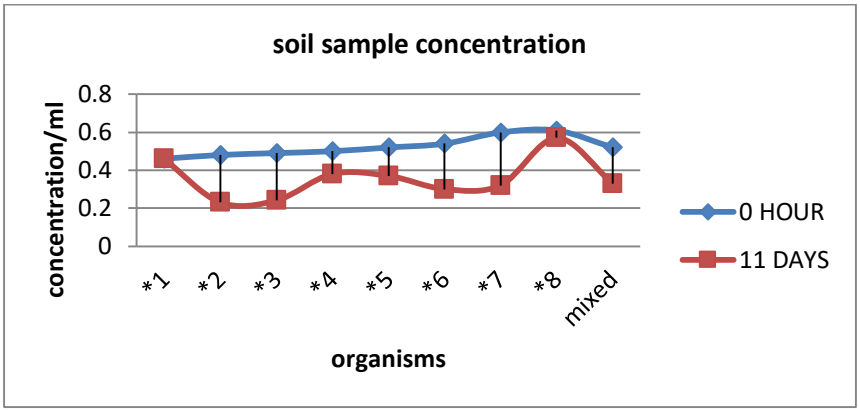


Fig .9: Bioremediation of Cypermethrin Using Soil

Soil Samples	0 Hour	11 Days
*1	0.46	0.46
*2	0.48	0.23
*3	0.49	0.24
*4	0.50	0.38
*5	0.52	0.37
*6	0.54	0.30
*7	0.60	0.32
*8	0.61	0.57
mixed	0.52	0.33

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[11].

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 ($C_{14}H_{42}O_7Si_7$)

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CONCLUSION

We have isolated cypermethrin degrading bacteria from agriculture soil of different areas. From the study, we confirm that these organisms are degrading the pesticide. we used two species of bacteria namely *Pseudomonas spp and Bacillus spp.* With this bacteria soil fertility can be increased and pesticide contamination could be removed as well. The degradation was checked by using Leuco crystal violet dye checking for the appearance of the dye and absorbance was measured. The plasmid was isolated and transformed into *E.coli DH5α*cells and colonies were inoculated to check degradation ability. Using, HPLC the ability of degradation capacity was monitored and the strains were able to degrade cypermethrin pesticide. From GC-MS study, it is found that the bacteria has a higher capability of degradation.

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