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ENTOMOLOGY

Biodegradation of Pyrethroid-Cypermethrin using *Pseudomonas aeruginosa* and Detection of its Plant Growth Promoting Properties

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ABSTRACT

Pyrethroids are the botanical origin pesticides commonly used in agriculture for fruit and vegetable protection against various pests. Cypermethrin belongs to fourth generation of pyrethroids which are effective at very low concentration. Cypermethrin has beneficial effects but at the same time shows toxicity to aquatic life, carcinogenicity to human beings. Different methods are available to remove the cypermethrin pesticides from the soil but these methods are expensive and time consuming. So, the focus of this paper is on degradation of this pesticide by using soil microorganisms. For this, microorganisms were isolated from soil and identified by 16S rRNA sequencing. The isolate *P. aeruginosan* showed tolerance up to 200mg/lit cypermethrin concentration. COD value was found to be decreased up to 67.74% and 77.47% after 14 days of incubation and at 100mg/lit and 150mg/lit cypermethrin concentration resp. GCMS analysis showed presence of degradation metabolites indicating cypermethrin degradation capacity of *P. aeruginosa. P. aeruginosa* also showed plant growth promoting activity indicated by ability of nitrogen fixation, siderophore production, HCN production and IAA production. Thus, the isolate *P. aeruginosa* might have a potential in terms of cypermethrin degradation up to 200mg/lit concentration and plant growth promotion.

Highlights

- Cypermethrin degrading organisms were isolated and identified.
- Isolates were checked for tolerance to increased cypermethrin concentrations up to 200mg/it.
- Cypermethrin biodegradation was confirmed by detecting decrease in COD values with incubation time and detection of degradation metabolites by GC MS analysis.

Keywords: Pesticide, Pyrethroid group, Cypermethrin, Biodegradation, GCMS analysis

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 micro flora (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. (Western *et al*. 2009). It has been said that "no pesticide is perfect, but the pyrethroids come close".

Pyrethroids are of botanical origin and have been obtained from dry flowers of *Chrysanthemum cinerariefolium* plant which is known from nineteenth century (Grant *et al.* 2001; Elliott 1980). Pyrethroids have four major generations and cypermethrin belongs to fourth generation of pyrethroids (Casida 1980).

Cypermethrin [(+/-)-α-cyano-3-phenoxybenzyl (+/-)-*cis*, *trans*-3 (2, 2dichlorovinyl)-2,

2-dimethylcyclopropane carboxyl ate] is a synthetic Pyrethroid pesticide, have uses in cotton, cereals, vegetables and fruit, for food storage, in public health and in animal husbandry. It is also used in home and garden pest control worldwide (Tallur *et al.* 2008; Lin *et al*. 2011).

Recently cypermethrin is studied extensively due to its aquatic life toxicity as well as high risk to human health. (Zhang *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 used which involves transformation of these chemical compounds in to non-Hazardous form. (Saraswat and Gaur 1995). Different attempts have been made for isolation of Pyrethroid degrading organisms from soil and water polluted with these pesticides. Different organisms have been isolated belonging to genera *Micrococcus, Klebsiella, Serratia* (Grant *et al*. 2002; Sakata *et al*.1992; Tallur *et al*. 2008; Murugesan *et al.* 2010; Maloney *et al*. 1993; Halden *et al*. Nirmali *et al*. 2005). These organisms degrade the pesticides and uses them as sole carbon source for their energy metabolism (Baxter *et al.* 1975).

Siderophores are produced by many micro-organisms as means of sequestering limiting iron. Pseudomonads produce a range of iron-chelating compounds including salicylic acid, pyochelins and fluorescent pseudobactins and pyoverdins. Fluorescent siderophores are unique to pseudomonads- a trait that has implicated these organisms as PGPR (S. Shivshakthi *et al*. 2014; Girija Ganeshan 2005).

Indole acetic acid (IAA), is also produced by many strains that exhibit biocontrol properties. Although IAA has not been directly implicated as a metabolite in disease control, it is bioactive and stimulates root elongation. IAA is produced by the nitrogen-fixing bacteria, *Azospirillum* and is thought to play a key role in the plant growth promoting effect that these bacteria have on germinaceous plants (Mehnaz *et al.* 2009).

Similar experiments have been performed by Shweta Sharma *et al.* (2014) to evaluate plant growth regulators *in vitro*.

The present study aims at isolation and identification of cypermethrin degrading organisms, checking the tolerance of isolates to increased cypermethrin concentration, detection of degradation products by GC MS analysis and checking plant growth promoting properties of isolates.

MATERIALS AND METHODS

Isolation of cypermethrin degrading microbes from the soil

Microorganisms capable of cypermethrin degradation were isolated by using the enrichment technique. For this the soil samples were inoculated in minimal medium broth (K_2HPO_4-7g, KH_2PO_4-3g, $MgSO_4$-0.1g, $(NH_4)_2SO_4$-1g, 5mL Trace element solution) containing cypermethrin as sole carbon source at concentrations of 1mg/lit, 2 mg/lit and 3 mg/lit. Minimal medium broth was incubated at room temperature for 72hrs. After every 24hrs, samples were taken from each broth and spread plated on minimal agar medium supplemented with same concentrations of cypermethrin as above. Individual colonies were sub cultured on minimal agar plates containing same concentrations of cypermethrin until pure cultures were obtained. These microorganisms were used for further study. These organisms were labeled as FCM1, FCM2, and FCM3 etc. The isolated pure cultures were maintained at 4°C and as glycerol stocks at -20°C and sub cultured after every three months.

Checking tolerance of isolates to increased cypermethrin concentration:

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

Identification of isolated organisms by biochemical characteristics and 16srRNA sequencing:

The isolates which showed tolerance to highest cypermethrin concentration (200mg/lit) were identified by using morphological, cultural and biochemical characteristics as described by Colins and Lyne (1985) up to the genus level. Further the isolate was identified by 16srRNA sequencing from Agharkar research institute Pune, India.

Detection of diazotrophic nature of isolate FCM68

The isolated organism FCM68 was grown on Nitrogen free Ashby's mannitol agar medium supplemented with cypermethrin. Mannitol sugar was deleted from the medium.

Diazotrophic nature of isolate was confirmed by streaking the isolate FCM68 on Nitrogen Free malate medium with Bromothymol blue (NFB) as pH indicating dye. Incubation was done at room temperature for 24 hrs. The presence of blue coloration on NFB plates indicated nitrogen fixing capacity of FCM68.

Detection of siderophore

Production of siderophores by *Pseudomonas aeruginosa* was assessed by $FeCl_3$ reagent. 24 hour old culture of *Pseudomonas* was centrifuged at 15,000 rpm for 10 min, 1ml of supernatant was taken and was treated with 1 ml of $FeCl_3$ reagent. Color change was observed. Un-inoculated broth with siderophore reagent served as blank.

Detection of hydrogen cyanide

Pseudomonas aeruginosa was grown on Tryptic-soy-agar (TSA) for production of hydrogen cyanide. Whatman filter paper strips or discs were soaked in a picric acid solution were placed in the lid of each Petri-plate. Petri-plates were then sealed with parafilm and incubated at 28°C for 48 hours. A color change was observed. Change in the color of filter paper from yellow to light brown, brown or reddish brown of the strips was served as an indication of weak, moderate or strong production of HCN.

Detection of IAA

IAA production by different *Pseudomonas aeruginosa* was determined using Salkowaski's reagent. The purified and freshly grown cultures on Luria-Bertani (LB) medium were transferred into tubes containing 5 ml LB broth supplemented with 100µg L-tryptophan and were incubated at 28±1°C for 2 days. The broth was then centrifuged for 5 min at 10,000 rpm and in the supernatant equal volume of Salkowaski's reagent was added. The contents were mixed and allowed to stand in the dark at room temperature for 30 min to develop color. Un-inoculated broth served as control.

Determination of % reduction in Chemical Oxygen Demand (COD) in presence of *Pseudomonas aeruginosa*

Open reflux method

Open reflux method was used for detection of COD changes as a measure of Cypermethrin degradation by *Pseudomonas aeruginosa*. The isolate was inoculated in minimal medium broth supplemented with 50mg/lit, 100mg/lit, 150mg/lit and 200mg/lit Cypermethrin concentration. Incubation was done at room temperature 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.

In this method, 20mL sample from each concentration was taken in refluxing flask. Initial COD was found to be very high. So, each of the samples were diluted as 1:10, 1: 100 and 1:1000. 20mL of diluted sample from each dilution was used for COD determination and 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 0.25 $NK_2Cr_2O_7$ was added and mixed. Remaining amount of sulphuric acid reagent was added and refluxing was done at 150°C for 2hrs. Sample from each refluxing flask was taken in 250ml flask and was diluted to 150mL with distilled water. Cooling was done to room temperature and excess $K_2Cr_2O_7$ was titrated with 0.10-0.15mL FAS with 2-3 drops of ferro in indicator, and point of titration was determined as sharp change in colour from blue-green to reddish brown color which persists for 1 min. 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:

$$COD\ (mg/L) = (A–B)^* M^* 8000/mL\ sample$$

Where,

A = mL FAS used for blank

B = mL FAS used for sample

M = Molarity of FAS

8000 = Mill equivalent weight of Oxygen*1000mL/L

GC-MS analysis for detection of degradation products

The isolate *Pseudomonas aeruginosa* was inoculated in minimal medium broth supplemented with cypermethrin at a concentration of 200mg/lit. Incubation was done at 30°C for 14 days (Nilesh P. Bhosle).

20mL medium was centrifuged at 10000rpm for 10 minutes. The cell free supernatant was separated and acidified with 2NHCl to pH 2. Then equal volume of acetone was added. The aqueous layer from the sample was removed by passing the sample through separating funnel containing Whatman filter paper and anhydrous 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.

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 mm 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 Fig. 2 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*.

RESULTS AN 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. The isolates were labeled as FCM1, FCM2, and FCM3……etc. Initially isolates were grown on Nutrient agar medium with cypermethrin. Then the isolates were screened by checking their ability to grow on minimal medium supplemented with cypermethrin as sole carbon source (Fig. 2).

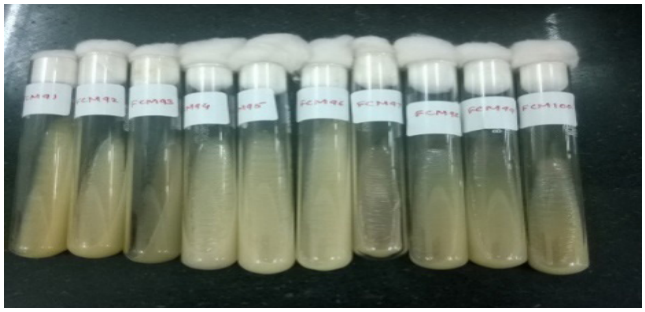


Fig. 1: Growth of isolates on minimal agar medium

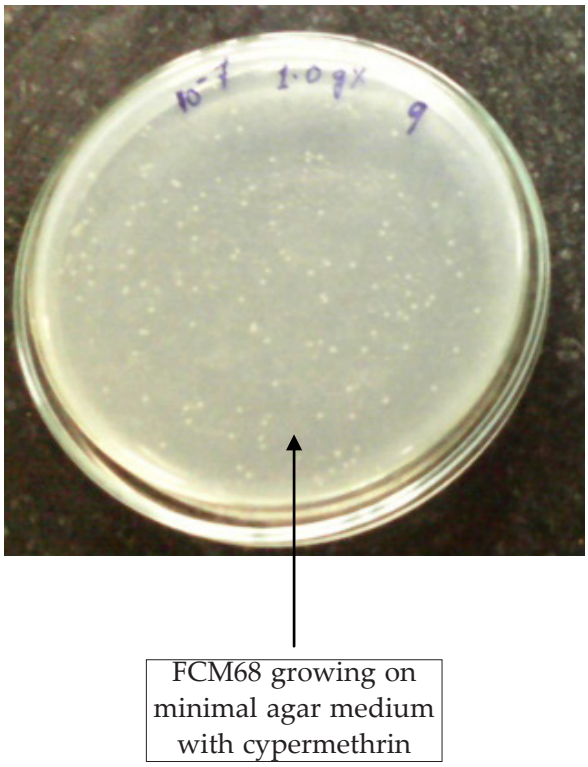


Fig. 2: Growth of the isolate FCM68on minimal agar plate

Checking tolerance of isolates to increased cypermethrin concentration

Isolated organisms were checked for the growth in presence of different cypermethrin concentrations. Isolate no. FCM68 and FCM82 showed growth in presence of 200mg/lit cypermethrin concentration. These isolates were used for further study. Similar observations are made by Nilesh Bhosale *et al.* (2013) and Manaswi Gurjar *et al*. (2018). The maximum concentration of cypermethrin degraded was 150mg/lit. However, present study indicated cypermethrin degradation by *P. aeruginosa* at 200mg/lit concentration.

Table 1: Tolerance of isolate to increased cypermethrin concentration

Isolate No.	Cypermethrin concentration (mg/lit)							
	0.25	0.50	0.75	0.9	1	10	100	200
FCM1	+++	++	+	-	-	-	-	-
FCM2	+++	++	++	++	-	-	-	-
FCM3	+++	++	++	+	-	-	-	-
FCM4	+++	++	++	-	-	-	-	-
FCM5	+++	++	++	-	-	-	-	-
FCM45	+++	++	++	+	-	-	-	-
FCM46	++	++	++	+	-	-	-	-
FCM49	++	++	++	+	-	-	-	-
FCM68	+++	+++	+++	++	+++	+++	+++	++
FCM82	+++	+++	+++	++	++	+++	+++	++

Key: *+- Absorbance value 0.04, ++ - Absorbance value 0.54, +++ -Absorbance value 0.89, - - No turbidity.*

Identification of FCM68

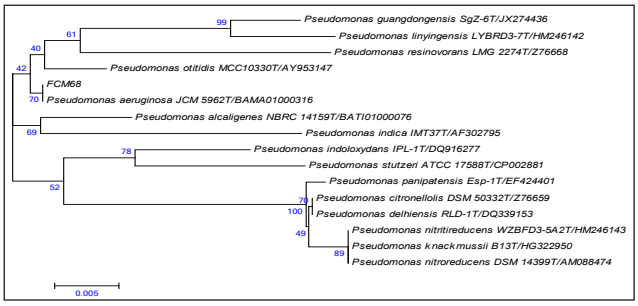
(i) Biochemical characteristics of FCM68

Table 2: Biochemical characteristics of FCM68

Sl. No.	Biochemical character	Observation	Inference
1	Catalase test	Effervescence of oxygen were seen	Positive test
2	Oxidase production	Purple coloration was observed on Whatman filter paper in presence of oxidase reagent	Positive test
3	Gelatin hydrolysis	Zone of gelatin hydrolysis around the colony was seen	Positive test
4	Nitrate reduction test	Nitrate reduction broth turned red	Positive test
5	Glucose utilization	Turbidity was seen in peptone water broth	Positive test
6	OF test	Yellow coloration was observed in OF tubes	Positive test
7	Growth on cetrimide agar plate	Growth was observed	Positive test

(ii) Identification of isolate FCM 68 by 16SrRNA sequencing

Strain Designation	Closest Phylogenetic affiliation	Max ident	Accession number
FCM68	*Pseudomonas aeruginosa*	100%	MF423469



By referring to biochemical tests and 16SrRNA sequencing the isolate FCM68 was identified as *P. aeruginosa* and nucleotide sequence was deposited in NCBI with accession no. MF423469.

Checking diazotrophic nature of *P. aeruginosa* (FCM68)

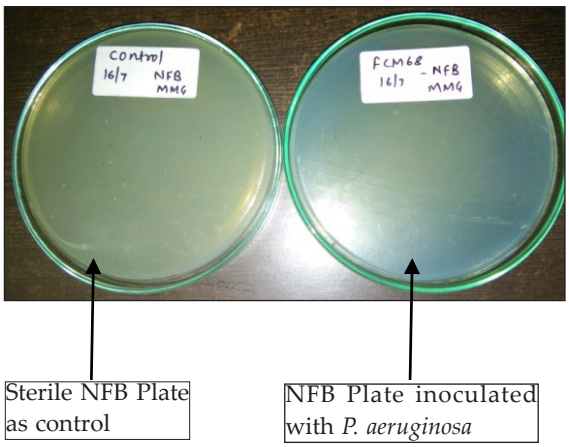


Fig. 3: Checking for diazotrophic nature of *P. aeruginosa*

P. aeruginosa showed growth on Ashby's nitrogen free medium and nitrogen free bromothymol blue medium. Growth on NFB medium was indicated by

blue coloration on the medium. The diazotrophic nature of isolate is useful for the plant growth.

Detection of siderophore

Siderophores are the small iron chelating agents produced by many microorganisms. Siderophore production would be helpful for the plant growth. This property of siderophore production will also be useful for the microorganisms to survive in competitive environment (Sayyad R.Z. *et al*. 2005).

Isolate *P. aeruginosa* showed production of siderophore indicated by pink coloration in the test in presence of $FeCl_3$ reagent. So, the property of siderophore production would be helpful for the plant as plant growth promoter.

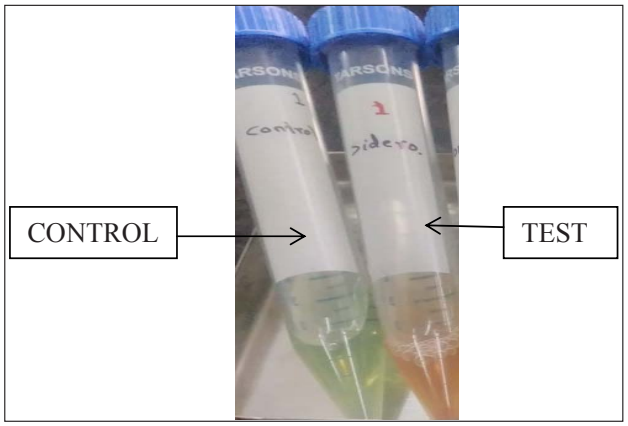


Fig. 4: Detection of siderophore production by *P. aeruginosa*

Detection of Hydrogen Cyanide (HCN) production

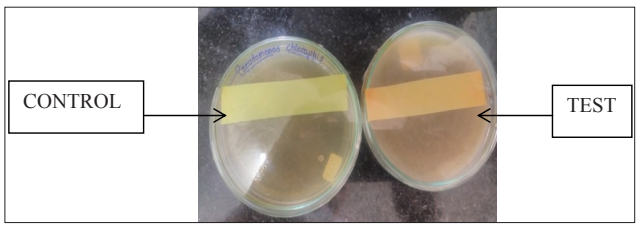


Fig. 5: Detection of HCN production by *P. aeruginosa*

Volatile compounds such as ammonia and HCN are produced by many rhizosphere strains and have been implicated as important metabolites in biocontrol. For example, some species of *Pseudomonas* can produce levels of HCN *in vitro* that are toxic to certain pathogenic fungi e.g. *Thielabiopsis basicola* and thus prevent black root-rot of tobacco (Jennifer S. William 2015).

Isolate *P. aeruginosa* showed HCN production test positive as indicated by brown colouration on the filter paper.

Detection of Indole Acetic Acid (IAA)

An important plant hormone, indole acetic acid (IAA), is also produced by many strains that exhibit biocontrol properties. IAA is normally useful ass metabolite in disease control, it is also known to stimulate the root elongation. IAA is produced by the nitrogen-fixing bacteria, *Azospirillum* and is thought to play a key role in the plant growth promoting effect that these bacteria have on germinaceous plants (Mahdiyeh Mansoori *et al.* 2013).

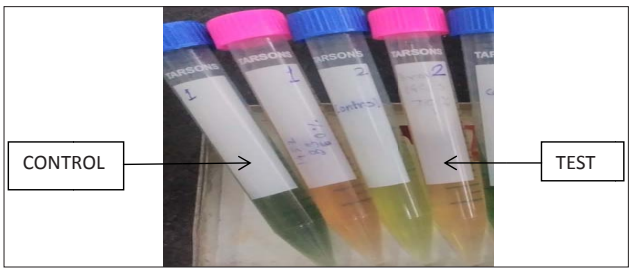


Fig. 6: Detection of IAA production by *P. aeruginosa*

Chemical oxygen demand of microbial degraded residues of cypermethrin and control after periodic time intervals

Detection of Chemical Oxygen Demand (COD) is helpful in determination of toxic component present in the sample. Minimal medium was supplemented with different cypermethrin concentrations and after different time intervals COD value was determined. It was observed that as the time of incubation was increased for every cypermethrin concentration, COD value was sound t be decreased. This is an indication of cypermetrhin degradation capacity of *P. aeruginosa.*

Table 4: Detection of percent reduction in COD

Cyperme-thrin Conc. (mg/lit)	Incubation time (Days)	Control	*P. aeruginosa*	% reduction in COD
50	2	45760	30515	33.32
	6	44760	23564	48.51
	10	44760	20480	55.25
	14	44300	18486	58.27

100	2	65560	33900	48.3
	6	64560	25890	59.9
	10	63560	22546	65.22
	14	63540	20500	67.74
150	2	85760	38320	55.32
	6	83760	28186	67.14
	10	82760	23960	71.05
	14	80760	18200	77.47
200	2	87360	65360	25.19
	6	86340	62360	27.78
	10	84350	59733	29.19
	14	84120	54000	35.81

Values expressed as mean of triplicates.

GC-MS analysis for detection of cypermethrin degradation products by *P. aeruginosa*

Gas chromatography–mass spectrometry (GC-MS) analysis is helpful in detection of metabolites, identification of unknown compounds, drug detection, , and identification of unknown samples. It can detect the presence of trace element in molecule.

In the present study, cypermethrin degradation products were detected using GC MS. Peak of first compound, GC1 (5.06 min) appeared during the cypermethrin biodegradation by *P.aeruginosa*. This compound was identified as 4,7 methanoindene, 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 identified as 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.

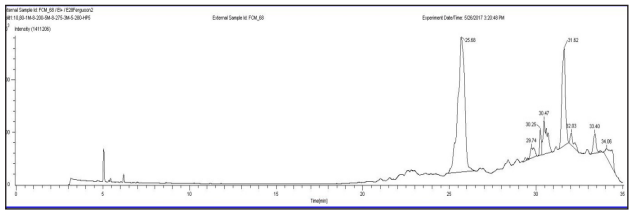


Fig. 7: GC MS chromatogram for analysis of degradation products produced by *P. aeruginosa*

CONCLUSION

The isolated organism *P. aeruginosa* showed capacity of growing at high concentration of cypermethrin indicating degradation. This organism showed plant growth promoting properties like IAA, siderophore and HCN production indicating its versatile nature in biodegradation and plant growth promotion.

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Conflict of Interest: Authors do not have any conflict of interest.

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