Degradation of Pyrethroid Insecticide- cypermethrin (25% EC) using Soil Microorganisms and Detection of Degradation Products

M.M. Gurjar¹, V.S. Hamde²

ABSTRACT

Background: Cypermethrin is used extensively to kill the pests that decreases the crop yield. Excessive use of cypermethrin results in soil and water contamination, thus affecting soil microflora and the aquatic life. Thus, removal of cypermethrin is particularly important. Different physical and chemical methods are available for the cypermethrin removal but are time consuming and costly. So, microbial degradation of cypermethrin is important. The present study aimed to isolate and identify cypermethrin degrading organisms, detection of the cypermethrin degradation product by GC-MS analysis and detection of residual amount of cypermethrin from the medium.

Methods: In this laboratory investigation, soil samples were collected from different farms and isolation and identification of microorganisms capable of cypermethrin degradation was performed. Further, detection of residual cypermethrin concentration, COD value and cypermethrin degradation products using GC-MS analysis was performed.

Result: Cypermethrin degrading microorganisms were isolated and identified as \textit{P. aeruginosa} and \textit{K. pneumoniae}. These microorganisms degraded 200mg/lit cypermethrin and the cypermethrin degradation products were detected and identified by comparing with the standard database. Residual amount of cypermethrin was detected from the medium by colorimetric method. Residual cypermethrin concentration was decreased with time in the test indicating cypermethrin degradation ability of the \textit{P. aeruginosa} and \textit{K. pneumoniae}. Further, COD value was determined and COD value was found to be decreased in the medium in presence of \textit{P. aeruginosa} and \textit{K. pneumoniae} indicating cypermethrin removal.

Key words: Biodegradation, Cypermethrin, GC-MS analysis, Pesticide, Pyrethroid group.

INTRODUCTION

The term pesticide refers to wide range of compounds like insecticides, herbicides, fungicides, rodenticides, plant growth regulators etc (Hayes, 1975). Pesticides are used to control different pests that are found to be harmful. It has been reported that extensive use of cypermethrin leads to undesired side effects on population and activity of useful soil microflora (Pandey and Singh, 2004). In recent years use of pyrethroids has increased extensively due to restrictions or ban over highly toxic organochlorine and organophosphate pesticides and it has become the dominant pesticide among retail sales to consumers. It has been said that “no pesticide is perfect, but the pyrethroids come close” (Pandey and Singh, 2004).

Pyrethroids are botanical origin insecticides and have been obtained from dry flowers of \textit{Chrysanthemum cinerariefolium} plant which is known from nineteenth century (Grant \textit{et al}, 2001). Pyrethroids have four major generations and cypermethrin belongs to fourth generation of pyrethroids (Casida, 1980). Cypermethrin [(+/-)-α-cyano-3-phenoxybenzyl (+/-)-cis, trans-3 (2,2-dichlorovinyl)-2, 2-dimethylcyclopropane carboxylate] is a synthetic pyrethroid pesticide. It is used in home and garden pest control worldwide (Tallur \textit{et al}, 2008; Lin \textit{et al}, 2011).

Recently; cypermethrin is studied extensively due to its aquatic life toxicity as well as high risk to human health (Zhang \textit{et al}, 2011). So, it is necessary to develop an effective and rapid method for degradation and removal of cypermethrin from the environment. For this, the biological treatment is important which involves transformation of these chemical compounds in to non-Hazardous form (Saraswat and Gaur, 1995). The present study aims at isolation and identification of cypermethrin degrading organisms, checking tolerance of isolates to increased cypermethrin concentration, detection of residual cypermethrin by colorimetric method and detection of cypermethrin degradation metabolites by GC-MS analysis.
Degradation of Pyrethroid Insecticide- cypermethrin (25% EC) using Soil Microorganisms and Detection of Degradation Products

**MATERIALS AND METHODS**

**Isolation of cypermethrin degrading microbes from the soil**

Microorganisms capable of cypermethrin degradation were isolated by using the soil enrichment technique (Bhadhkhade B. J. 2002) in 2014 from the soil obtained from Theur and Manjari Dist. Pune. Microorganisms were grown on minimal agar medium containing 1mg/lit cypermethrin. Individual colonies were sub-cultured on minimal agar plates containing same concentrations of cypermethrin until pure cultures were obtained. These organisms were labeled as FCM1, FCM2, FCM3 etc. The isolated pure cultures were maintained at 4°C and as glycerol stocks at -20°C and sub cultured every three months.

**Checking tolerance of isolates to increased cypermethrin concentration**

Isolated organisms were grown in presence of minimal medium containing increasing cypermethrin concentrations as- 1mg/lit, 10mg/lit, 100mg/lit, 150mg/lit, 200mg/lit.

**Identification of isolates by biochemical characteristics and 16SrRNA sequencing**

The isolates which showed tolerance to 200mg/lit cypermethrin were identified by using morphological, cultural and biochemical characteristics as described by Collins and Lyne (1985) up to the genus level. Further the isolates were identified by 16SrRNA sequencing.

**Checking diazotrophic nature of isolates**

Diazotrophic nature of isolates was confirmed by streaking the isolates (200mg/lit cypermethrin tolerating) on Nitrogen Free malate medium with Bromo thymol blue (NFB). NFB medium plates were incubated at 37°C for 24 hrs.

**Chemical Oxygen Demand (COD) determination Open reflux method**

Open reflux method was used for detection of COD changes as a measure of cypermethrin degradation by FCM68 and FCM82. These isolates were inoculated in minimal medium broth supplemented with 50mg/lit, 100mg/lit, 150mg/lit and 200mg/lit cypermethrin. Samples were incubated at 30°C and samples were obtained at different time intervals like 2, 6, 10 and 14 days. COD from each sample and control was determined by standard open reflux method for COD estimation as described by Pitwell, L.R. (1983).

**Colorimetric estimation of residual cypermethrin**

For residual cypermethrin estimation, 1 mL of the sample (Cypermethrin residue dissolved in methanol) was mixed with 1 mL reagent- A (O-dinitrobenzene-P-Nitrobenzaldehyde dissolved in 80% 2- methoxyethanol), 25 µl reagent- B (Sodium hydroxide solution) was added as described previously (Kaur et al. 2013, Patent no.US8609428b2). This mixture was incubated for 5-20 minutes and purple colour developed was detected colorimetrically at 530-580nm.

For calibration curve, double dilutions of cypermethrin were prepared as 160, 80, 40, 20, 10, 5, 2.5 mg/ mL in acetone (Stock- 160 mg/lt). Absorbance from each tube was taken at 530-580 nm using colorimeter. Calibration curve of cypermethrin concentration against absorbance was plotted.

**GC-MS analysis for detection of degradation products**

The isolates FCM68 and FCM82 were inoculated in minimal medium broth supplemented with cypermethrin at a concentration of 200mg/lit. Flasks were incubated at 30°C for 14 days and the cypermethrin degradation products were detected by method described by Nilesh P. Bhosle (2013).

**RESULTS AND DISCUSSION**

**Isolation of pesticide degrading microorganisms**

Microorganisms capable of cypermethrin degradation were isolated from different soil samples. 96 different isolates were obtained by soil enrichment technique. Isolates were labeled as FCM1, FCM2, FCM3 up to FCM100.

**Checking tolerance of isolates to increased cypermethrin concentration**

Two isolate FCM68 and FCM82 showed growth at 200mg/lit cypermethrin. These isolates were used for further studies. Bhosale et al (2013) showed ability of *Pseudomonas* strains to tolerate cypermethrin up to 150mg/lit.

**Identification of FCM68 and FCM 82**

As per morphological and biochemical characteristics, the isolates FCM68 and FCM82 were identified as genera *Pseudomonas* and *Klebsiella* respectively. Further identification was done using 16SrRNA sequencing.

Isolates FCM68 and FCM82 were identified by amplification of 16SrRNA using universal primers FPP2 and RPP2 resulting in final product of 1,500 base pairs. The PCR products were purified and sequenced using only SRV31 primer (hypervariable region). A consensus sequence of 607 base pairs and 615 base pairs products were evidenced for *P. aeruginosa* (Fig 3.1) and *K. pneumoniae* (Fig 3.2), respectively and a standard phylogenetic tree was deduced by using standard sequences available with Gene Bank (Fig 3.3 and 3.4). The BLAST analysis of isolate FCM68 found to be 97.19%, 98.68%, 99.17% and 99.67% identical with *Pseudomonas indologlyxans*, *Pseudomonas alcaligenes*, *Pseudomonas otitidis* and *Pseudomonas aeruginosa*, respectively (Fig 3.3).

The BLAST analysis of isolate FCM82 found to be 97.42%, 98.22%, 98.38% and 98.70% identical with *Klebsiella singaporensis*, *Klebsiella pneumoniae* subspecies *ozaenae,*
Degradation of Pyrethroid Insecticide- cypermethrin (25% EC) using Soil Microorganisms and Detection of Degradation Products

*Klebsiella pneumoniae* subspecies *rhizascleromatis* and *Klebsiella pneumoniae* subspecies *pneumoniae*, respectively. The nucleotide sequence data of FCM68 and FCM82 has been deposited in GenBank under accession numbers MF 423469 and MF 423470; respectively.

### Checking diazotrophic nature of isolates

*P. aeruginosa* and *K. pneumoniae* grew on NFB medium, blue coloration was observed. This indicated diazotrophic nature of these isolates. The property of diazotrophy that is nitrogen fixation of these isolates would be helpful in plant growth along with other plant growth promoting properties. Naher (2013) detected the diazotrophic nature of isolates by using NFB solid medium and detected the growth and blue coloration on the NFB medium indicating diazotrophic nature.

### Chemical Oxygen Demand (COD) detection

Chemical Oxygen Demand was found to be decreased in presence of *P. aeruginosa* and *K. pneumoniae*. *P. aeruginosa* showed higher percentage of COD reduction indicating its greater efficiency in cypermethrin degradation. However, at 200mg/lit cypermethrin, maximum COD reduction was 35.81%. This might be due to increased toxicity of cypermethrin at higher concentration.

![Fig 2.1: Sequence of 16S rRNA gene of isolate FCM68.](image)

![Fig 2.2: Phylogenetic tree for FCM 68.](image)

![Fig 2.3: 16S rRNA gene sequence of FCM 82.](image)
Degradation of Pyrethroid Insecticide- cypermethrin (25% EC) using Soil Microorganisms and Detection of Degradation Products

Fig 2.4: Phylogenetic tree for FCM 82.

Sterile NFB Plate as control
NFB Plate inoculated with P. aeruginosa
NFB Plate inoculated with K. pneumoniae

Fig 2.5: Growth of P. aeruginosa and K. pneumoniae on nitrogen free medium.

Table 2.2: Identification of isolate FCM 82 by 16SrRNA sequencing.

| Strain Designation | Closest Phylogenetic affiliation                     | Max ident | Accession number |
|--------------------|-----------------------------------------------------|-----------|------------------|
| FCM82              | Klebsiella pneumoniae subsp. Pneumoniae             | 99%       | MF423470.1       |

Table 2.3: Cypermethrin degradation metabolites produced by P. aeruginosa.

| Retention time (min.) | Molecular formula | Name of metabolite                                                                 |
|-----------------------|-------------------|----------------------------------------------------------------------------------|
| 5.06                  | C_{10}H_{14}       | 4,7-Methanoindene,39,4,5,6,7-hexahydroendo                                        |
| 25.69                 | C_{14}H_{20}O_{5}  | Triacantonic acid methyl ester                                                    |
| 29.74                 | C_{12}H_{24}S_{2}  | Disulphide, di-tert-dodecyl                                                        |
| 30.25                 | C_{12}H_{24}ClNO_{2} | Cyclopropane carboxylic acid,3-62,2-dichloro ethynlyl0-2,2 dimethyl-cyano       |
| 31.62                 | C_{14}H_{20}O_{3}  | Silane, dimethyl0docosyloxy butoxy                                                |
| 33.40                 | C_{16}H_{30}O_{5}  | Phenol,2,6-bis (1,1 dimethyl ethyl)                                                |

Table 2.4: Cypermethrin degradation metabolites produced by K. pneumoniae.

| Retention time (min.) | Molecular formula | Name of metabolite                                                                 |
|-----------------------|-------------------|----------------------------------------------------------------------------------|
| 5.04                  | C_{10}H_{14}       | 4,7-Methanoindene,39,4,5,6,7-hexahydroendo                                        |
| 6.38                  | C_{12}H_{20}O_{4}  | Dibenzo, biphenylene-5,6,11,12 tetronene,5a,5b,11a,11b -tetrahydro              |
| 17.49                 | 2H-Indole-2-one,1,3, dihydro-3,3-bis(4-hydroxy-3-methyl phenyl)                 |
| 18.20                 | C_{10}H_{20}O_{2}  | Benzene,1,1-(1 phenyl-2- ethenediy1) bis(P-methoxy phenyl)-1-phenyl               |
| 18.63                 | C_{12}H_{24}NO_{2} | 3(2-ethyl-piperdin-1-methyl)-8a-methyl-5 methylene decahydro-naphtho(2,3-b)-furan-2-one |
| 19.01                 | C_{12}H_{20}O       | 1.4-Epoxyphthalene-1(2H) methanol,4,5,7-tris(1,1dimethyl)3,4, dihydro            |
| 27.39                 | C_{10}H_{20}O      | n-Heptadecylbenzene                                                               |
| 27.45                 | C_{12}H_{24}O_{2}  | 16-pregnenolone                                                                   |
| 30.70                 | C_{12}H_{24}ClNO_{3} | Cyclopropane carboxylic acid,3-62,2-dichloro ethynlyl0-2,2 dimethyl-cyano      |
| 31.12                 | C_{16}H_{30}       | Octadecane,3-ethyl-5-(2-ethyl-butyl)                                              |
Degradation of Pyrethroid Insecticide- cypermethrin (25% EC) using Soil Microorganisms and Detection of Degradation Products

As per the study, when the minimal medium was inoculated with *P. aeruginosa* in presence of 50, 100, 150 and 200 mg/lit cypermethrin, reduction in COD was 58.27, 67.74, 77.47 and 35.81% respectively; whereas when the minimal medium was inoculated with *K. pneumoniae*, in presence of 50, 100, 150 and 200 mg/lit cypermethrin, reduction in COD was 33.97, 46.29, 41.43 and 37.29 respectively after 14 days of incubation. However, at 200mg/lit COD reduction was not significantly increased (Fig 2.6). In presence of *K. pneumoniae*, highest COD reduction (46.29 %) was seen at 100mg/lit cypermethrin. However, at 150 and 200mg/lit cypermethrin, % COD reduction was lesser than at 100mg/lit cypermethrin for *K. pneumoniae* (Fig 2.7).

This may be due to toxicity of increased cypermethrin concentration in the medium or due to accumulation of toxic components in the medium that interferes with the cypermethrin degradation process. Pesticide degradation and decreased COD was reported by Jilani and Altaf Khan, (2006). Also, it was reported that reduction in COD value is directly proportional to degradation of the parent compound into possible nontoxic compound (Bhosale et al, 2013).

It was noted that decrease in COD value as a means of removal of organic load was proportional to the removal of cypermethrin from the medium. Similar correlation between 2,4, - DAT biodegradation and COD decrease was observed by Berchtold et al. (1995). Pesce and Wunderlin, 1997 also observed correlation between 2,4 and 2,6 diamino toluene degradation by acclimated bacteria and decrease in COD value. Similarly, Jilani S. et al (2006) showed decrease in COD value with disappearance of cypermethrin from the medium.

### Estimation of residual amount of cypermethrin in presence of *P. aeruginosa* and *K. pneumoniae*

For residual cypermethrin estimation, samples were extracted from the minimal medium inoculated with *P. aeruginosa* and *K. pneumoniae* at different time intervals (0, 24, 48, 72 and 96 hrs). Absorbance of all the test samples (15 for *P. aeruginosa* and 15 for *K. pneumoniae*) was noted at 530 nm and extrapolated on calibration curve to determine the residual cypermethrin concentration. At every cypermethrin concentration used in the test, residual cypermethrin concentration was decreased as incubation time increases from 0 - 96 hrs (Fig 2.8 and Fig 2.9).

When minimal medium was inoculated with *P. aeruginosa*, as the incubation time is increased from 0 hrs to 96 hrs, residual cypermethrin concentration was found to be decreased from 48.5 to 3.4, 99.79 to 17.48, 146.14 to 45.53 and 167.48 to 77.84 mg/lit at 50, 100, 150 and 200mg/lit cypermethrin; respectively. Similarly, when minimal medium was inoculated with *K. pneumoniae*, as the incubation time is increased from 0 hrs to 96 hrs, residual cypermethrin concentration was found to be decreased from 49.6 to 7, 99.79 to 18.09, 146.14 to 57.11 and 167.48 to 91.26mg/lit at 50, 100, 150 and 200mg/lit cypermethrin; respectively. Previous studies showed 81% cypermethrin
degredation by *Bacillus* species up to 15 days post incubation compared to control (50ppm) as evidenced by HPLC analysis (Pankaj *et al.*, 2016). In present research work, first time residual cypermethrin concentration from the minimal medium is detected by using colorimetric assay and approx. 85% cypermethrin was degraded from the medium supplemented with 50mg/lit cypermethrin.

**GC-MS analysis for detection of cypermethrin degradation products**

Peak of first compound, GC1 (5.06 min) appeared during the cypermethrin biodegradation by *P. aeruginosa*. This compound was identified as 4,7methanoindene, based on its retention time and molecular weight with those of corresponding authentic compounds in the database. Peaks of GC3 (25.69 min) and GC4 (30.25) were observed and the corresponding compounds were triaconatanoic acid methyl ester and cyclopropane carboxylic acid as per the database. Similarly, some other metabolites were also identified as they showed different retention times. Test organism, *P. aeruginosa* degraded cypermethrin up to 200mg/lit as evidenced by GC-MS results. Comparison of GC-MS results of standard cypermethrin with degradation products in presence of *P. aeruginosa* showed production of degradation metabolites such as Triaconatanoic acid, methyl ester Disulphide, di, tert-dodecyl cyclopropane carboxylic acid, 3,2 Silane, dimethyl-(docosyloxy)butoxy Sulfurous acid, octadecylpentyl ester Phenol, 2,6, bis[1,1 dimethyl ether] etc. However, at 200mg/lit cypermethrin concentration, percent decrease in COD is up to 40% indicating decreased capacity of *P. aeruginosa* to degrade cypermethrin. This might be due to increased accumulation of toxic products or may be due to nutrient depletion from the growth medium. Similar observations were made by Nilesh Bhosale *et al.* (2013) and Manaswi Gurjar *et al.* (2018).

**CONCLUSION**

The isolated organisms *P. aeruginosa* and *K. pneumoniae* showed capacity of growing at high concentration of cypermethrin. Cypermethrin degradation using *P. aeruginosa* and *K. pneumoniae* was revealed by GC-MS analysis and by determination of changes in COD.

**ACKNOWLEDGEMENT**

We are thankful to all those who helped to complete this research work. We are also thankful to Principal Fergusson College, Pune and Head department of Microbiology for allowing the work to be done in the department.
REFERENCES

Berchtold, S.R., Vanderloop, S.L., Suidan, M.T., Maloney, S.W., (1995). Treatment of 2,4-diaminotoluene using a two-stage system: fluidized-bed anaerobic granular activated carbon reactor. Wat. Environ. Res. 67. 1081-1091.

Bhadhade, B.J., Dhakephalkar, P.K. (2002). Plasmid associated degradation of an Organophosphorous pesticide, Monocrotophos, by Pseudomonas mendocina. Biotechnology Letters. 24 (8): 647-650.

Bhosale Nilesh P. and Sahera Nasreen (2013). Remediation of Cypermethrin-25 EC by Microorganisms. European Journal of Experimental Biology. 3(1): 144-152.

Casida, J.E. (1980). Environ. Health. Prospect. 34: 189-202.

Collins CH, Lyne PM. (1985). Microbiological methods. 5th edition. Butterworth and Co (Publisher) Ltd. P 1606.

Grant, R.J., Daniell and W.B. Betts (2001). Biodegradation of synthetic pyrethroid insecticides in formulated compounds. Letters in Applied Microbiology. 36: 173-176.

Hays, W.J. (1975). Toxicology of pesticides. The Williams and Wilkins, Baltimore. 37-106.

Jilani, S. and Altaf Khan (2006). Biodegradation of Cypermethrin by Pseudomonas in a batch activated sludge process. Int. J. Environ. Sci. Tech. 3(4): 371-380.

Lin, Q.S., Chen (2011). Biodegradation of cypermethrin by newly isolated actinomycetes HU-S-01 from wastewater sludge. International Journal of Environmental Science Tech. 8 (1): 229-311.

Navprabhjot Kaur and Poonam Sharma (2013). Screening and characterization of native Pseudomonas sp. as plant growth promoting rhizobacteria in chickpea (Cicer arietinum L.) rhizosphere. African Journal of Microbiology Research. 7(16): 1465-1474.

Nilesh, P. Bhosle, Zafar, S. Khan and Sahera Nasreen (2013). In vitro degradation of Cypermethrin through microorganisms by scale up technique. International Journal of Advanced Research. 1(7): 229-238.

Pandey, S. and D.K. Singh (2004). Total bacterial and fungal populations after chlorpyrifos and quinalphos treatments in groundnut (Arachis hypogaea L.) soil. Chemosphere. 55(2): 197-205.

Pesc, S.F. and Wunderlin, D.A., (1997). Biodegradation of 2,4- and 2,6-diaminotoluene by acclimated bacteria. Water Res. 31(7): 1601-1608.

Pitwell, L.R. (1983). Standard COD. Chem. Brit. 19:907.

Saraswat, R., Gaur (AK) (1995). Bioremediation of β endosulfan by Rhodococcus sp. Int. J. Microbiol. 35(3): 249-253.

Tallur Preeti, N. and Veena, B. Megadi (2008). Biodegradation of Cypermethrin by Micrococcus sp. strain CPN 1. Biodegradation. 19: 77.