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RESEARCH ARTICLE

***In-vitro* degradation of Cypermethrin through microorganisms by scale up technique**Nilesh P. Bhosle¹, Zafar S. Khan² and Sahera Nasreen³

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Abstract

In present *in-vitro* study the potential of four microorganisms used were *Pseudomonas aeruginosa* (Accession No. JF708942.1, Strain IRMD-2010), *Alcaligenes faecalis* (Accession No. HQ202537.1), *Trichoderma viride* and *Rhizopus stolaniferous* were assessed for degradation of cypermethrin by scale up technique under controlled environmental conditions. These four microorganisms primarily isolated from cypermethrin treated cotton field soil and they were capable of utilizing pyrethroid pesticide cypermethrin as sole source of carbon and energy. Bioremediation of cypermethrin was analyzed in three varying concentration such as 50ppm, 100ppm, 150ppm in µg/ml ratio after 14 days period of incubation by dry weight of mycelia, Gas chromatography-Electronic capture detector (GC-ECD) and Gas chromatography-Mass spectroscopy (GC-MS) profiling for the metabolite detection. Simultaneously chemical oxygen demand, P^H detection and growth kinetics were also carried out and recorded each 3, 7, 11, 14 days of incubation period. The GC-ECD profiling revealed that *Pseudomonas auroginosa* potent one to degrade the cypermethrin 81.35%, 70.35%, 53.32% at tested 50ppm, 100ppm, 150ppm concentrations respectively after 14 days incubation period. While *Alcaligenes faecalis*, *Trichoderma viride* and *Rhizopus stolaniferous* showed moderate degradation of cypermithrin than *Pseudomonas aeruginosa*. The GC-MS result sheet showed that Pathalic acid and Proponic acid are the two primary metabolites obtained. Therefore *Pseudomonas auroginosa* was more potential to degrade the toxic compound like cypermethrin in soil and also beneficial for soil remediation for high yield production of crops.

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1. Introduction

India is an agriculture based country in which about 60-70% of its population is dependent on agriculture. Presently a huge portion of land already under cultivation is being rapidly depleted by industries and urban encroachments. On the other hand, the demand for agricultural crops is increasing day by day due to the rapidly increasing in population. Hence, there is a need for a high increase in the quantity of agricultural crop yield as well as improvement in its quality. To meet these objectives agrochemicals like insecticides, fungicides, and

herbicides are being used on a large scale in agricultural lands for controlling pest. Similarly about 30% of agricultural yield is lost due to pests. Hence, the use of pesticides has also become indispensable as pesticide has become an integral and economically essential part of modern agriculture. Besides combating insect pests, insecticides also affect the population and activity of beneficial microbial communities in soil (Pandey and Singh, 2004), Pesticides are generally categorized as insecticides, herbicides and fungicides according to the type of pest which they have shown efficacious action

(Miligi *et al.*, 2006). The biological treatment of chemically contaminated soil involves the transformation of complex chemical compound into non hazardous form (Saraswat and Gaur, 1995).

Cypermethrin is more effective against insects, pest including moths, pest of cotton, fruit and vegetable crops. Extensive and improper use of chemical leads to greater health risk to plants, animal and human and soil texture which had been reviewed time to time by several workers. Cypermethrin has moderate persistence in soil. Under laboratory conditions cypermethrin degrades more rapidly than soil (Sarswat and Gaur, 1995) and in aerobic condition the half life of cypermethrin is four days to eight weeks (Wauchope *et al.*, 1992). *In-vitro* studies has shown two soil bacteria that are able to degrade this insecticide, they are member of genera *Pseudomonas* and *Serratia* (Grant *et al.*, 2002). Pesticides are utilized by some bacteria as a sole carbon source for their energy metabolism and degraded by them (Baxter *et al.*, 1975). Despite earlier findings, the microbial population of soil is affected by cypermethrin the ammonification and nitrification in treated soils is affected, a sign of the environmental impact of cypermethrin (Rangaswamy and Venkateswarlu, 1993).

Soil microorganisms particularly soil fungi, represent biogeosphere component to determine the level of their real toxic effect, since they take part in their detoxification and mineralization by using carbon as energy source (Dukie and Mandic, 1998; Nunez *et al.*, 2001). The bioremediation of pesticide using potential microorganisms will be a beneficial technique for the degradation of pesticides waste into less toxic compounds. The present study has been taken to analyze bioremediation of pesticide with special reference to cypermethrin by using *P. auroginosa* by scale up process at under Controlled environmental conditions.

2. Materials and Methods:

2.1. Insecticides:

The Cypermethrin-25 EC (BASF India Lid.) was purchased locally from Mondha market, Agriculture Shop, Aurangabad.

Composition: Cypermethrin-25 EC Technical: (70% basis) 36% w/w, Surfactants- Cresilax AE1, AE2, AE3, (Calcium salt of Alkyl benzene sulphonate, Alkyl phenol Ethoxylate, Tri glyceride Ethoxylate): 10.00% w/w., Solvent- C-IX :54.00 w/w, Total 100.00% w/w.

Cypermethrin IUPAC Name: [(RS)- α -cyano-3-phenoxybenzyl (1RS)-*cis-trans*-3-(2,2-dichlorovinyl)-1,1-dimethyl-cyclopropanecarboxylate.]

2.2. Collection of soil samples:

The soil samples were collected from the different sites of Cotton cultivated field of Belora Dist. Jalna (M.S) India. These fields were already sprayed with cypermethrin for past few years. The soil samples were collected from these fields brought in laboratory in sterile polythene bag and stored at 15°C temperature for further study.

2.3 Soil enrichment Technique for isolation of Cypermethrin degrading bacteria:

Soil samples taken from the top 0-15 cm of field plots were air dried to 20% (w/w) moisture content. Fifty grams of each sample was placed in six glass plates and covered to maintain moisture conditions. The soil samples were treated with aqueous solution of Cypermethrin-25 EC to get final concentration 1000 ppm and incubated at room temperature for two weeks by mixing gently. The moisture content was maintained using distilled water. The insecticide treatment was repeated three times at every two week of time interval.

2.4 Isolation of Cypermethrin degrading microorganisms:

The enriched culture method was used to isolate the cypermethrin degrading microorganisms from enriched soil. Pour plate method was used for isolation of bacteria using nutrient agar (beef extract 3g, dextrose 5g, NaCl 5g, pepton 5g, Agar-agar 15g, distilled water 1 liter, P^H 7.00) with 1 ml cyprmethrin and for fungi potato dextrose agar (Potato peeled 200g, Dextrose 20g, Agar-agar 15g, distilled water 1 liter, P^H 7.00) with 1 ml cyprmethrin concentration and incubated at room temperature.

2.5 Identification of Microorganisms:

Several microorganisms were isolated and identify but only those microorganisms which showed more growth rate were used for further degradation study. Particularly two bacteria and two fungi were selected for the cypermethrin degradation. Isolated bacteria were identified on the basis of colony morphology, staining character and 16s rRNA gene sequencing from NCCS laboratory Pune (MS) India. And the fungi were identified in Department of Botany, Research laboratory, Government institute of science, Aurangabad (MS) India, using a manual of soil fungi - Joseph C. Gilman.

2.6 Minimal minerals salt nutrient culture medium for Bactria to degradation:

The medium FTW media (Herman and Frankerberger, 1999) comprised of K₂HPO₄- 0.255, KH₂PO₄-0.255, (NH₄)₂SO₄-0.255, MgSO₄.7H₂O-0.05, CaCO₃-0.005 and FeCl₂.4H₂O-0.005 was

blended with 1 ml of trace elements solution (Focht, 1994). The Focht trace element solution contained (in mg/l): $MgSO_4 \cdot H_2O$ -169, $ZnSO_4 \cdot 7H_2O$ -288, $CuSO_4 \cdot 5H_2O$ -250, $NiSO_4 \cdot 6H_2O$ -26, $CoSO_4$ -28, and $Na_2MoO_4 \cdot 2H_2O$ -24; all in gm/l ratio. The P^H was adjusted at 7 and media were sterilized at $121^\circ C$ for 15 minutes.

2.6 Minimal minerals salt nutrient culture medium for fungi to degradation:

A synthetic medium was used containing $(NH_4)_2SO_4$ -2 g, KH_2PO_4 -3g, $MgSO_4 \cdot 7H_2O$ - 0.5g, glucose- 3g, microelements minerals solution -2 ml (Cooney and Levine, 1972) and distilled water 1 liter. The P^H was adjusted at 7 and media was sterilized at $121^\circ C$ for 15 minutes.

2.7 Scale-up technique:

1ml of 24 hours old two bacterial sub cultured suspension inoculated in separate 250ml Erlenmeyer flasks containing bacterial nutrient culture media with 50 ppm concentration of cypermethrin and control was served without bacteria suspension and 1ml four days old two fungus mycelia/spore suspension was inoculated into separate 250ml Erlenmeyer flasks containing fungal nutrient culture media with 50 ppm concentration of cypermethrin and control was served without fungus spore suspension. The microbial culture inoculated flasks were kept in orbital shaker at $30^\circ C$ with 160 rpm (rotation per minute) for 14 days. After 14 days of incubation period, 1 ml of this 14 days old cypermethrin degraded culture media and 100 ppm of cypermethrin concentration was added into another 250 ml of Erlenmeyer flasks containing nutrient culture medium and control was served without microorganism. The flasks were again kept on orbital shaker at $30^\circ C$ with 160 rpm for another 14 days incubation period. Likewise, the microbial culture was sub cultured into other nutrient culture media containing Erlenmeyer flasks with a cypermethrin concentration of 150ppm and was kept on orbital shaker at $30^\circ C$ with 160 rpm for increasing a total incubation period of 42 days. At this stage, the isolated microorganisms were found adapted to Cypermethrin-25 EC and by assessing the cypermethrin as a sole source of carbon for growth and maintenance.

2.7 Analytical procedure:

After every 0,3,7,11 and 14 days interval samples were removed and analyzed by COD analysis, pH, Growth kinetics study took O.D. at 600 nm using U.V. Spectrophotometer (Elico). The fungal mycelial dry weight was also recorded after 14 days incubation period. Filter residue

mycelia dry weights obtained by using whatman paper No.1. Dry weight of mycelium was obtained by drying whatman paper in oven at $60^\circ C$ after 24 hrs. And COD analysis as per standard procedure laid down in Book of Chemical and Biological Methods for water pollution study- R. K. Trivedy & P.K. Goel.

2.8 Residual quantification analysis:

After every 14 day's interval the final concentration of cypermethrin was determined by GC-ECD. The solution mixture was extracted with dichloromethane; the organic layer was obtained and it was dried and re dissolved in *n*-hexane. The GC conditions were as follows: electron capture detector with SPB-5 capillary column, injector/interface temperature $260^\circ C$, oven temperature $240^\circ C$, detector temperature- $300^\circ C$, and N_2 carrier gas 1 ml/min.

2.9 Detection of cypermethrin metabolites:

Culture filtrate of medium containing cypermethrin was extracted with dichloromethane. The dichloromethane extract was evaporated and the residue dissolved in acetone. The extracts were analyzed by GC-MS (Doctors Analytical laboratory Pvt. Ltd. Pune). The GC-MS analysis was performed in electron ionization (EI) mode (70 eV) with an Agilent gas chromatograph equipped with an MS detector. A HP-1701 capillary column (30 m length \times 0.25 mm id \times 0.25 μm film thickness) was used with a initial temperature program of $80^\circ C$ for 1 min; increased up to $200^\circ C$ at $8^\circ C/min$ and held for 2 min. and finally increased up to $260^\circ C$ at $15^\circ C/min$ and held at $260^\circ C$ for 10 min. Nitrogen was used as the carrier gas at a constant flow of 1.0 ml/min. The samples were analyzed in split mode (1:20) at an injection temperature of $260^\circ C$ and an EI source temperature of $230^\circ C$ and scanned in the mass range from 50 *m/z* to 450 *m/z*.

3.0 Results:

3.1 Identification of cypermethrin degrading microorganisms:

Microorganisms which showed more growth and resistance against cypermethrin were identified such as *Pseudomonas aeruginosa* (Accession No. JF708942.1. Strain IRMD-2010) and *Alcaligenes faecalis* (Accession No. HQ202537.1) and *Trichoderma virid* and *Rhizopus stolaniferus* were the fungus which showed more growth than other fungus therefore only these fungi were used for further degradation study.

3.2 Growth Kinetics Studies:

The comparative growth rate of *P. aeruginosa* and *A. faecalis* in presence of

cypermethrin with control revealed that *P. aeruginosa* showed faster growth rate than *A. faecalis* at 50ppm, 100ppm and 150ppm concentration of cypermethrin respectively. While comparative growth rate of two identified fungus showed that dry mycelial weight of *T. viride* was more than the *R. stolaniferus*; it means that *T. viride* growth rate was faster than *R. stolaniferus* (Table-2). Therefore *P. aeruginosa* and *T. viride* were more potent microorganisms for utilizing cypermethrin as a sole source of carbon and break down it into different metabolites. This result revealed that the concentration of cypermethrin increases the growth rate of microorganism was decreased but not completely inhibited.

3.3 pH Detection:

In the cypermethrin degradation study P^H played a vital role. At the time of degradation assay, *P. aeruginosa* & *A. faecalis* cultural medium P^H was increased at 50 ppm and 100 ppm but at 150 ppm concentration P^H get slowly increased as compare to previous concentration while fungus culture medium revealed that P^H slowly decreased as concentration of cypermethrin increased (Table-1). The result showed that the alkaline P^H was favorable for cypermethrin degradation in bacteria cultural medium while in fungi cultural medium descending P^H scale than the control. This result revealed that acidic P^H of fungal culture medium was favorable for the degradation of cypermethrin.

3.4 Chemical oxygen demand Analysis (COD):The concentration of cypermethrin in culture filtrate after fourteenth day's time intervals was evaluated by chemical oxygen demand analysis method. The result revealed that as the concentration of cypermethrin increased the percentage of degradation was reduced. A bacteria *P. auroginosa* and *A. faecalis* showed 90.33% and 69.00% removal of cypermethrin and the fungi *T. viride* and *R. stolaniferus* showed 78.00 % and 67.34 % removal of cypermethrin at 50 ppm concentration of cypermethrin. At the 100ppm concentration of cypermethrin the microorganism's *P. auroginosa*, *A. faecalis*, *T. viride* and *R. stolaniferus* showed 76.39 %, 66.78 %, 61.18 % and 59.61 % degradation of cypermethrin respectively. While at 150 ppm concentration, *P. auroginosa*, *A. faecalis*, *T. viride* and *R. stolaniferus* showed 50.23 %, 38.55 %, 42.99 % and 32.59 % degradation of cypermethrine respectively (Table-1). The result revealed that the concentration was scale up in decreasing the rate of degradation, therefore higher concentration of cypermethrin was harmful for the growth of microorganism but *P. auroginosa* was also showed moderate degradation in higher concentration

of cypermethrin. Therefore *P. auroginosa* was more potent microorganism for the cypermethrin degradation.

3.5 Gas Chromatography-Electron capture detector Analysis (GC-ECD):

The strain of *P. auroginosa*, *A. faecalis*, *T. viride* and *R. stolaniferus* were enriched and adapted by scale up process in minimal salt medium (MSM) containing Cypermethrin as a sole source of carbon and energy. The cypermethrin degradation studied at varying concentration such as 50ppm, 100ppm and 150ppm respectively. The scale up process was carried out with successive microbial sub-cultures from lower concentration to higher concentration of cypermethrin after 14 days time interval under at 30 °C with 160 rpm. The GC-ECD data illustrates that, after a period of 14 days interval *P. auroginosa* had degraded cypermethrin up to 81.35%, 70.35%, and 53.32% at 50 ppm, 100 ppm and 150 ppm concentration of cypermethrin respectively. Secondly *T. viride* showed degradation of cypermethrin up to 66.06%, 58.59%, 41.01% at all three tested concentration respectively. While *A. faecalis* degraded cypermethrin up to 62.45%, 57.07% and 35.03% with all three tested concentration of cypermethrin respectively, followed by *R. stolaniferus* with less degradation up to 47.89%, 35.00% and 13.63% at 50 ppm, 100 ppm and 150 ppm concentration of cypermethrin respectively (Table- 1). The COD, GC-ECD analysis data showed that *P. auroginosa* had more potential for cypermethrin break down into simple compounds followed by *T. viride*. While *A. faecalis* and *R. stolaniferus* showed minimum degradation of cypermethrin.

3.6 Identification of metabolite (GC-MS Analysis):

The GC-MS chromatogram revealed that primary metabolites of degraded cypermethrin were found Phthalic acid -isobutyl 2-pentyl ester, Phthalic acid-butyl 4-octyl ester, 2-Propenic acid-3-(4- methoxyphenyl)-2-ethylhexyl ester, Azuleno(7,9-Dihydroxy-6,9a-dimethyl)-3-methylenedecarhydroazuleno[4,5-b]furan-29(3H)-one) and Cyclopropane carboxylic acid was detected by the GC-MS analysis.

From these result it can be concluded that the isolated microorganisms were effective for breakdown of toxic compound like cypermethrin especially *P. auroginosa* which was more potent for cypermethrin degradation and obtained primary metabolites were less hazardous to the environment than cypermethrin.

Table 1. COD, GC-ECD analysis & P^H detection after 14 days incubation.

Concentration	Parameter	Control	<i>P.a.</i>	<i>A.f.</i>	<i>T.v.</i>	<i>R.s.</i>	C.V.
50 ppm	pH	7.00	8.53	7.93	5.93	5.66	
	COD mg/l	1000±23.59	967 ±8.023	3100±23.59	2200±11.61	3266±15.57	0.982
	Removal %	00.00	90.33	69.00	78.00	67.34	
	GC-ECD µg/ml	3.09±0.010	0.58±0.0080	1.16 ±0.0092	1.05 ±0.0092	1.61±0.012	1.446
	Degradation%	00.00	81.35	62.45	66.01	47.89	
100 ppm	pH	6.99	8.02	8.11	5.13	5.04	
	COD mg/l	19066 ±7.54	3833 ±12.48	4666 ±7.54	7400 ±4.71	7700 ±9.433	0.229
	Removal %	00.00	76.39	66.78	61.18	59.61	
	GC-ECD µg/ml	6.54 ±0.0046	1.97 ±0.012	2.82±0.026	2.72 ±0.012	3.11 ±0.011	0.839
	Degradation%	00.00	70.35	57.07	58.59	35.00	
150 ppm	pH	6.95	7.50	7.34	6.21	5.94	
	COD mg/l	28533±10.38	14200 ±7.075	17533 ±13.73	16266 ±4.114	19233 ±6.132	0.076
	Removal %	00.00	50.23	38.55	42.99	32.	
	GC-ECD µg/ml	9.02 ±0.019	4.21±0.020	5.86 ±0.0092	5.32 ±0.0080	7.79 ±0.020	0.438
	Degradation%	00.00	53.32	35.03	41.01	13.63	

Value expressed as mean of triplicates ± S.E.M., C.V.= Coefficient variance and *P.a.* - *Pseudomonas auroginosa*, *A.f.*- *Alcaligenes faecalis* *T.v.*- *Trichoderma viride*, *R.s.*- *Rhizopus stolaniferus*.

Table 2. Growth Kinetics & Mycelia dry weight

Growth kinetics		50ppm (O.D)	100ppm (O.D)	150ppm (O.D)
Sr. No.	Microorganisms			
1	Control	0.042 ±0.00092	0.046±0.00080	0.057±0.00010
2	<i>Pseudomonas auroginosa</i> ,	1.027±0.034	0.927±0.0058	0.413±0.0028
3	<i>Alcaligenes faecalis</i>	0.814±0.0060	0.625±0.010	0.315±0.0028
	C.V.	3.605	2.394	1.982
	Mycelia dry weight	Weight in grams	Weight in grams	Weight in grams
1.	Control	0.097±0.00098	0.097±0.00069	0.0974±0.0012
2.	<i>Trichoderma viride</i>	0.407±0.0025	0.289±0.020	0.236±0.021
3.	<i>Rhizopus stolaniferus</i> .	0.299±0.00069	0.218±0.011	0.186±0.006
	C.V.	1.119	7.507	8.092

Value expressed as means of triplicates ±S.E.M. C.V. = Coefficient variance.

Fig :- GC-MS Analysis of Microbial concertium degraded sample.

Fig.1 :Azuleno (7, 9-Dihydroxy-6,9a-dimethyl3methylenedecarhydroazuleno[4,5-b]furan-29(3H)-on

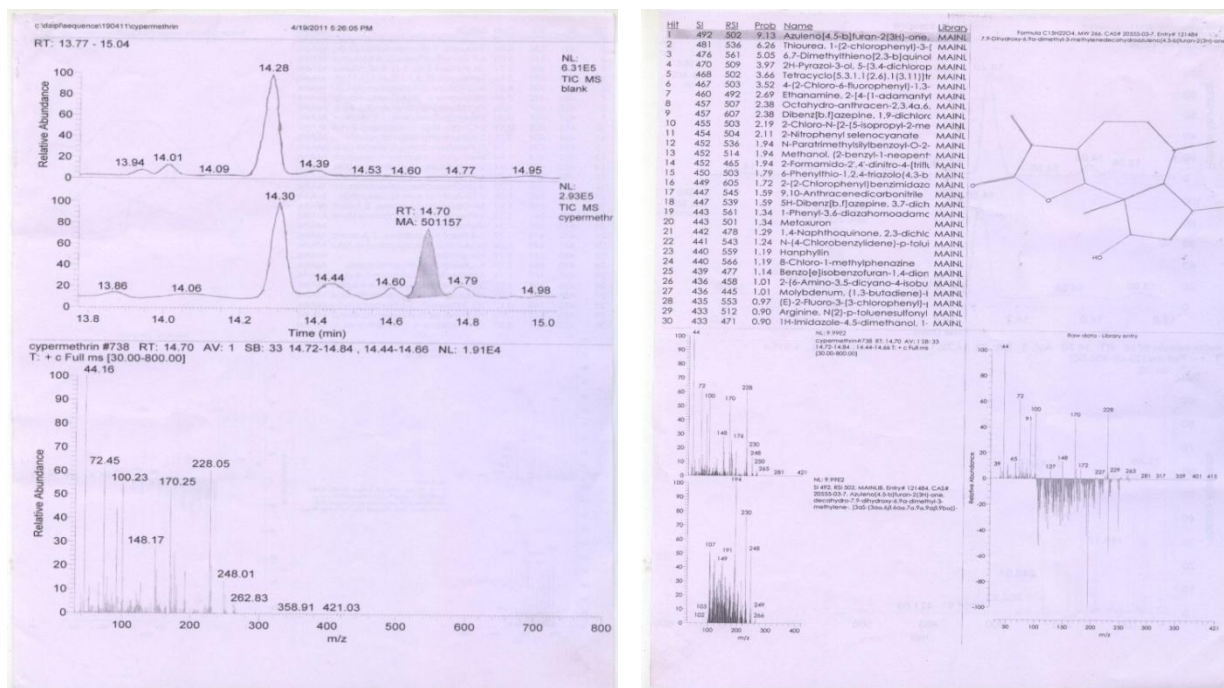


Fig.2: Phthalic acid-isobutyl 12-pentyl ester.

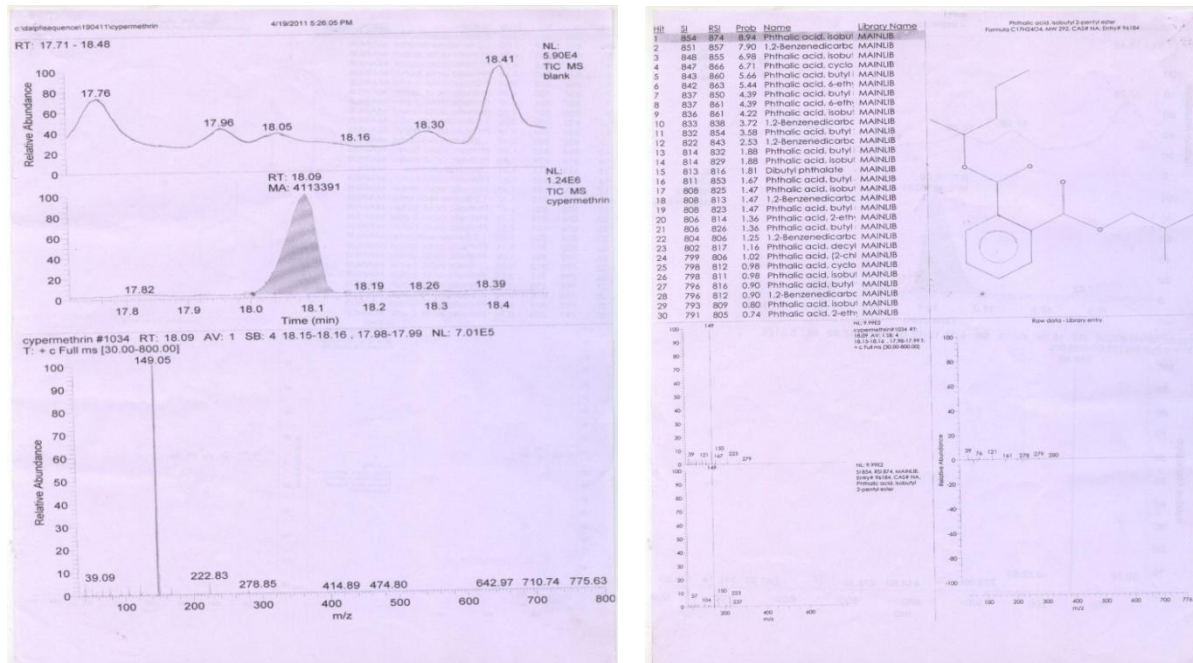


Fig.3: Phthalic acid-butyl 4-octyl ester.

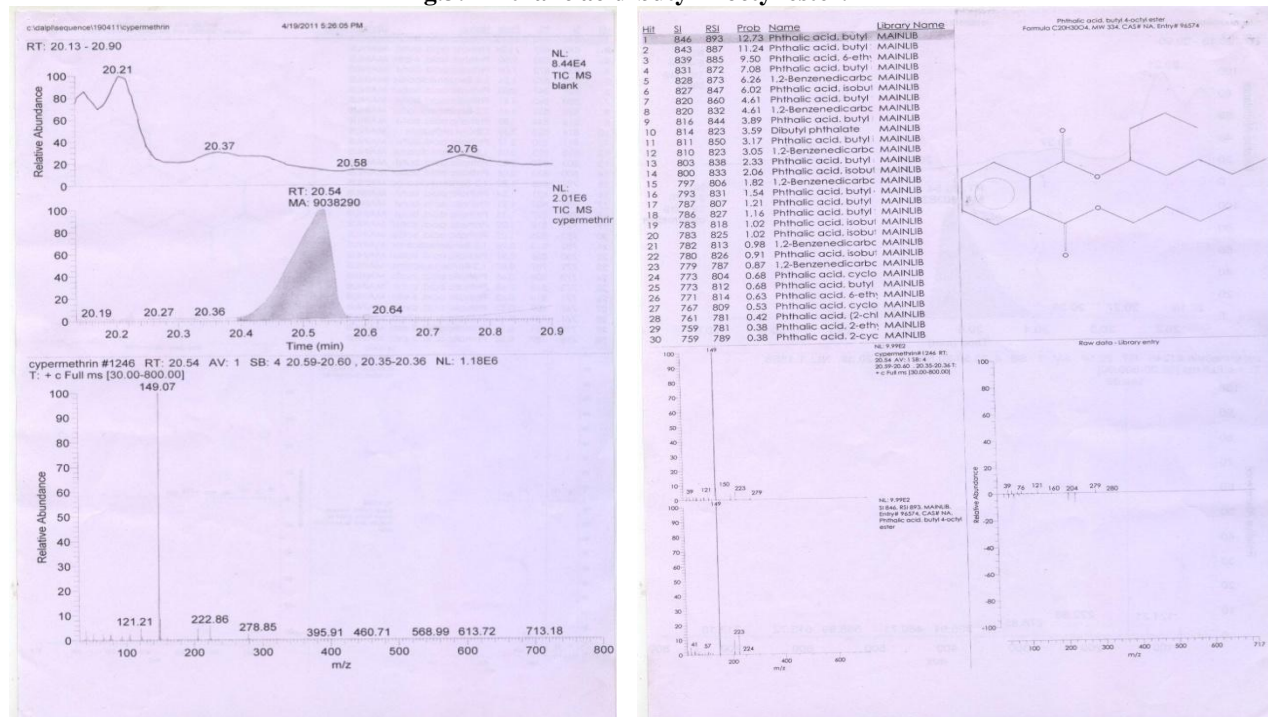


Fig 4: 2-Propenoic acid-3-(4- methoxyphenyl)-2-ethylhexyl ester.

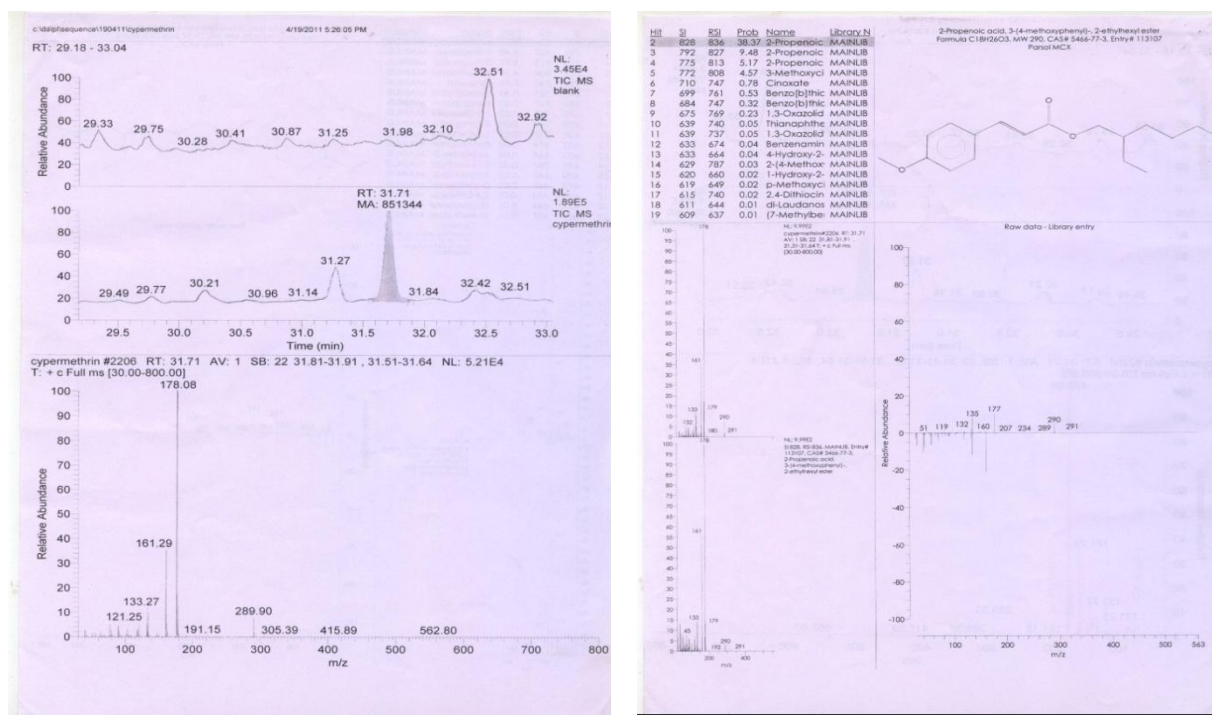
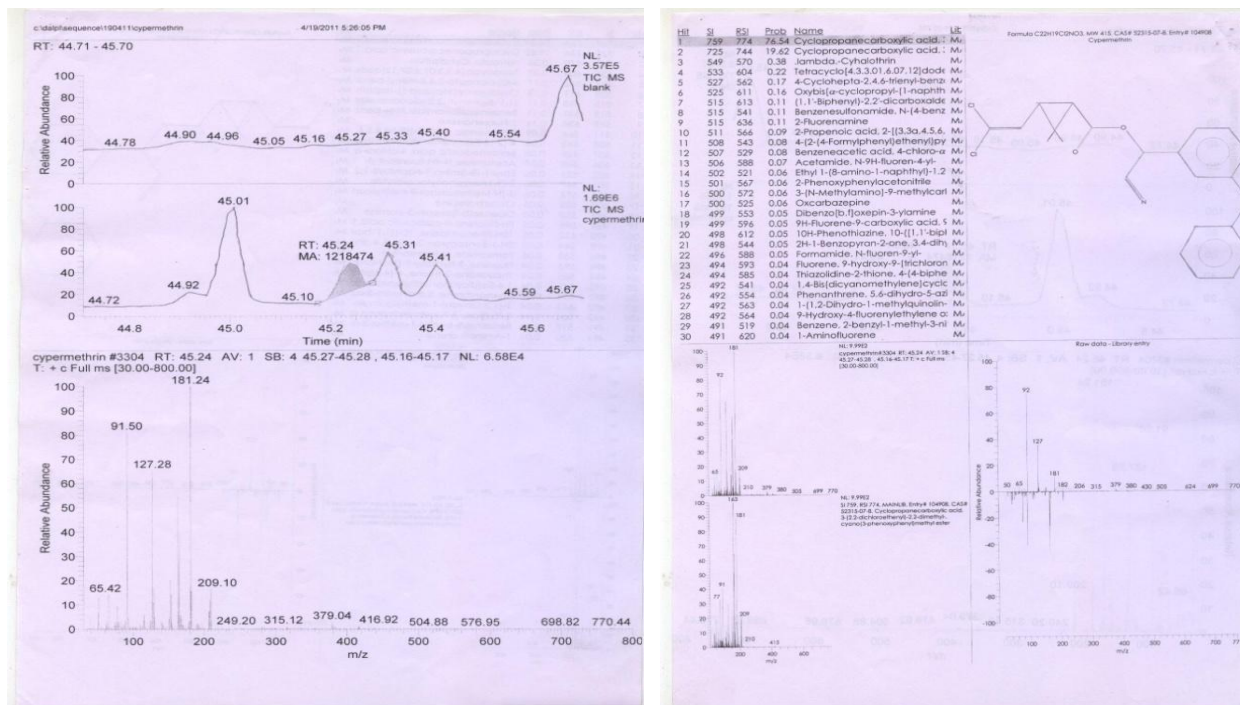


Fig. 5 : Cyclopropanecarboxy acid.



3.7 Discussion:

The potential of microorganisms isolated from the cotton cultivated field to mineralize the cypermethrin into primary metabolites were evaluated by using GC-MS method. The finding of present study revealed that four different microorganisms' i.e two fungal genera and two bacteria genera showed varied degradation abilities at several tested concentration of commercial base cypermethrin. The bacterial growth density was calculated at optical density 600nm showed that the *P. aeruginosa* had faster growth than *A. faecalis* and in fungi the mycelial dry weight showed that the *T. viride* was more effective than *R. stoloniferous*. Similar findings were reported by Lee *et al.* 1992 that the genus *Pseudomonas* which was gram negative, rod shaped, highly oxidative and metabolically versatile due to which it was able to degrade aromatic hydrocarbons and pesticides and showed more growth than other microorganisms. At the higher concentration of cypermethrin the optical density of bacteria and dry weight of fungi decreased. As the Similar findings were reported by Jilani and Khan, 2006 that increasing in concentration of insecticide decreases the activity of microorganisms. However, at increased concentration of cypermethrin from 50ppm to 150ppm a marked negative effect on the rate of degradation was observed in higher concentration of cypermethrin. Several researchers

also reported similar result of lower degradation at high concentration of hazardous organic compounds (Smith and Adkins, 1996; Collins and Daugulis, 1996; Silvia and Wunderlin, 1997; Lee, *et al.*, 1998; Goudar and Strevett, 2000).

The result of pH analysis indicated that the cypermethrin degradation by *P. aeruginosa* showed satisfactory degradation even at alkaline P^H (7.5-8.54) and was found to degrade the cypermethrin at 50ppm to 100ppm effectively and less degradation found at 150ppm concentration. It was noted that biodegradation was not significantly affected by because of changing the P^H value from 7.5- 8.54. The Hanel, (1988) reported that the tolerable limits for pH is between 6.0-9.0 and even the influent pH values outside this range are of little or no practical significance. Therefore the similar results were obtained in this study. Furthermore, according to literature, cypermethrin hydrolyzes slowly in water at pH 7 and below, while hydrolysis being more rapid at pH 9 (Walker and Keith, 1992).

During experimental study conducted in scale up process revealed *P. aeruginosa* had retained their degradation capacity at wide range of P^H 7.5 to 8.53. The COD result showed that removal of organic load was proportional to the cypermethrin disappearance. Similar correlation between COD removal and degradation were also observed by S.

Jilani and M. Altaf Khan (2006) and Berchtold, *et al.* (1995).

In Present work *Trichoderma viride* and *Alcaligenes faecalis* grows at 50ppm, 100ppm more effectively but at 150ppm it had lost degradation capacity and *Rhizopus stoloniferus* showed less tolerance against cypermethrin but *Pseudomonas aeruginosa* was very potent at 150ppm concentration. GC-ECD result evaluated that *Pseudomonas aeruginosa* showed more potential against the various concentration of cypermethrin. The various researchers also showed that *Pseudomonas aeruginosa* was more potent against the cypermethrin degradation (R.J. Grant, T.J. Daniell and W.B. Betts 2002, S.Jilani and M. Altaf Khan 2004, D. Malik, M. Singh, & P. Bhatia 2009, M.H. Fulekar 2009, A.G Murugesan *et al* 2010).

The GC-MS result showed that the ester metabolite was found during the bioremediation of cypermethrin pesticide which can eventually disintegrate into simpler compounds, which would be mineralized further as nutrient and inorganic forms. This metabolite may be less hazardous than cypermethrin to aquatic life and soil environment.

3.8 Conclusion:

The biodegradation study of cypermethrin by scale up process showed that the isolated microorganism from the cotton cultivated field a very useful for the mineralization of cypermethrin. The COD, GC-ECD analysis showed that *P.aeruginosa* having more capacity to degrade the cypermethrin than other isolated microorganisms. The ester metabolite is detected by GC-MS analysis it is eco- friendly for aquatic life and soil environment. In the present work the isolated cypermethrin degrading microorganisms could be used successfully for the removal of cypermethrin and other hazardous organic compound from contaminated soil.

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