

**CYPERMETHRIN DEGRADATION USING *KLEBSIELLA PNEUMONIAE*
AND CHECKING TOXICITY OF DEGRADATION PRODUCTS ON
AQUATIC LIFE**

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ABSTRACT

Agricultural loss due to different pests is increasing extensively. Various chemical pesticides like organophosphorus pesticides, organochlorines, carbamates are being used for control of the various pests. But these pesticides are found to be associated with different side effects and toxicity to human beings and to aquatic life. So, these pesticides have been banned for use by many countries. Therefore, synthetic pyrethroids are being used extensively in the agriculture after the ban over organochlorine and organophosphorous pesticides.

Cypermethrin is the fourth generation pyrethroid useful against pests of fruits, vegetables and household insects. Cypermethrin shows affectivity against different pests at low concentration but at the same time shows toxicity to human beings and to aquatic life. So, removal of these pesticides from soil is very important for crop protection.

Focus of this paper is degradation of cypermethrin by using rhizospheric isolates. For this, different isolates were obtained from soil samples and checked for their capacity of cypermethrin degradation. Two isolates showing capacity of cypermethrin degradation were identified by 16S rRNA. One of the isolate *K. pneumoniae* was used for cypermethrin degradation. Cypermethrin degradation was confirmed by detecting reduction in the COD values. COD value was found to reduced more than 50%. Cypermethrin degradation metabolites were detected by GC-MS

analysis and different degradation metabolites were identified by comparing with standard database.

Toxicity of the cypermethrin degradation product was tested by fish bioassay. It was found that guppy fishes could survive up to 48 hrs in presence of cypermethrin degradation product whereas in control fishes were died within half hour.

Keywords: Cypermethrin, Rhizospheric isolates, Synthetic pyrethroids, Fish bioassay

1. INTRODUCTION

Pesticides can be classified in to different types like insecticides, herbicides, fungicides, rodenticides etc. (Hayes, 1975). Pesticides are useful to control different types of pests which are toxic to plants and to human beings. But upon prolonged application and due to accumulation in high concentrations, pesticides are found to be toxic to soil microflora. (Pandey and Singh, 2004). Pyrethroids being botanical in origin and synthetic, these have been used extensively as compared to other pesticides. Cypermethrin is a fourth generation pyrethroid being effective at low concentration and shows toxicity to aquatic life and human health. (Zhang *et al.*, 2011).

Different methods are available for removal of these pesticides from the soil but are time consuming and costly. So, biodegradation process plays an important role in pesticide removal from soil.

By using micro organisms these pesticides can be transformed in to non- hazardous form. (Saraswatandand Gaur, 1995). Different organisms have been isolated belonging to genera *Micrococcus*, *Klebsiella*, *Serratia*. (Grant *et al.*, 2002; Tallur *et al.* 2008; Murugesan *et al.*, 2010.; Maloney *et al.* 1993, Nirmali *et al.*, 2005). These organisms degrade the pesticides and uses them as sole carbon source for their energy metabolism.

Gurjar M.M. and Hamde also have detected ability of *P. Aeruginosa* to degrade the cypermethrin. Many researchers have reported the ability of mixed bacterial cultures in biodegradation of pyrethroid pesticides. For e.g Chen *et al.* (2012b) have made use of mixed culture of *Bacillus cereus* ZH-3 and *S. aureus* HP-S-01 to increase the capacity of cyermethrin degradation up to 73.1% as compared to the individual cultures. As the incubation time was increased from 48 to 72 hrs. Cypermethrin degradation was up to 95.7-100% respectively. Other researchers Liu *et al.* (2014) made use of mixed culture of *Bacillus licheniformis* B-1 and *Sphingomonas* sp. SC-1 and cypermethrin half-life was found to be shortened from 71.5-35 in 7h. Akbar *et al.* (2015b) have reported degradation of more than 70% of pyrethroids by *Bacillus megaterium* Jcm2 in 7 days. Also Yuanfan *et al.* (2010) have reported ability of *Sphingobium* sp.

JQL4-5 to degrade only 36.5% cypermethrin, 25% bifenthrin and 13.3% cyhalothrin. So the present paper focuses on isolation and identification of cypermethrin degrading organisms from soil, detection of degradation metabolites by GC-MS analysis and COD estimation as a means of cypermethrin degradation.

2. MATERIAL AND METHODS

2.1 Insecticide

Cypermethrin-25 EC was purchased from local market shop from Hadapsar and Swargate, Pune M.S. India. All other required chemicals were purchased from Hi-media Mumbai and Sigma Aldrich, USA.

2.2 Isolation of Cypermethrin degrading organisms by soil enrichment technique

Soil samples were air dried to 20% (w/w) moisture content. Fifty grams of each soil 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 1000mg/lit 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 (Bhosle *et al.* 2013)

2.3 Primary and secondary screening of isolates

Primary screening of isolates was done using nutrient agar medium supplemented with cypermethrin at various concentrations like 1mg/lit, 2mg/lit, 3mg/lit. Microorganisms capable of growing on nutrient agar medium with different cypermethrin concentrations were selected for further study. Spread plate technique was used to isolate microorganisms capable of cypermethrin degradation. Isolated organisms were spread plated on minimal medium supplied with cypermethrin as sole carbon source. Organisms showing growth up to 200mg/lit were used for further study.

2.4 Identification of isolates

Isolates were identified using morphological, biochemical characteristics and by 16S rRNA sequencing from Agharkar Research Institute, Pune.

2.5 Detection of chemical oxygen demand

Open reflux method

Open reflux method was used for detection of COD changes as a measure of Cypermethrin degradation by FCM82, FCM82 was inoculated in minimal medium broth supplemented with 50mg/lit, 100mg/lit, 150mg/lit and 200mg/lit concentration of Cypermethrin. Incubation was done at 30⁰C and samples were obtained at different time intervals like 24 hrs, 48 hrs, 72 hrs and 96 hrs. COD from sample and control was determined by standard open reflux method for COD estimation.

In this method, 20 mL sample from each concentration was taken in refluxing flask. Initial COD was found to be high. So each of the samples were diluted as 1:10, 1: 100 and 1:1000, 20 mL of diluted sample from each dilution was used for COD determination. 1:1000 dilution was found to be useful for COD determination. Samples were taken in refluxing flask. Then 1g Mercuric sulphate, few glass beads and 5mL sulphuric acid was added in the refluxing flask. Refluxing flask was cooled while mixing to avoid loss of volatile materials. Then 10 mL of 0.25N K₂Cr₂O₇ was added and mixed. Then slowly 25mL sulphuric acid reagent was added and refluxing was done at 150⁰C for 2 hrs. Sample from each refluxing flask was taken in flask and was diluted to 150mL with distilled water. Cooling was done to room temperature and excess K₂Cr₂O₇ was titrated with 0.10-0.15N FAS with 2-3 drops of ferroin indicator, and point of titration was determined as sharp change in colour from blue-green to reddish brown color which persists for 1 minute or longer. Blank was titrated with the reagents and distilled water was added at the place of sample.

COD was determined by using the formula:

$$\text{COD(mg/L)} = (A-B) \times M \times 8000/\text{mL sample}$$

Where,

A= mL FAS used for blank

B= mL FAS used for sample

M= Molarity of FAS

8000=Mill equivalent weight of Oxygen x1000mL/L

2.6 Detection of degradation metabolites by GC- MS analysis

Minimal medium was used for growth of *K. pneumoniae*. Cypermethrin was used as sole carbon source at a concentration of 150mg/lit. Incubation was done at 30⁰C for different time intervals. 20mL of incubated minimal medium was centrifuged at 10000rpm for 10 minutes. Supernatant

was separated and acidified with 2NHCl to pH 2. Then equal volume of acetone was used for extraction of Cypermethrin metabolites. The aqueous layer from the sample was removed by passing the sample through separating funnel containing Whatmann filter paper and unhydrous sodium sulphate. The remaining sample was collected in amber colored screw cap bottle and it was used for GC-MS analysis. GC-MS analysis of samples was done from IIT Pawai Bombay.

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 m film thickness) was used with an 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*.

2.7 Fish Bioassay

Fish bioassay was performed to check the toxicity of cypermethrin degradation products on aquatic life.

For this, 50 healthy guppy fishes of similar weight and age were obtained from local breeder from Ghole road Pune. Fishes were transported to laboratory in the aerated plastic bags. These fishes were added in 10 litre beaker containing aerated water and ambient water temperature. (20-25⁰C). Stock solutions of the test compounds and their dilutions were made as per the standard methods (ECD, 1993; APHA, AWWA, WEF, 1998). The test species were also selected as per these standards. The water sample was continuously aerated before putting the fishes in to beaker to remove residual chlorine. Aeration was stopped during dosing period. These fishes were fed daily during acclamatisation period. The pure cypermethrin at 200mg/lit concentration was used as a standard. Isolate *K. pneumoniae* was inoculated in minimal medium containing cypermethrin at 150mg/lit concentration and incubation was done on rotary shaker at 150rpm for 48 hrs. The broth was centrifuged at 10000 rpm for 10 minutes. Cell pellet was separated and supernatant was used for fish assay. Supernatant was diluted tenfold as 1:10/1:100 and 1:1000. 10mL sample from each dilution was added in the beaker containing one-liter aerated water. Ten fishes were added in each beaker and were observed for survival for 96 hrs. Positive control group was used with cypermethrin as standard and negative control was used without cypermethrin.

3. RESULTS

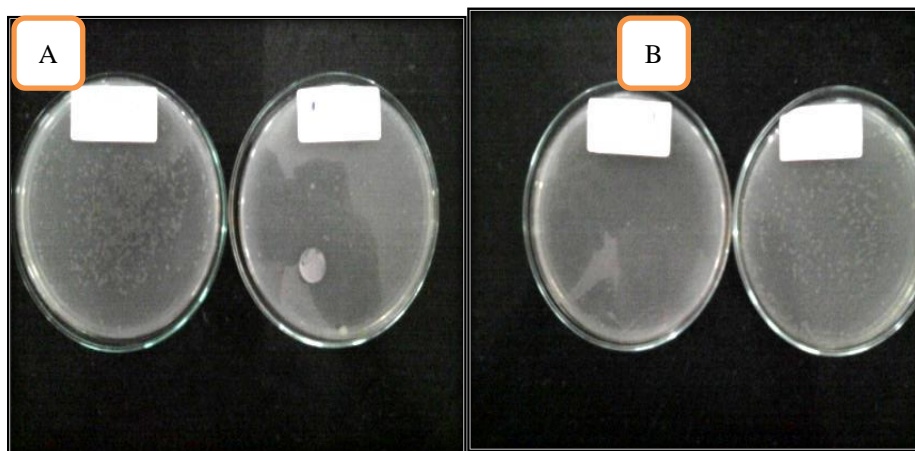
3.1 Isolation of cypermethrin degrading organisms

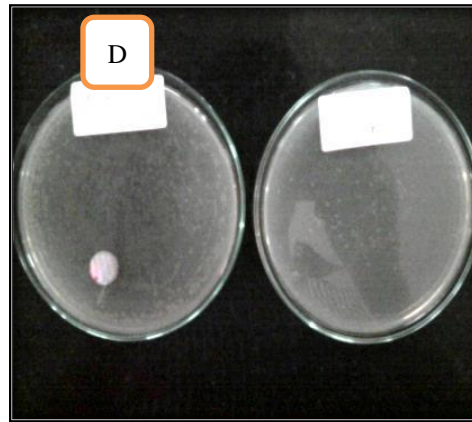
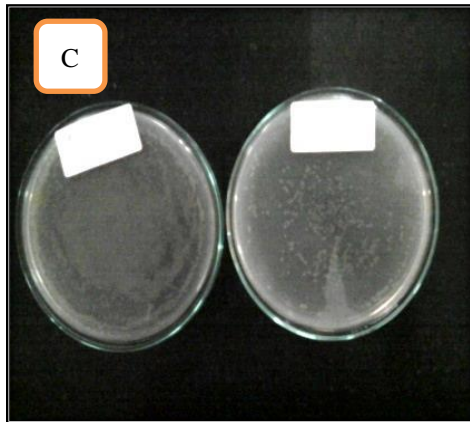
Different isolates were obtained on minimal agar medium with cypermethrin. These isolates were labelled as FCM1, FCM2 etc. Organisms capable of growth up to 200mg/lit cypermethrin concentration were used for further study.

Fig. 1: Growth of isolates on nutrient agar medium



Fig. 2 Growth of FCM82 on minimal medium with different cypermethrin concentrations





- A- Growth on minimal medium with 50mg/lit cypermethrin concentration
- B- Growth on minimal medium with 100mg/lit cypermethrin concentration
- C- Growth on minimal medium with 150mg/lit cypermethrin concentration
- D- Growth on minimal medium with 200mg/lit cypermethrin concentration

Table 1: Colony characteristics of FCM 82

Sr.No.	Colony character	FCM82
1	Size	2-3mm
2	Shape	Circular
3	Colour	Cremishyellow
4	Margine	Entire
5	Opacity	Transluscent
6	Elevation	Elevated
7	Consistency	Smooth
8	Gram staining	Gram negative rods
9	Motility	Non motile rods

3.2 Biochemical characteristics of FCM 82

Table 2: Biochemical tests of FCM82

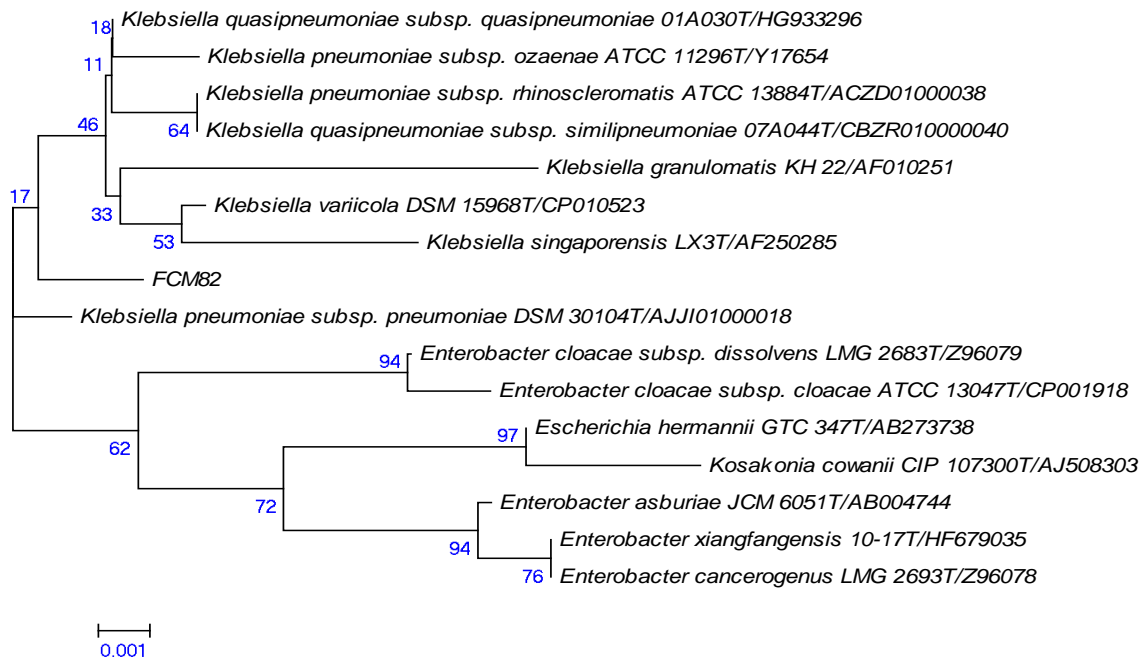
Sr. No.	Biochemical character	Observation	Inference
1	Glucose fermentation test	Sugar fermentation tube turns yellow due to acid production with gas production	Positive test
2	Lactose fermentation	Sugar fermentation tube turns yellow due to acid production with gas production	Positive test
3	Mannitol fermentation	Sugar fermentation tube turns yellow due to acid production with gas production	Positive test
4	VP test	GPB turned red	Positive test

FCM82 was identified by using morphological and biochemical characteristics. Tentatively isolate was identified as *K. pneumoniae*. Further identification was done by 16S rRNA sequencing method.

3.3 Identification of FCM 82 by 16S rRNA sequencing

Table 3: Phylogenetic tree of *Klebsiella pneumoniae*

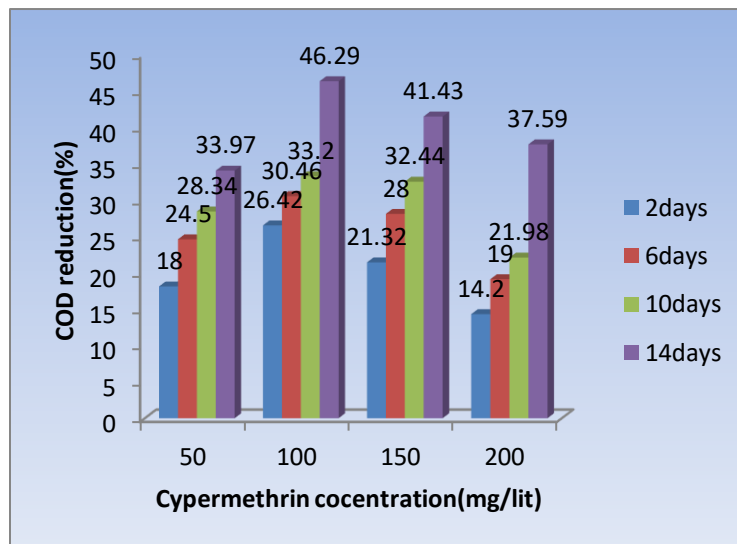
Strain Designation	Closest Phylogenetic affiliation	Max ident
FCM82	<i>Klebsiella pneumoniae subsp. Pneumoniae</i>	99%



By using 16S rRNA sequencing isolate was confirmed as *Klebsiella pneumoniae* subsp. *pneumoniae*.

3.4 Detection of Chemical Oxygen Demand (COD)

Fig. 4: Detection of COD reduction by *K. pneumoniae* at different time intervals.



It is seen from the graph that as the time interval was increased concentration of reduced COD increases. Maximum COD reduction was found to be at 100mg/lit cypermethrin concentration. In contrast, at 200mg/lit concentration, percentage COD reduction was found to comparatively low. This might indicate possible toxicity of cypermethrin to *K. pneumoniae* at higher concentration.

3.5 Detection of degradation metabolites by GC-MS analysis

Samples were extracted from the minimal medium supplemented with cypermethrin as sole carbon source after 14 days of incubation and were used for GC- MS analysis.

Fig. 5: Chromatogram showing cypermethrin degradation products by *K. pneumoniae*

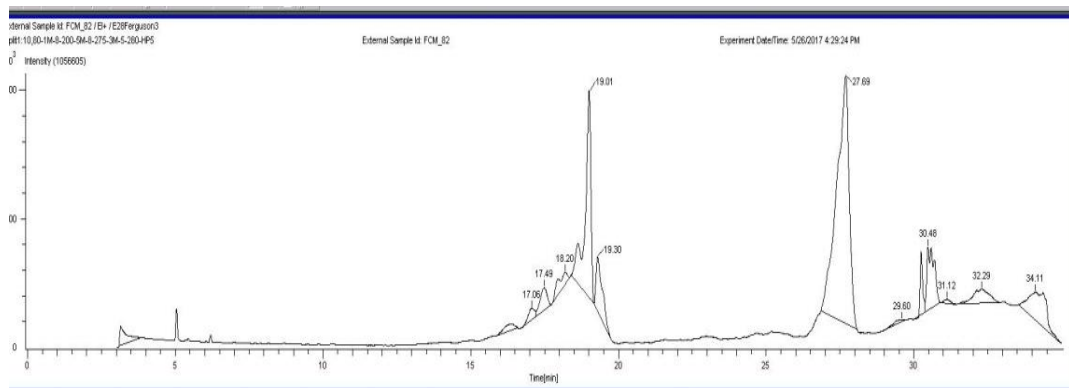
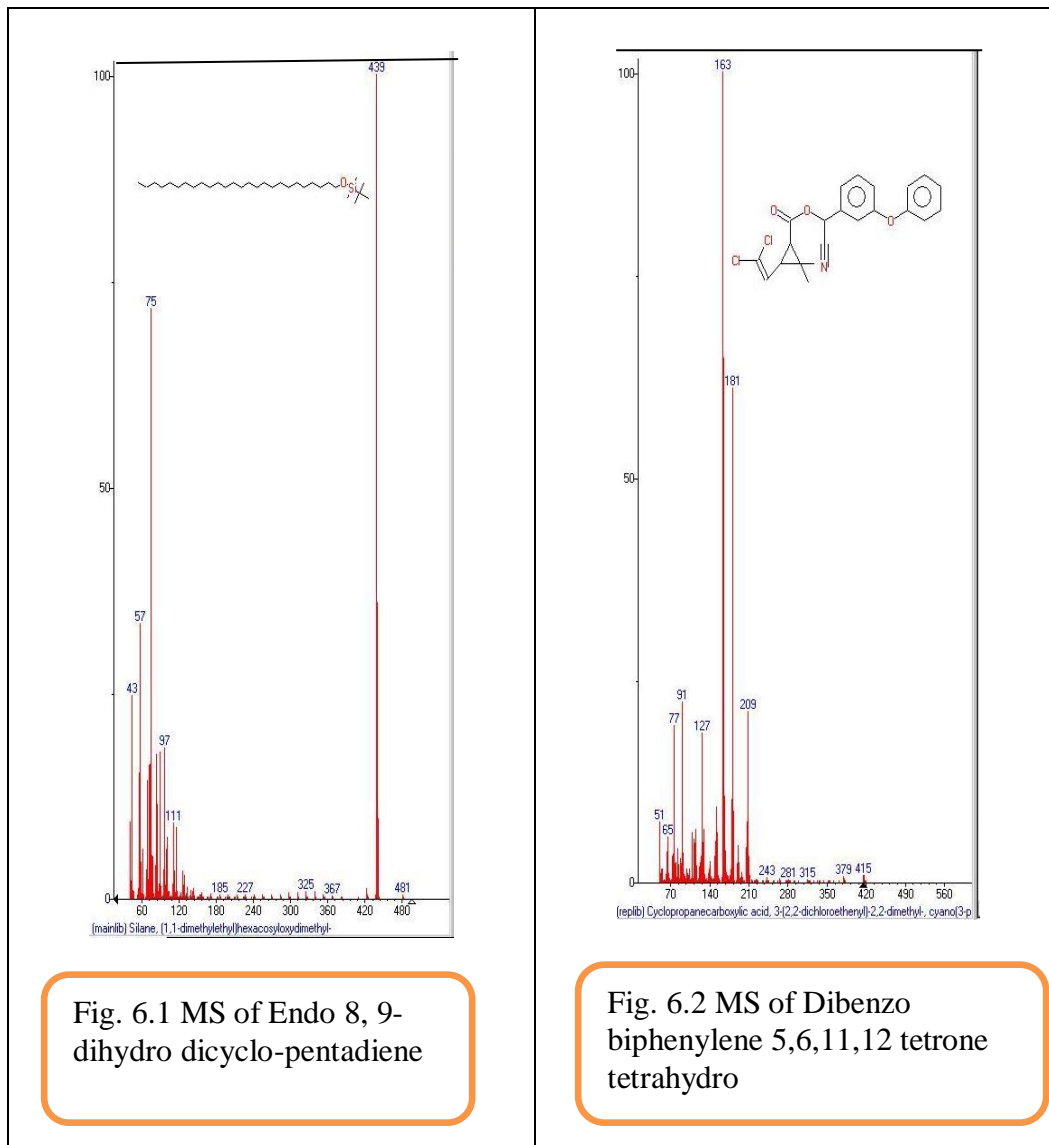


Table 4: Different cypermethrin degradation metabolites detected by GC- MS analysis

Sr. No.	Metabolite	Retention time(min.)	Name
1	A	5.04	Endo 8,9-dihydro dicyclo-pentadiene
2	B	6.38	Dibenzo biphenylene5,6,11,12 tetrone tetrahydro
3	C	17.49	Indole -2-one 1,3 dihydro 3,3-bis [4-hydroxy-3-methyl-phenyl]
4	D	18.20	Ethylele 1,2,bis[P-methoxy phenyl[-1-phenyl]

Table 3 shows different cypermethrin degradation metabolites and these were identified by comparing with the authentic database.

Fig. 6: Degradation metabolites of cypermethrin



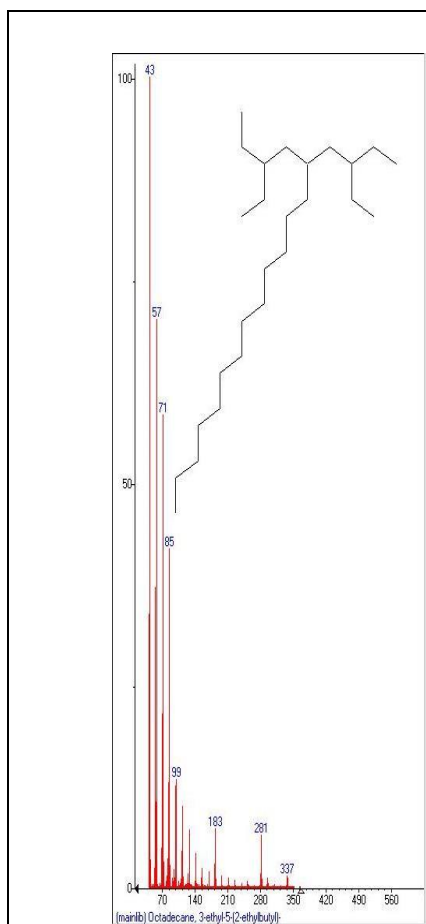


Fig. 6.3 MS of Indole -2-one
1,3 dihydro 3,3-bis [4-
hydroxy-3-methyl-phenyl

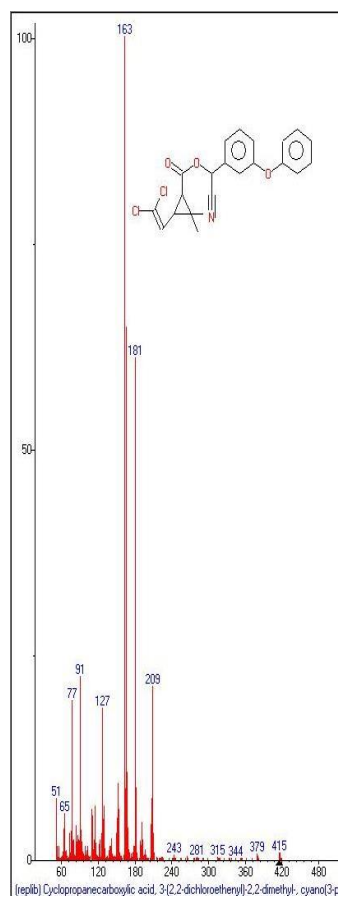


Fig. 6.4 MS of Ethylele
1,2-bis[P-methoxy
phenyl[-1-phenyl]

In the experiment when *K. pneumoniae* was inoculated in the minimal medium with cypermethrin, different degradation metabolites were obtained at different retention times. Fig. 6 shows MS spectra of representative cypermethrin degradation products detected by GC-MS analysis.

3.6 Fishbioassay

Fig. 7: Experimental set up for fish bioassay



No. of fishes added in each test = 10

Table 5: Fish survival at different time intervals

Sample after Days of incubation	No .of fishes survived			
	After 3 hrs	After 6 hrs	After 8 hrs	After 24 hrs
7	5	3	1	0
14	7	5	3	1
21	6	4	4	3
Control(100mg/lit)	0	0	0	0

As per table 4.19 when cypermethrin degradation products produced by *K. pneumoniae* after 7 days incubation were added in the beakers, only 10% fishes could survive after 8 hrs. But when degradation products were obtained after 21 days of incubation, 25% fishes could survive after 8 hrs and 30% fishes survive after 24 hrs of incubation. This observation also indicates that

degradation products produced by *K. pneumoniae* are less toxic to aquatic life as compared to control.

DISCUSSION

Isolate FCM 82 identified as *K. pneumoniae* subspecies *pneumoniae* showed growth up to 200mg/lit cypermethrin concentration. So it was used for further study. As reported by Bhosale *et al.* (2013), soil isolates showed growth up to 150mg/lit. cypermethrin concentration.

Different pesticide degrading organisms have been isolated by many researchers. Naphade *et al.* (2013) have isolated organisms from garden soil and were identified as *Paracoccus chinensis* KS-11(T), *Planococcus rifietoensis* and *Pseudomonas aeruginosa*. These isolates showed ability of degradation of different groups of pesticides like Endosulfan (35% Thiodan), Chlorpyrifos (21.5% E.C. Godrej Chlorvip) and Cypermethrin.

Isolated organisms showed ability to grow up to 3mg/lit as per the previous experiments. So the scale up technique was used to increase the ability of isolates to adapt to the higher cypermethrin concentrations, (Naphade *et al.* 2012). The isolated organisms were transferred on minimal agar medium containing cypermethrin up to 200mg/lit concentration. Organisms which showed growth up to 200mg/lit cypermethrin concentration were selected.

Fulekar (2009) has used the scale up technique for bioremediation of Fenvalerate by *Pseudomonas aeruginosa*. Fulekar and Geetha, (2008) used the scale up technique for degradation of Chlorpyrifos by *Pseudomonas aeruginosa* using different Chlorpyrifos concentrations as 10, 20, 50, 75, 100 mg/lit. As per the study conducted by Bhosale *et al.*, (2013) the scale up technique has been used for two fungal isolates *Penicillium chrysogenum* and *Rhizopus stolonifer*. These fungi were found to be effective in cypermethrin degradation up to 150mg/lit concentration. Fulekar and Geetha; (2009) carried out biodegradation of Trichlorpyr Butoxy ethyl Ester (TBEE) in bioreactor.

As per the present research work, isolate FCM82 showed growth at 200mg/lit. concentration. This indicated possibility of using this isolate in cypermethrin degradation at higher concentration as compared to earlier studies.

Highest decrease in COD percentage was seen at 100mg/lit cypermethrin concentration for *K. pneumoniae*. At 200 mg/lit cypermethrin concentration, percent reduction in COD was found to be decreased. This might be due to increased cypermethrin concentration in the medium may be toxic to isolate or may be due to accumulation of toxic components in the medium which interferes with the degradation process.

Similar relation between pesticide degradation and decreased COD value have been observed by Jilani and Altaf Khan (2006). As per the previous studies of Bhosale (2013), reduction in COD value was directly proportional to degradation of the parent compound in to nontoxic compound.

In presence of cypermethrin degradation product produced by *K. Pneumoniae* after 21 days of incubation, 25% fishes could survive after 8 hrs and 30% fishes survive after 24 hrs of incubation. This observation indicates that degradation products produced by *K. pneumoniae* are less toxic to aquatic life as compared to control.

Similar work has been done by Prasanth *et al.* (2005). Toxicity of monocrotophos was detected on *L. rohita* fishes and it was found that 100% mortality of these fishes occurred at 0.0044ppm and within 96 hrs, 50% fishes were died.

4. CONCLUSION

From the above experiments it can be concluded that *K. pneumoniae* shows the ability of cypermethrin degradation. These degradation products were separated by GC-MS analysis and degradation products showed least toxicity to the aquatic life.

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