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Research Article

PYRETHROID-CYPERMETHRIN DEGRADATION USING SOIL MICRO FLORA AND PARAMETER OPTIMIZATION FOR THE DEGRADATION

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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 effective at very low concentration. Cypermethrin shows insecticidal activity by interfering with the nervous system functioning of insects. Generally Cypermethirin is applied in the form of sprays on the crops. Along with the beneficial effects of this pesticide, it also shows toxicity towards aquatic life, shows carcinogenic effects on human beings. So removal of the remaining amount of Cypermethrin is important and microorganisms are found to be important in removal of this pesticide from the soil.

Present study focuses on removal of Cypermethrin from the soil by using microorganisms. For this the soil samples were obtained from different farms sprayed with cypermethrin for last few years. Microorganisms capable of utilizing pyrethroid insecticide -cypermethrin were isolated from this soil by enrichment method. These microorganisms were identified by morphological, biochemical characterization and 16srRNA sequencing.

The isolated microorganism *P. aeruginosa* showed capacity of growing in presence of 200mg/L concentration of cypermethrin.Parameters such as pH, temperature and incubation time were optimized by single parameter at a time for cypermethrin degradation. The organism degraded cypermethrin as evidenced by isolation and identification of metabolite extracts by thin layer chromatography. Thus, *P.aeruginosa* was found to be versatile in degradation of pyrethroid - cypermethrin.

INTRODUCTION

India is an agro based country. But nowadays for industrialization and urbanization, maximum land is being used. Due to this there is reduction in the land surfaces available for plantation. But at the same time there is an increase in population and hence increase in demand for the quality and quantity of agricultural crops. In order to achieve this, farmers are making increased use of chemical and biological fertilizers. Also there is an increase in use of different pesticides to control the crop losses and to control the human diseases like malaria, dengue spread by mosquito bite. (Pandey and Singh, 2004)

Pesticides are used to control pests that are considered to be harmful. For example, they are used to kill mosquitoes that can transmit potentially deadly diseases like viruses, malarial parasites and number of other viral infections.(Miligiet *al.,*2006). In recent years use of cypermethrin 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). 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). It has been said that "no pesticide is perfect, but the pyrethroids come close".

Recently cypermethrin is studied extensively due to its aquatic life toxicity as well as high risk to human health. (Zhang *et al*.,2011). Cypermethrin has been classified as a possible human carcinogen by US Environmental protection agency (EPA) of the USA (Zhang *et al*.,2010) and also it has neurotoxicity, (Wolansky and Harrill,2008)**,** genotoxicity (Ansari *et al*.,2011). So, it is very necessary to develop an

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effective and rapid method for degradation and removal of cypermethrin from the environment.

Different methods like use of ozone for oxidation, photodegradation, incinerationetc. are available to remove cypermethrin from the environment. (Segal Rosenheimer and Dubowski, 2007;Xie *et al*.,2011). But these methods are found to be expensive and less effective.(Yang *et al*.,2011). Therefore microbial degradation is found to be the most significant method for removal of cypermethrin.(Fenlon *et al*.,2011).

However, to remove these chemicals from soil, biological treatment is used which involves transformation of these chemical compounds in to non-Hazardous form. (Saraswat and Gaur, 1995).References shows that different bacterial genera like *Micrococcus, Klebsiella, Serratia* etc. have capacity of cypermethrin degradation.(Grant *et al*., 2002).These organisms degrades the pesticides and uses them as sole carbon source for their energy metabolism (Baxter *et al.*, 1975).It is also found that cypermethrin affects the soil flora as well as the ammonification and nitrification processes. (Rangaswamy and Venkateswarlu, 1993). The present study aims to isolate cypermethrin degrading organisms from the soil samples, identification of isolate by biochemical characterization and 16srRNA sequencing and optimization of parameters like P^H, temperature, incubation time for cypermethrin degradation.

MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected from different sites of Brinjal cultivated sites from Baramati, Theur and Manjari, Dist. Pune. These farm fields were sprayed with cypermethrin for past few years. Soil samples were taken from the top 0-15 cm of field plots collected in sterile polythene bags with the sterile scalpel. These soil samples were then brought to the laboratory and stored at room temperature for further study.

Chemicals used

Pesticide

The cypermethrin -25EC was purchased from local market shop from Hadapsar and Swargate, Pune M.S. India.
All other solvents and reagents were purchased from Hi media and Sigma Aldrich, USA.

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

Isolation of pesticide degrading microbes

Primary screening of isolates

Microorganisms capable of cypermethrin degradation were isolated by using the enrichment technique. For this the soil samples were inoculated into nutrient broth containing cypermetrhin at a concentration of 1mg/lit, 2 mg/lit and 3 mg/lit .Broth was incubated at room temperature. After every 24hrs, sample was taken from each broth and spread plated on nutrient agar medium supplemented with cypermethrin at same concentrations as 1mg/lit, 2 mg/lit, 3 mg/lit. Individual colonies were sub cultured on nutrient agar plates containing same concentration of cypermethrin until pure culture was obtained. Microorganisms capable of growing on nutrient agar medium with different cypermethrin concentrations were selected for further study. These organisms were labeled as FCM1, FCM2; FCM3 etc. The isolated cultures were maintained at 4^0C and as glycerol stocks at -20^0C and sub cultured after every three months.

Secondary screening of isolates

Isolates obtained by primary screening were further used for secondary screening. Secondary screening was performed by checking growth of isolated organisms on minimal medium (K_2HPO_4-7g, KH_2PO_4-3g, $MgSO_4$-0.1g, $(NH_4)_2SO_4$-1g, 5mL Trace element solution) supplemented with cypermethrin. These isolates were maintained as stock cultures and working cultures were used for further study.

Checking tolerance of isolates to increased cypermethrin concentrations

Isolated organisms were grown in presence of minimal medium containing Cypermethrin as sole carbon source. These isolated organisms were checked for growth in presence of increased cypermethrin concentrations as 1mg/lit, 10mg/lit, 100mg/lit, 150mg/lit, 200mg/lit.

Morphological, Biochemical characterization and identification of isolated organism

The pure bacterial cultures obtained were identified using morphological, cultural and biochemical characteristics as described by Colins and Lyne (1985) up to the genus level. Initial identification was done by referring to Bergey's manual of determinative bacteriology. Further the isolate was identified by 16srRNA sequencing from Agharkar research institute Pune, India.

Optimization of parameters for cypermethrin degradation:

Optimization of pH

For optimization of pH, minimal medium was used with cypermethrin at a concentration of 50mg/lit as a sole source of carbon. Different pH values selected were 5, 5.5, 6, 6.5, 7, 7.5, and 8. Isolated organism *P. aeuruginosa* was inoculated in100ml minimal medium broth with cypermethrin and different pH values. Incubation was done at room temperature on rotary shaker at 150rpm for 24 hrs. After incubation, absorbance was taken at 600nm and TVC was determined from each flask by spread plate technique.

Optimization of incubation temperature

For optimization of temperature, minimal medium was used with cypermethrin at a concentration of 50mg/lit as a sole source of carbon. Different temperature values selected were 20^0c, 25^0c, 30^0c, 35^0c, 40^0c. Isolated organism *P.aeuruginosa* was inoculated in100ml minimal medium broth with cypermethrin. Incubation was done at different temperatures on rotary shaker at 150 rpm for 24 hrs. After incubation, absorbance was taken at 600nm at different temperatures and TVC was determined from each flask by spread plate technique.

Optimization of incubation time

For incubation time optimization, minimal media with 50mg/lit concentration of cypermethrin in each flask was inoculated with *P.aeruginosa* and incubation was done for 24hrs, 48hrs,

72hrs and 96 hrs. Absorbance was taken at 600nm each time interval and TVC/mL was determined by spread plate technique.

Growth kinetic studies for *P. aeuruginosa*

P. aeuruginosa was inoculated in minimal medium broth with different cypermethrin concentrations as 50mg/lit, 100mg/lit, 150mg/lit and 200mg/lit. Incubation was done at 30^0C for 48 hrs. Absorbance was determined for each cypermethrin concentration at 600nm.

Detection of cypermethrin degradation products

An isolated organism *P.aeuruginosa* was inoculated in 100ml minimal medium with cypermethrin. Incubation was done at 30^0C temperature on rotary shaker at 150rpm for 48 hrs. After incubation centrifugation was done at 10000 rpm for 10 minutes to separate pellet and supernatant. Then the supernatant was used for detection of metabolite of cypermethrin degradation by thin layer chromatography. The solvent system used was chloroform: acetic acid (95:5). Detector system used contain $K_3Fe(CN)_6$ - 1% w/v & FeCl.6H2O -1%w/v.

RESULTS

Soil sample collection

Soil samples were collected from different sites of Brinjal cultivated sites from Baramati, TheurandManjari, Dist. Pune

Isolation of pesticide degrading microorganisms

1Primary screening of isolates

Microorganisms capable of cypermethrin degradation were isolated from different soil samples. 96 different isolates were obtained by soil enrichment technique and out of them ten cultures showing growth in presence of minimal medium are used for further study.

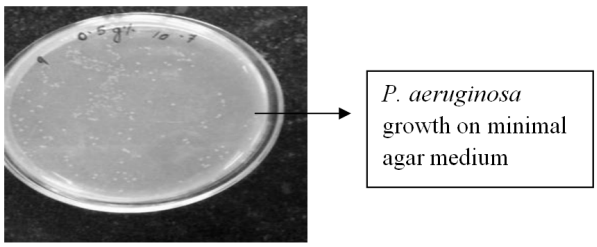


Fig 1 Growth of isolates on minimal agar plate

Secondary screening of isolates

Isolated organisms were screened by checking growth of the isolates on minimal medium containing cypermethrin as sole carbon source. Isolates were primarily labeled as FCM1, FCM2 and so on. Isolated organisms were primarily characterized by Gram staining and motility.

Table 1 Gram staining and motility characteristics of isolates

Isolate no.	Gram character	Motility
FCM1	Gram negative short rods	Motile
FCM2	Gram positive cocci	Non motile
FCM3	Gram negative rods	Motile
FCM4	Gram positive cocci in clusters	Non motile
FCM5	Gram positive roods	Non motile
FCM45	Gram positive cocci	Non motile
FCM46	Gram positive rods	Non motile
FCM49	Gram positive cocci	Non motile
FCM68	Gram negative rods	Motile

Checking tolerance of isolates to increasing concentrations of cypermethrin

Table 2 Checking tolerance of isolates to increased concentration of cypermethrin

Isolate No.	Cypermethrin concentration(mg/lit)					
	1	10	50	100	150	200
FCM1	+++	++	+	-	-	-
FCM2	+++	++	++	++	-	-
FCM3	+++	++	++	+	-	-
FCM4	+++	++	++	-	-	-
FCM5	+++	++	++	-	-	-
FCM45	+++	++	++	+	-	-
FCM46	++	++	++	+	-	-
FCM49	++	++	++	+	-	-
FCM68	+++	+++	+++	++	++	++
FCM93	+++	+++	+++	++	+	+

Key: +++ : Maximum turbidity ++ : Less turbidity
+ : Minimum turbidity - : No turbidity

Morphological characterization of isolated organism

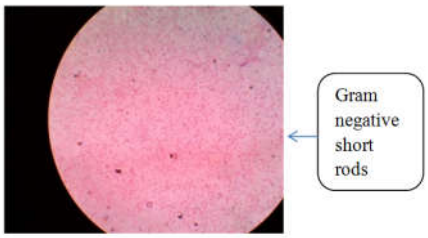


Fig 2 Gram staining photo of FCM68

Biochemical characterization

Table 3 Biochemical test characteristic for *P.aeruginosa*

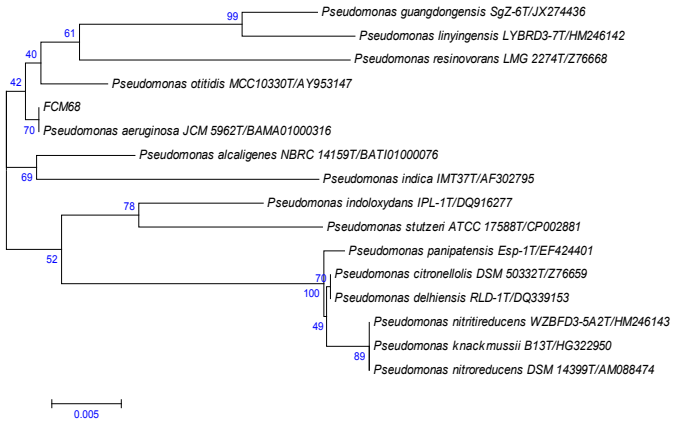
Test	Catalase	Oxidase	Gelatin hydrolysis	Nitrate reduction	Glucose utilization	OF test	Growth on cetrimide agar plate
Result	Positive	Positive	Positive	Positive	Positive	Positive	Positive

Identification of isolate FCM 68 by 16SrRNA sequencing

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

Identification report by 16SrRNA gene sequencing approach

GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCA
CTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTT
GCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTA
ACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCA
AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTA
GGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTC
AACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGT
ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGA
AATGCGTAGATATAGGAAGGAACACCAGTGGCGAAG
GCGACCACCTGGACTGATACTGACACTGAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATC
CTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCG
ACCGCCTGGGGAGTACGGCCGCAAGGTTNAAAACTC
AAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACNCGAAGAACCTTACC
TGGCCTTGACATGCTGA



Optimization of parameters for cypermethrin degradation

Optimization of pH

The *P.aeruginosa* showed maximum TVC/mL and Absorbance at 600nm at P^H near 7±0.2 indicating organisms capacity of using cypermethrin as carbon source and thus showing its degradation.

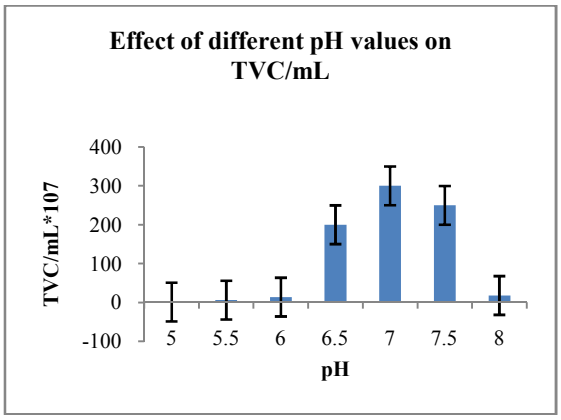


Fig 3 A) Effect of different pH values on TVC/mL

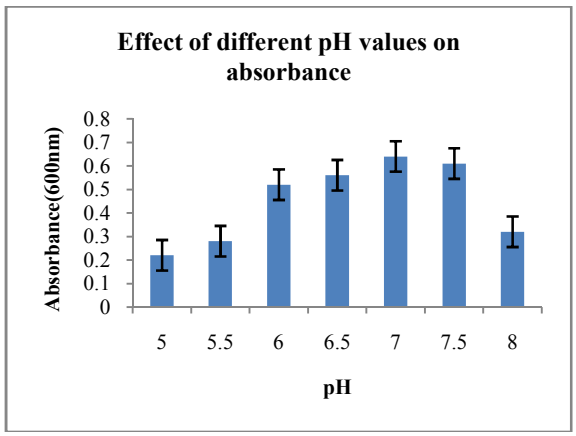


Fig 3 B) Effect of different pH values on absorbance

Optimization of temperature

The isolated organism *P. aeruginosa* showed maximum TVC/mL and Absorbance at 600nm at 30^OC of incubation temperature indicating organisms capacity of using cypermethrin as carbon source and thus showing its degradation.

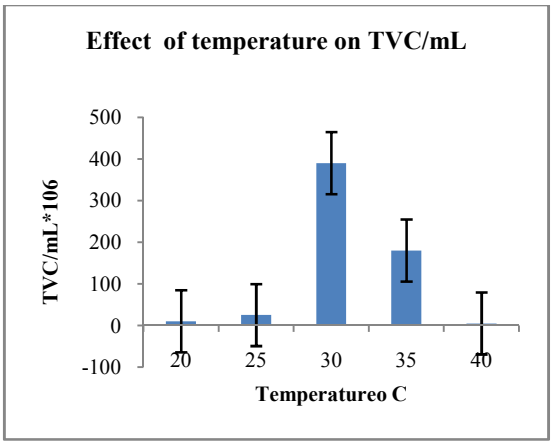


Fig 4 A) Effect of different temperature values values on TVC/mL

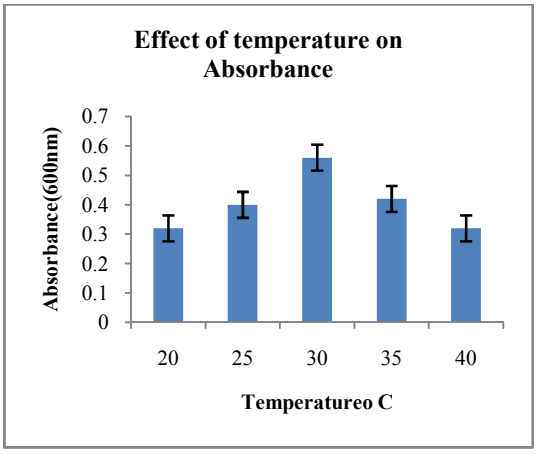


Fig 4 B) Effect of different temperature absorbance

Optimization of incubation time

The isolated *P. aeruginosa* showed maximum pesticide degradation at 48 hrs of incubation time.

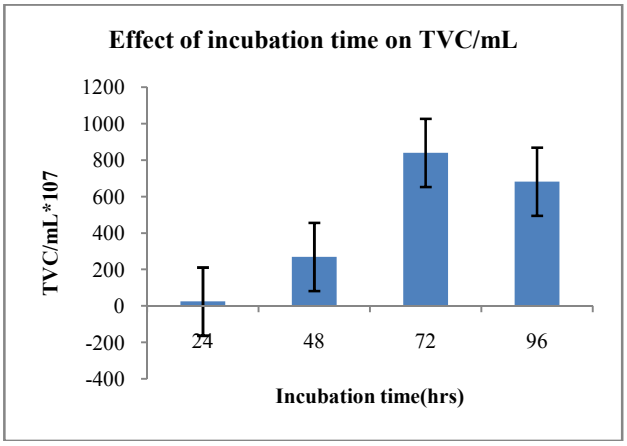


Fig 5 A) Effect of different incubation time on TVC/mL

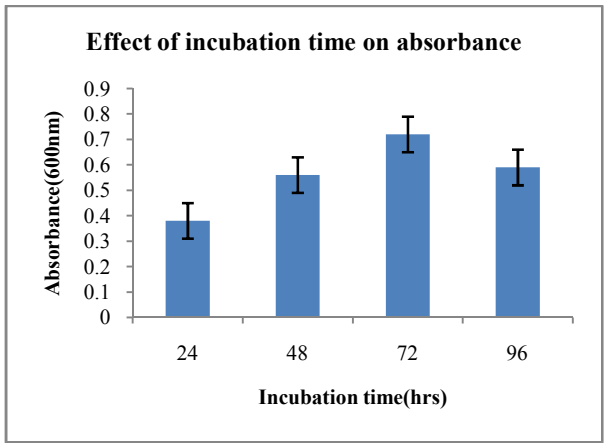


Fig 5 B) Effect of different incubation time on absorbance

Growth kinetic studies

Growthkinetic studies with *P.aeruginosa* showed increase in absorbance at 600nm as the cypermethrin concentration was increased. After 150mg/L concentration of cypermethrin, absorbance was decreased indicating that at higher cypermethrin concentration, growth rate was decreased not inhibited.

Table 4 Absorbance of *P. aeruginosa in* presence of different cypermethrin concentrations

Sr.No.	Inoculum	50mg/L (Ab600nm)	100mg/L (Ab600nm)	150mg/L (Ab600nm)	200mg/L (Ab600nm)
1	Control	0.032± 0.000472	0.039± 0.00124	0.042± 0.000816	0.049± .001247
2	*P.aeruginosa*	0.68± 0.024495	0.76± 0.020548	0.82± 0.012472	0.54± 0.012472

Value expressed as means of triplicates ±S.D

Detection of cypermethrin degradation products by Thin Layer Chromatography

Chromatographic properties

Rf values of metabolites of cypermethrin degradation by *Pseudomonas aeruginosa* are as Follows:

Table 5 Rf values of compounds separated by thin layer chromatography

Solvent system used	Isolated compound I	Control (Cypermethrin)
chloroform: acetic acid (95:5)	0.33	0.35

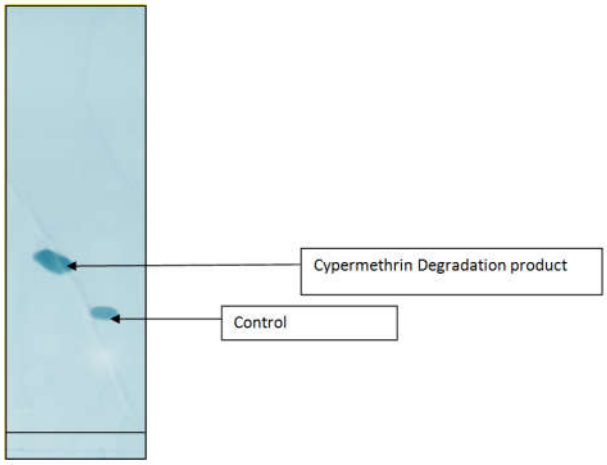


Fig 6 Thin layer chromatogram showing different spots for standard and cypermethrin degradation product

DISCUSSION

In the present study microorganisms were isolated from soil samples sprayed with cypermethrin for last few years. These isolates were screened by checking ability to grow on minimal medium containing cypermethrin as sole carbon source. Growth of organisms was studied initially in presence of 1-3mg/lit of cypermethrin. Isolate no FCM68 showed maximum growth at higher cypermethrin concentration up to 200mg/lit and hence was studied further. On the basis of morphology, biochemical characteristics as per Bergey's manual of determinative Bacteriology 9th edition and 16srRNA, the isolated organism was identified as *P. aeruginosa.*

P. aeruginosa showed good growth at higher cypermethrn concentration as indicated by increasing absorbance at 600nm and increased TVC/mL. In 1992, similar observations were reported Lee *et al.* stating that the genus *Pseudomonas* which was Gram negative, rod shaped, highly oxidative and metabolically versatile and it shows ability to degrade aromatic hydrocarbons and pesticides up to 100mg/lit concentration. It was seen from the present study that at higher concentration of cypermethrin that is above 200mg/lit, *P. aeruginosa* showed decreased growth indicated by decrease in absorbance and decreased TVC/mL. As the Similar findings were reported by Jilani and Khan, 2006 that increasing in concentration of insecticide decreases the activity of microorganisms.

The result of pH analysis indicated that the cypermethrin degradation by *P. aeruginosa* showed satisfactory degradation even at alkaline P^{H} (6.5-7.5) and was found to degrade the cypermethrin at 50mg/lit to 150mg/lit and200mg/lit effectively and less degradation was found above this concentration. 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. The similar results were obtained in this study.

During experimental study conducted in scale up process revealed *P. aeruginosa* had retained its degradation capacity at wide range of P^{H} 6.5 to 7.53.

Various researchers also showed that *P.aeruginosa* was more potent for cypermethrindegradation (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).

Kinetic studies with *P.aeruginosa* showed maximum absorbance at 200mg/lit and then absorbance has decreased. Similar findings are also made by Nilesh P. Bhosle eat.al in 2013. *P. aeruginosa* degraded cypermethrin producing end product. Some end products can be detected from TLC by comparing the Rf value of degraded product with standard compounds.

The analysis of culture extracts of *P. aeruginosa* on cypermethrin containing media by thin layer chromatography (TLC) revealed presence of degradation products.

CONCLUSION

The isolated organism *P. aeruginosa* showed capacity of growing at high concentration of cypermethrin. The microbial degradation using *P. aeruginosa* showed capacity of

cypermethrin degradation as revealed by thin layer chromatography. This is the primary stage work related to cypermethrin degradation.

Future prospects

Other parameters are to be optimized for cypermethrin degradation and the metabolites are to be found out by GC-MS. The isolated organisms are to be used in association with the natural soil microorganisms and cypermethrin degradation capacity of the isolate is to be found out. The toxicity of the degraded products is to be checked.

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