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Remediation of Cypermethrin-25 EC by Microorganisms

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ABSTRACT

In present study the degradation potential of eight microorganisms used were Pseudomonas aeruginosa, Pseudomonas fluorescens, Bacillus cereus, Alcaligenes faecalis, Trichoderma viride, Trichoderma koningii, Penicillium chrysogenum and Rhizopus stolanifer 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 Cypermitherin 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 (COD) was 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 81.22%, 69.88%, 53.32% followed by Pseudomonas fluorescens 79.28%, 66.97% and 51.21% at tested 50ppm, 100ppm, 150ppm concentrations respectively after 14 days incubation period. While Bacillus cereus, Alcaligenes faecalis, Trichoderma koningii, Penicillium chrysogenum, Trichoderma viride and Rhizopus stolanifer 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. The fish tolerance also recorded with residue and Cypermethrin, result showed that fish tolerance was more in microbial degraded residue of Cypermethrin than the 100ppm Cypermethrin. 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.

Key words: Biodegradation, Cypermethrin, Scale up technique, *Pseudomonas aeruginosa*, COD GC-ECD, GC-MS, Pathalic acid, Proponic acid. Fish tolerance.

INTRODUCTION

The first organic synthetic pesticide potassium dinitro-cryslate was used as insecticide The term pesticides covers a wide range of compounds like insecticides, fungicides, herbicides, rodenticides, molluscides, nematicides, plant growth regulators and heavy metals like copper, zinc, arsenic, lead, cadmium and mercury (Hayes, 1975). Among these, organo-chlorine (OC) insecticides which were used to control insects attack were banned or restricted after the

1960s in most of the technologically advanced countries because of its hazardous effect on environment. Thereafter introduction of other synthetic insecticides like organophosphate (OP) in the 1960s, carbamates in 1970s and pyrethroids in 1980s, herbicides and fungicides in the year 1970s-1980s contributed greatly in pest control and agricultural output. Ideally a pesticides must be lethal to the targeted pests but not to non-target organism including man. Unfortunately controversy and abuse use of pesticides soil surface was contaminated. The rampant use of these chemicals, under the adage, "if little is good, a lot more will be better" has played havoc with chronic health problems of humans and other life forms along with soil and environment pollution.

There were enormous studies conducted for degradation of such harmful pesticides. The potential of microorganisms to degrade and remove pesticide from soil has also been successes fully attempted (Doris *et al.*, 1990). Therefore the present study is based on the degradation of pesticides by using biological source such as soil microorganisms. There are many microorganisms present in the soil but only few microorganisms have the potential for pesticides degradation. Isolation of these microorganisms was carried out from the cotton, Brinjal and wheat cultivated soil by enrichment soil methods in laboratory with addition of pesticides. Three different pesticides belonging to insecticide, fungicide and herbicide group were selected and used for microbial degradation. The technical grade Cypermethrin-25EC, Bavistin and 2, 4-D were selected for the degradation because these pesticides are widely used for controlling the different pest on crop in India.

In cypermethrin-25 EC belonging to pyrethroide family used as insecticide in large scale commercial agriculture applications as well as consumer products for domestic purpose. It was synthesized in 1974 and firstly marketed in the year 1977. Pyrethroide have four major a generation among which Cypermethrin belongs to fourth generation (Casida, 1980). The synthetic pyrethroid insecticides are analogs of naturally occurring pyrethrins as per botanical origin (Elliott, 1980) and derived from dry flower of *Chrysanthemum cinerariaefolium* plant which have been known since the nineteenth century (Grant *et al*, 2001). It is unlikely to contaminate the ground water because it binds tightly to soil particles. The huge amount of pesticide application causes desired side effects on population and activity of beneficial microorganisms in soil (Pandey and Singh, 2004). Soil microbes showed different response against different types of insecticides. Cypermethrin and monocrotophos had adverse effects on the total number of soil bacteria in the soil while fenvalerate had very low effect on the soil microbes (Rangaswamy and Venkateswarlu, 1992; Ajaz *et al*., 2005). Therefore soil fertility is decreased and due to which crop production is also affected. Among the different genera of microorganisms for degradation of pesticides, genus *Pseudomonas* has a special status, as the strain of *P. cepacia* are known to metabolize broad range of organic compounds and utilizes more than 100 different substrates as the sole source of carbon, nitrogen, sulfur (Dagley, 1986). Many efforts have been undertaken to isolate bacteria, capable of biodegradation of synthetic pyrethroid insecticides and a lot of pyrethroid-degrading microorganisms have been isolated from polluted soil and water (Sakata *et al*.,1992; Maloeny *et al*., 1993; Halden *et al*.,1999; Nirmali *et al*., 2005; Jilani and Khan, 2006; Tallur *et al*., 2008; Murugesan *et al*., 2010a). *In vitro* studies have shown that microorganisms have capacity to degrade cypermethrin mainly *Pseudomonas aeruginosa* more efficacious than other microorganisms (Murugesan, *et al.*, 2010a and 2010b; Jilani and Khan, 2006). These microorganisms were isolated from soil of the pre treated Cypermethrin cotton and Brinjal crop field (Murugesan, *et al.*, 2010b). *In vitro* study showed two soil bacteria that are able to degrade cypermethrin insecticide; they are the member of the genera *Pseudomonas* and *Serratia* (Grant, 2001).

MATERIALS AND METHODS

Chemicals:
Cypermethrin-25EC.
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.

Chemical formula: $C_{22}H_{19}O_3NCl_2$
Cypermethrin IUPAC Name: [(RS)-α-cyano-3-phenoxybenzyl (1*RS*)-*cis-trans*-3-(2,2-dichlorovinyl)-1,1-dimethyl-cyclopropanecarboxylate.]

Collection of soil samples:
The soil samples were collected from the different sites of Cotton, Brinjal and wheat cultivated field from Belora Dist. Jalna (M.S) India. These fields were already sprayed with Pesticides mainly Cypermethrin-25 EC, Carbendazim and 2, 4-D for past few years. The soil samples were collected in sterile polythene bags for further study.

Soil enrichment Technique for isolation of Pesticides degrading bacteria:
Soils samples collected from the top 0-15 cm of field plots and were air dried and maintain 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 mix aqueous solution of Cypermethrin-25 EC, Carbendazim and 2, 4-D to get final concentration 1000 ppm by mixing gently and incubated at room temperature for two weeks. The moisture content was maintained using distilled water. The pesticides treatment was repeated three times for every two week of time interval.

Isolation of Pesticides degrading microorganisms:
The enriched culture method was used to isolate the Pesticides degrading microorganisms from soil. Enrichment of pesticides degraders were carried out in 150 ml minimal minerals salt medium in 250 ml conical flask. The medium was sterilized at 121^0C for 30 min. Followed by addition of 1% (w/v) of pesticides separately as a sole source of carbon and energy. Consequently, second enrichment was carried out by transferring 1 ml of from first enriched flask culture into freshly prepared and sterilized 50 ml of minimal minerals salt medium and was incubated for same growth conditions. Isolation was done on sterile minimal minerals salt agar media plates with 0.25% pesticides. After 3 days incubation different types of colony were observed and pick up isolated colonies and maintain on slants for further study.

Minimal minerals salt nutrient culture medium (Bactria):
The medium FTW media (Herman and Frankerberger, 1999) comprised of K_2HPO_4- 0.255, KH_2PO_4-0.255, $(NH_4)2SO_4$-0.255, $MgSO_4.7H_2O$-0.05, $CaCO_3$-0.005 and $FeCl_2.4H_2O$-0.005 was blended with 1 ml of trace elements solution (Focht. 1994). The Focht trace element solution contained (in mg/l): $MgSO_4.H_2O$-169, $ZnSO_4.7H_2O$-288, $CuSO_4.5H_2O$-250, $NiSO_4.6H_2O$-26, $CoSO_4$-28, and $Na_2MoO_4.2H_2O$-24; all in gm/l ratio. The P^H was adjusted at 7 and media were sterilized at 121^0 C for 15 minutes.

Minimal minerals salt nutrient culture medium (Fungi):
A synthetic medium was used containing $(NH_4)2SO_4$-2 g, KH_2PO_4-3g, $MgSO_4$. $7H_2O$- 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 121^0 C for 15 minutes.

Identification of Microorganism:
Several microorganisms were isolated and identified but only those microorganisms which showed high growth rate were used for further pesticides degradation study. Particularly four bacteria and four fungi were selected for the Pesticides degradation. The fungi were identified in Department of Botany, Research laboratory, Government institute of science, Aurangabad (MS) India, using manual of soil fungi - Joseph C. Gilman. A total of four bacteria capable of fast growing in the presence of pesticides using them source of carbon and energy were identify on the basis of 16s rDNA sequencing using universal primers. The molecular identification of bacterial isolates was done by National center for cell science (NCCS) Pune.

Scale-up technique:
1ml of 24 hours old four bacterial sub cultured suspension inoculated in separate 250ml Erlenmeyer flasks containing bacterial nutrient culture media with 50 mg/L concentration of Cypermethrin and control was served without bacteria suspension and 1ml four days old four fungus mycelia/spore suspension was inoculated into separate 250ml Erlenmeyer flasks containing fungal nutrient culture media with 50 mg/L concentration of Cypermethrin and control was served without fungus spore suspension. The microbial culture inoculated flasks were kept in orbital shaker at 30 º C with 160 rpm (rotation per minute) for 14 days. After 14 days of incubation period, 1 ml of these 14 days old Cypermethrin degraded culture media and 100 mg/L 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 ºC 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 Cypermethrtin concentration of 150 mg/L and was kept on orbital shaker at 30 ºC 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.

Chemical oxygen demand (COD):
COD was determined as the oxygen required for chemical oxidation of organic matter with the help of strong chemical oxidant. (Reflex condensation with $k_2cr_2o_7$ methods using manual of water and waste water analysis, NEERI). COD was determining by refluxing sufficient diluted culture filtrate of microorganisms for 2 hours, in the presence of $HgSo_4$ and Ag_2So_4 in concentrated H_2So_4. After refluxing residual $K_2Cr_2O_7$ was estimated by titrating content with .01N ferrous ammonium sulphate using a ferroin as indicator with wine red color which was the end point.

Residual quantification analysis of Cypermethrin by Gas Chromatography with electronic capture detector (GC-ECD):
After every 14 day's interval the final concentration of cypermithrin was determined by GC-ECD methods. 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°C, oven temperature 240°C, detector temperature-300°C, and N2 carrier gas 1 ml/min.

Detection of cypermethrin metabolites by Gas chromatography and Mass spectroscopy (GC-MS):
Culture filtrate of medium containing cypermethrin was extracted with dichloromethane. The dichloromethane extract was evaporated and the residue was 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 × 0.25 mm id × 0.25 ìm film thickness) was used with a initial temperature program of 80 °C for 1 min; increased up to 200 °C at 8 °C/min and held for 2 min. and finally increased up to 260 °C at 15°C/min and held at 260 °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 °C and an EI source temperature of 230 °C and scanned in the mass range from 50 *m*/*z* to 450 *m*/*z*.

Fish Assay:
Healthy (10±0.5 g) were brought to the laboratory from the freshwater pond of the Harsool Lake, Aurangabad. The fish were acclimatized to the laboratory environment for about 5 days. They were regularly fed with commercial food pellets and given aeration. Feeding was stopped before 24 hours of the commencement of the test. After 24 hours a pair of fish was added into aquaria. Only healthy fish were used in the experiments. Fish were exposed in one liter glass aquaria containing 100 mg/L of pesticides and in other aquaria containing pesticides residue (1:1). Regular feed was given fishes and oxygen supply was maintained with mini aerator pumps. The behavior of fishes was observed closely for the first five hours and later extended up to 3 hours with intervals observations. This finding test gave an idea to bring about mortality and tolerance in the test species of fishes.

RESULTS

On the basis of morphological characterization the four fungal cultures were identified as *Trichoderma viride*, *Trichoderma koningii*, *Penicillium chrysogenum* and *Rhizopus stolanifer* and bacteria were identified through 16s rDNA sequencing as *Alcaligenes faecalis* (Accession No. HQ202537.1), *Pseudomonas aeruginosa* (Accession No. JF708942.1. Strain IRMD-2010), *Pseudomonas fluorescens* (Accession No. AF094731.1. strain ATCC 17574) and *Bacillus cereus*. (Accession No. HM752769.1. Strain IMAUB1022 CS-2010). The identified Fungi and bacteria were used further for biodegradation of Cypermethrin-25 EC.

Chemical Oxygen Demand:
During the experiment, a good correlation was established between Chemical Oxygen Demand removal and Cypermethrin degradation rates. It was observed that microorganisms showed degradation of Cypermethrin at different concentrations of 50mg/l, 100mg/l and 150mg/lin minimal minerals salt medium at 3rd, 7th, 11th and 14th day's intervals. The percentage decreased during the biodegradation Cypermethrin following result was obtained.

A) Bacteria:- *P. auroginosa* recorded maximum degradation at 50mg/l concentrations as 14.80%, 32.80%, 71.20% and 90.33%, at 100 mg/l concentration 14.16%, 28.21%, 73.89% and 76.39% removal of Cypermethrin was recorded, at 150 mg/l 16.57%, 28.24%, 32.55%, and 50.23% respectively. Followed by *P. fluorescens* at 50 mg/l as 13.89%, 30.28%, 56.03% and 84.37%, in 100 mg/l 11.53%, 20.94%, 70.64%, and 72.37%, at 150 mg/l as 09.66%, 27.55%, 32.09 % and 48.48% and in *B. cereus* at 50 mg/l 11.17%, 28.39%, 71.20% and 72.00% respectively, at 100 mg/l 10.31%, 23.98%, 67.57% and 72.02% respectively and in 150 mg/l 15.99%, 28.58%, 30.69% and 44.39% respectively while *A. faecalis* at 50 mg/l 9.36%, 27.44%, 53.03% and 69.00% , at 100 mg/l 11.53%, 13.84%, 62.97% and 66.78% respectively and at 150 mg/l concentration it showed the lowest removal of Cypermethrin with COD as 07.40%, 24.56%, 33.95% and 38.55% degradation was observed and recorded.(Table no. 1)

B) Fungi:- The four fungi selected for degradation study as *T. viride*, *T. koningii*, *P. chrysogenum* and *R. stolaniferous* among the *T. viride* had recorded maximum degradation potential at 50 mg/l 14.34%, 30.59%, 56.03% and 78.00%, at 100 mg/l 9.613%, 18.91%, 48.80% and 61.08% and at 150 mg/l 13.00%, 23.65%, 32.44% and 32.47% respectively. In *T. koningii* at 50mg/l it was 12.98%, 26.17%, 54.80% and 77.00%, at 100 mg/l 12.40%, 18.91%, 49.49% and 60.31% respectively and lastly at 150 mg/l 09.32%, 23.65%, 31.51% and 41.23% respectively. In *P. chrysogenum* at 50 mg/l 11.62%, 26.50%, 47.98% and 70.67%, at 100 mg/l 10.13%, 18.58%, 48.12% and 60.84% and at 150 mg/l 11.39%, 29.91%, 32.79% and 41.47%. Finally *R. stolaniferus* at 50 mg/l 11.17%, 29.96%, 43.96% and 67.34% respectively, at 100 mg/l it was showed 0.173%, 15.54%, 39.42% and 59.61% respectively and at 150 mg/l showed very less degradation was recorded 06.42%, 20.32%, 30.23% and 32.59% respectively. (Table no. 1)

Cypermethrin detection by GC-ECD:

The Bactria of *P. aeruginosa*, *P. fluorescens*, *B. cereus* and *A. faecalis* and fungi *T. viride*, *T. koningii*, *P. chrysogenum* and *R. stolaniferous* were enriched and adapted by scale up process in minimal salt medium (MSM) containing Cypermethrin as a sole source of carbon and energy at varying concentration as 50mg/l, 100mg/l and 150mg/l respectively. The scale up process was carried out with successive frequent microbial sub-cultures from lower concentration to higher concentration of Cypermethrin after 14 days time interval under continuous incubation at 30^{o} C with 160 rpm shaking speed.

The GC-ECD data illustrates that, after a period of 14 days intervals at 50 mg/l, 100 mg/l and 150 mg/l. The *P. aeruginosa* had degraded Cypermethtrin up to 81.35%, 70.

and 53.32% respectively than control. Followed by *P. fluorescens* degraded Cypermethrin up to 79.28%, 66.97% and 52.66% respectively than control. The *B. cereus* showed 66.34%, 57.07% and 51.21% degradation of Cypermethrin respectively and *A. faecalis* showed 62.45%, 57.07% and 35.03% degradation respectively.

While fungi *T. viride* had maximum degradation up to 66.06%, 58.59 % and 41.01% respectively than the control. In *T. koningii* showed 61.81%, 56.01% and 36.36% degradation respectively. In *P. chrysogenum* recorded 55.01%, 54.69% and 35.80% degradation respectively and finally least degradation was by R. *stolaniferous* up to 47.89%, 35.00% and 13.63% at 50 mg/l, 100 mg/l and 150 mg/l concentration of cypermethrin respectively (Table No. 2). The COD, GC-ECD analysis data showed that the Bacteria *P. aeruginosa* had more potential for cypermethrin break down into simple compounds. While Bacteria *A. faecalis* and fungi *R. stolanoferous* had recorded minimum degradation of cypermethrin at all three selected concentrations. Therefore Bacteria *P. aeruginosa* had highest potential for removal of cyermethrine and less potential was recorded by the fungus *R. stolanoferous* in minimal minerals salt medium. The result indicates that as the concentration of Cypermethrin increased the percentage rate of degradation was decreased, indicating that higher concentration of Cypermethrin was harmful for the growth of microorganisms, but *P. auroginosa* showed significant degradation at higher concentration of Cypermethrin. Therefore the Bacteria *P. auroginosa* was the potent only potent microorganism for the Cypermethrin degradation than the other microorganism studied.

Table No.-1: Chemical oxygen demand of Cypermethrin microbial degraded Residues and control after periodic day's intervals

Conc. (mg/l)	Days	Cont.	*P.a*	%	*P.f.*	%	*B.c.*	%	*A.f.*	%	*T.v.*	%	*T.k.*	%	*P.c.*	%	*R.s.*	%	C.V.
50	3	11033 ±15.57	9400 ±4.71	14.80	9500 ±9.433	13.89	9800 ±7.075	11.17	10000 ±4.719	9.36	9450 ±23.59	14.34	9600 ±9.433	12.98	9750 ±20.43	11.62	9800 ±16.51	11.17	0.2091
	7	10566 ±15.57	7100 ±16.04	32.80	7366 ±2.831	30.28	7566 3.094	28.39	7666 ±3.303	27.44	7333 ±1.248	30.59	7800 6.242	26.17	7766 ±2.06	26.50	7400 ±8.173	29.96	0.1431
	11	10766 ±3.775	3100 ±4.719	71.20	4733 ±3.303	56.03	3100 ±9.433	71.20	5066 ±7.549	53.03	4733 ±6.132	56.03	4866 ±5.191	54.80	5600 ±6.606	47.98	6033 ±3.402	43.96	0.1804
	14	10000 ±12.48	967 ±8.023	90.33	1566 ±7.549	84.34	2800 ±17.01	72.00	3100 ±9.433	69.00	2200 ±6.242	78.00	2300 ±10.8	77.00	2933 ±6.132	70.67	3266 ±6.606	67.34	0.5007
100	3	9066 ±5.191	16366 ±1.887	14.16	16866 ±4.325	11.53	17100 ±3.775	10.31	16866 ±5.191	11.53	17233 ±6.132	9.613	16700 ±2.359	12.40	17133 ±1.415	10.13	19033 ±3.775	0.173	0.0376
	7	19733 ±6.132	14166 ±1.415	28.21	15600 ±12.48	20.94	15000 ±8.173	23.98	17000 ±11.68	13.84	16000 ±10.28	18.91	16000 ±2.359	18.91	16066 ±6.606	18.58	16666 ±5.191	15.54	0.0762
	11	19533 ±8.491	5100 ±10.28	73.89	5733 ±6.537	70.64	6333 ±3.303	67.57	7233 ±3.303	62.97	10000 ±11.79	48.80	9866 ±2.831	49.49	10133 ±4.719	48.12	11833 ±5.663	39.42	0.1148
	14	19066 ±1.887	4500 ±4.719	76.39	5267 ±3.303	72.37	5333 ±1.415	72.02	6333 ±2.831	66.78	7400 ±3.303	61.08	7566 ±3.303	60.31	7466 ±6.132	60.84	7700 ±6.982	59.61	0.0829
150	3	28966 ±2.359	24166 ±4.719	16.57	26166 ±2.359	09.66	24333 ±4.719	15.99	26666 ±3.686	07.40	25200 ±6.242	13.00	26266 ±1.415	09.32	25666 ±2.452	11.39	27100 ±6.572	06.42	0.0254
	7	29033 2.87	20833 ±8.173	28.24	21033 ±2.359	27.55	20733 ±3.303	28.58	21900 ±5.564	24.56	22166 ±1.701	23.65	22166 ±1.248	23.65	21800 ±3.303	29.91	23133 ±7.768	20.32	0.0309
	11	28666 ±4.032	19333 ±4.719	32.55	19466 ±1.248	32.09	19866 ±2.947	30.69	18933 ±5.191	33.95	19366 ±5.721	32.44	19633 ±6.982	31.51	19266 ±2.452	32.79	20000 ±4.194	30.23	0.0351
	14	28533 ±1.634	14200 ±9.421	50.23	14700 ±4.719	48.48	15866 ±9.82	44.39	17533 ±3.749	38.55	19266 ±2.87	32.47	16766 ±4.501	41.23	16700 ±6.947	41.47	19233 ±5.318	32.59	0.0520

Value expressed as mean of triplicates± S.E.M= Stan dard error of mean. C.V.= Coefficient variance of P.a. - Pseudomonas aeruginosa, P.f.- Pseudomonas fluorescens ,B.c.- Bacillus cereus, A.f.- Alcaligenes faecalis, T.v.- Trichoderma viride, T.k.- Trichoderma koningii, P.c.- Penicillium chrysogenum and R.s.- Rhizopus stolanifer.

Table No-2:. GC-ECD Analysis of Cypermethrin microbial degraded residues.

Pesticides Conc.	M.O.	Area of Sample	Area of Std.	Dilution factor	Concentration mg/l	Degradation %
50mg/l	Control	25.702	166.394	500	3.09	00.00
	P.a.	42.36	291.892	100	0.58	81.22
	P.f.	219.859	137.348	10	0.64	79.28
	B.c.	356.528	137.348	10	1.04	66.34
	A.f.	84.469	291.892	100	1.16	62.45
	T.v.	180.812	137.348	20	1.05	66.01
	T.k.	48.886	166.394	100	1.18	61.81
	P.c.	101.237	291.892	100	1.39	55.01
	R.s.	117.234	291.892	100	1.61	47.89
100 mg/l	Control	47.739	291.892	1000	6.54	00.00
	P.a.	81.742	166.394	100	1.97	69.88
	P.f.	45.015	166.394	200	2.16	66.97
	B.c.	23.503	166.394	500	2.82	57.07
	A.f.	58.687	166.394	200	2.82	57.07
	T.v.	37.184	273.71	500	2.72	58.59
	T.k.	240.707	166.394	50	2.89	56.01
	P.c.	722.474	291.892	100	2.97	54.79
	R.s.	70.847	273.71	300	3.11	35.00
150 mg/l	Control	61.724	273.71	1000	9.02	00.00
	P.a.	307.278	291.892	100	4.21	53.32
	P.f.	292.012	273.71	100	4.27	52.66
	B.c.	150.543	273.71	200	4.40	51.21
	A.f.	401.119	273.71	100	5.86	35.03
	T.v.	77.715	291.892	500	5.32	41.01
	T.k.	196.328	273.71	200	5.74	36.36
	P.c.	211.432	291.892	200	5.79	35.80
	R.s.	53.278	273.71	1000	7.79	13.63

P.a. - Pseudomonas aeruginosa, P.f.- Pseudomonas fluorescens , B.c.- Bacillus cereus, A.f.- Alcaligenes faecalis, T.v.- Trichoderma viride, T.k.- Trichoderma koningii, P.c.- Penicillium chrysogenum and *R.s.- Rhizopus stolanifer*.

Detection metabolite of Cypermethrin-25 EC through Gas Chromatography and mass spectrometry (GC-MS):

The GC-MS chromatogram revealed that primary metabolites of degraded cypermethrin were found as 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-dimethyl3-methylenedecarhydroazuleno[4,5-b]furan-29(3H)-one) and Cyclopropane carboxylic acid which were detected by the GC-MS analysis.

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

Fish assay against the Cypermethrin pure and Cypermethrin degraded microbial residue.

Tolerance and mortality of fish against Cypermethrin and their microbial residue was examined after one hour time intervals up to three hours time duration. In between one hour fish was feeling uncomfortable in Cypermethrin, mortality was increased and tried to reach the surface of water. One fish was dead after 35 minutes time duration in 100 mg/l Cypermethrin concentrated medium. At residue concentration (1:1 ratio) fish was very unstable and no movement was seen. After 120 minutes fish of residual solution was very uncomfortable and movement was increased while in Cypermethrin solution contain aquarium fish died after 60 and 65 minutes. After 180 minutes time duration residue contains aquarium fish movement was increased and fishes gathered near aerator, one was dead after 150 min and one resist up to 3 hours (Table No. 3). Result revealed that microbial degraded residue fishes showed tolerance and more time survival capacity than Cypermethrin solution and it had proved that degraded residue of Cypermethrin was not harmful for the aquatic life, therefore microbial degradation of Cypermethrin is eco-friendly and non toxic to the environment.

Table No.-3: Effect of pesticides alone and their degraded residue on fish mortality.

Pesticides	Time (Min.)	Degraded residual liquid (1:1)	Pure Pesticides (100mg/L)
Cypermethrin-25EC,	60min	Fish was very unstable and no movement was observed.	Very unstable and immediately tried to reach the water surface. one fish dead after 35 min.
	120min.	Survival rate was increased by movements in the fishes.	Both two fishes was dead after 60 and 65 min.
	180min.	Survival rate was increased and fishes gathered near the aerator and one was dead after 150 min while two fishes resist up to 3 hours.	-

DISCUSSION

During experimental study conducted in scale up process bacteria *P. aeruginosa* had retained their degradation capacity at wide range of P^H 7.5 to 8.53. The chemical oxygen demand result showed that removal of organic load was proportional to the cypermethrin disappearance. The *P. aeruginosa* showed highest COD reduction (90.33%, 76.39% and 50.23%) at 50 mg/l, 100 mg/l and 150 mg/l respectively while fungi *R. stolaniferous* recorded minimum reduction of COD as 67.34%, 59.61% and 32.59% at 50 mg/l, 100 mg/l and 150 mg/l respectively. Similar correlation between COD removal and degradation was also observed by S. Jilani and M. Altaf Khan (2006) and Berchtold, *et al*. (1995), who noticed the same correlation between the COD removal and Biodegradation of increased concentrations of Cypermethrin from 20-125 mg/l gradually decreased the degradation performance of IES-PS-1 and 2, 4-DAT and also 2,4 and 2,6 diamino toluene degradation by acclimated Bacteria (Pesce and Wunderlin, 1997). The COD monitored during bioremediation showed that the reduction in COD concentration was directly proportional to the degradation of the parent compound into its intermediates or less harmful compounds with increasing period of time. Previous research studies also reported that COD is a direct indicator of bioremediation (Singh and Fulekar,2007).

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 eight different microorganisms' i.e. four fungal genera and four bacteria genera showed varied degradation abilities at several tested concentration of commercial based Cypermethrin. The bacterial growth density was calculated at optical density 600nm showed that the *P. aeruginosa* had faster growth than other Bacteria and in fungi the mycelial dry weight showed that the *T. viride* was more effective than other selected fungi. Similar findings were reported by Lee *et al*. 1998 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 (Goudar and Strevett, 2000; Lee, *et al*., 1998; Pesce and Wunderlin, 1997; Smith and Adkins, 1996).

In Present work *Trichoderma viride Pseudomonas fluorescens*, *Bacillus cereus* and *Alcaligenes faecalis* grows at 50ppm, 100ppm more effectively but at 150ppm it had lost degradation capacity and *Rhizopus stolanioferus* recorded as less tolerance against Cypermethrin but *Pseudomonas aeruginosa* was very potent at 150ppm concentration. GC-ECD result evaluated that *Pseudomonas aeruginosa* showed more potential as 81.22%, 69.88% and 53.32% at 50 mg/l, 100 mg/l and 150 mg/l concentrations of Cypermethrin respectively (Table No. 10, Fig-). 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* 2010a). The *P. aeruginosa* and other bacteria was grown as shake culture in minimal mineral salt medium containing Cypermethrin as sole source of carbon and energy. Bacterial degradation of Cypermethrin in pure cultures has been reported by several researchers such as Maloney *et al.*1998; Lee *et al.* 1998 and Grant *et al.* 2002.

The susceptibility and resistance of fish were studied against 100 mg/l concentration alone Cypermethrin and degraded residue of Cypermethrin. Three fish were used for assessing fish against pesticides and their degraded

residue. Fish resistance and susceptibility against pesticides and degraded residue was examined after 60 minutes, 120 minutes and 180 minutes time intervals. The result revealed that microbial degraded residue of Cypermethrin was eco-friendly and less toxic than pure Cypermethrin therefore the microbial degradation of Cypermethrin eco-friendly and there was no side effect on environment, aqueous life, soil.

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