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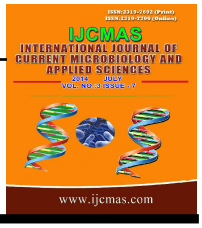

Original Research Article

Bioremediation of pesticide (Cypermethrin) using bacterial species in contaminated soil

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A B S T R A C T

In the present investigation, soil sample was collected from Kuruman Street, Thanjavur district. Some bacterial species such as *E.coli, P.aeruginosa, B.subtils* and *S.aureus* were isolated from pesticide contaminated soil by using serial dilution and Agar plating method. After isolation and identification, the bacterial colonies were maintained on nutrient agar medium and minimal medium in a petridish and test tubes. The sample were measured between 600 nm using UV – visible spectrophotometer for respective pesticide cypermethrin was estimated. Pot culture of *Vigna radiata* were performed. The morphological parameters such as Germinating ability, shoot length, root length were analysed. The growth of green gram and biochemical component was also changed. In case of cypermethrin highly increased the Protein, Chlorophyll content of green gram and also Carotenoid content. Invitro testing of cypermethrin act against *E.coli , S.aureus, P.aeruginosa* and *B.subtilis* culture, were examined. Cypermethrin cause increases the effect of plant growth and the soil components. It was natural, inexpensive, and eco – friendly microbes endowed with pesticide degrading potential could be an ecologically good alternative in detoxifying soil residues encourage the farmers to use natural pesticides rather than chemical pesticides.

Keywords

Cypermethrin, UV- visible spectro-photometer, *E.coli, S.aureus, P.aeruginosa, B.subtilis.*

Introduction

Bioremediation can be defined as any process that uses microorganisms (Bacteria, fungi), green plants or their enzymes to return the natural environment altered by contaminants of original condition. Bioremediation may be employed to attack specific soil contaminants, such as degradation of chlorinated hydrocarbons by bacteria, an example of a more general approach is the cleanup of oil spills by the addition of nitrate and sulphate fertilizers the decomposition of crude oil by indigenous or exogenous bacteria.

Pesticide have made a great impact on human health protection and preservation of foods fibre and other cash crops by controlling disease vectors and by keeping in check many species of unwanted insects and plants more than 55% of the land used

for agricultural production in developing countries uses about 26% of the total pesticides produced in the world[1]. However the rate of increase in the use of pesticides in developing countries. Pesticides are necessary to protect crops and losses that may amount to about 45% of total food production worldwide. Cypermethrin is a synthetic pyrethroid insecticide that has high insecticidal activity, low avian and mammalian toxicity, and adequate stability in air and light [2]. It is used to control many pests including lepidopterous pests of cotton, fruit and vegetable crops and is available as an emulsifiable concentrate or wettable powder. According to the label for a Ammo®2.5 E.C insecticide, which contains 2.5 pounds of cypermethrin per gallon, the product should not be applied directly to water to areas where surface water is present. Also, cypermethrin should not be applied when wind may cause beyond the intended treatment area.

Hence, the present study was carried out with the analysis of the physico - chemical parameters of Cypermethrin contaminated soil. Isolation and identification of the test organisms (*P.aeruginosa, E.coli, B.subtilis* and *S.aureus*) in contaminated soil. Identify the pesticide degrading activity of test organisms, study the effect of Cypermethrin degradation by pot experiment and to estimate the growth measurement, chlorophyll, protein level and carotenoid in experimental plant.

Materials and Methods

Sample collection

Soil samples were collected from pesticide and non pesticide contaminated field of agriculture from Kuruman Street, Orathanadu Taluk, Thanjavur District, Tamilnadu, South India.

Analysis of Physico - chemical Parameters [3]

The physic-chemical properties of the soil samples namely pH, temperature, moisture content, phosphate, magnesium and chloride were analyzed by standard methods

Isolation of Cypermethrin Degrading Bacteria

10g of soil sample was suspended in 250ml minimal medium supplemented with 0.33mg cypermethrin and incubated at 30^0C on plate form shaker at 200 rpm.

After 5 days of incubation, 5ml culture was used to inoculate into the fresh 0.35mg cypermethrin containing minimal medium. Subsequently three rounds of enrichment process was carried out in minimal medium supplemented with higher concentration of cypermethrin 0.45mg. Enriched medium was serially diluted and 0.1ml of aliquots was plated on nutrient agar plates supplemented with 0.45mg cypermethrin for the isolation of bacterial cultures. Morphologically different types of colonies were streaked for their purification on nutrient agar plates containing 0.45mg cypermethrin[4].

Identification and Growth Conditions of Isolates

The identification of bacterium exhibiting the activity of cypermethrin degradation was carried out by its morphological, physiological and biochemical features of using the Bergey's Manual of systematic Bacteriology[5].

Minimal medium with 0.4mg of Cypermethrin was inoculated in 1% seed culture of isolated strain and incubated at 30^0C under shaking conditions (200 rpm). Growth was observed by measuring

absorbance (O.D.) at 600nm.

Optical density=A/L

A= Absorbance and where L denotes thickness of sample

Enumeration of Cypermethrin utilizing bacteria

In the enumeration of cypermethrin utilizing bacteria, mineral salt medium was used in which cypermethrin act as a carbon source. The microbial strains of cypermethrin resistant bacteria were streaked in triplicates on the mineral salt media containing cypermethrin at different concentration (0.01% to 1%). After incubation, the cypermethrin utilizing colonies were isolated[6].

Growth kinetic studies of Cypermethrin resistant organisms

Growth of the isolates was determined by viable cell enumeration immediately after inoculation of various time interval such as 2,4,6 and 24 h later. Sample of bacterial culture (1ml) was drawn at regular intervals and serial dilutions ($10^{-5} – 10^{-8}$) of bacterial culture with and without addition of pesticide (control) was performed using 9 ml of sterile saline blank (0.85% NaCl: pH = 7). Appropriate dilutions of bacterial samples were plated in triplicate on nutrient agar medium. After incubation, the total viable colonies were counted using the method[7].

Effect of pesticide-planting method [8]

Effect of pesticide in the growth of plant was analyzed in pot culture experiment.

Estimation of morphometric parameters

The effect of pesticide was analyzed based on the morphometric characteristics of each treatment.

Percentage of germination[9]

The percentage of germination ability was calculated for each treatment by the following formula:

$$\text{Percentage of germinating ability} = \frac{\text{Total no. of seed germinate}}{\text{Total no. of seed shown}} \times 100$$

Root and shoot length

Plants were collected from each pot at 14th day. The length of the root and shoot was measured individually for plant and expressed in cm.

Estimation of biochemical parameters

Estimation of Chlorophyll [10]

Chlorophyll were computed using Arnon's formula .

V mg chlorophyll /g tissue = 20.2 (A_{645}) + 8.02 (A_{663}) × 1000

Where

A = absorbance at specific wavelengths.

V = final volume of chlorophyll extract in 80% acetone.

W = fresh weight of issue extracted.

Estimation of Proteins [11]

The amount of protein was calculated with a strandard graph prepared by using Bovine Serum Albumin (BSA).

Estimation of carotenoids[12]

The amount of carotenoids absorbancy was measured at 450nm in against acetone as blank.

$$\text{Carotenoids} = \frac{D \times V/10}{2500} \times \mu g ml^{-1}$$

Statistical Analysis

Random sampling was used for the entire test. The data of all values were statistically analyzed and expressed as mean ± standard deviation by using the formula [13].

$$\text{Mean } \overline{X} = \frac{\sum X}{N}$$

Where,

$\sum X$ = Sum of all values of variable

N = Number of observation

$$\text{Standard Deviation} = \sqrt{\frac{\sum (X-\overline{X})^2}{N}}$$

Where,

$\sum (X-X^2)$ = The sum of the square of the deviations of each value from the mean.

N = Number of Observation.

Results and Discussion

In the present study, bioremediation of pesticide (Cypermethrin) in contaminated soil by using the bacteria *S.aureus, B.subtilis, E.coli* and *P.aeruginosa.*

Physico- chemical Characteristics of soil sample analysis

The Physico-chemical Characteristics of soil sample revealed that the pH (7.7), Temperature (27^0 C), Moisture content (88%), Phosphate (31.6±0.0 mg), Magnesium (13.0 ±0.7 mg) and Chloride (11.5±0.5 mg) (Table 1).

Isolation of Cypermethrin degrading Bacteria

The bacterial species were isolated from pesticide contaminated soil by serial dilution method. Different bacterial colonies were observed in Nutrient Agar medium and Minimal medium. This colonies were identified by Gram's staining and biochemical tests.

Identification and growth conditions of isolates

The identification characteristics of these isolated organisms growth were observed by measuring absorbance (OD) at 600nm in *E.coil* (0.56) *S.aureus* (1.26) *P.aeruginosa* (0.62) and *B.subtilis* (1.28) respectively (Table 2).

Enumeration of cypermethrin utilizing bacteria

In this result of cypermethrin degradation of *E.coli, S.aureus,P.auroginosa* and *B.subtilis*. in *E.coli,* maximum zone of accumulate in 0.1%, 0.5%, 1.0% at 2.19±0.40, 1.95±0.3 and 0.78±0.20 respectively, followed by *S.aureus* maximum zone of accumulate in 0.1%, 0.5% and 1.0% at 2.09±0.3, 2.14±0.8, 0.67±0.15. *P.aeruginosa* maximum zone of accumulate in 0.1%, 0.5% and 1.0% at 2.06±0.3, 2.05±0.30, 1.08±0.10 and *B.subtilis* maximum zone of accumulate in 0.1%, 0.5% and 1.0% at 2.29±0.50, 1.96±0.4, 0.87±0.10 (Table 3).

Growth kinetic studies of cypermethrin resistant organisms

In the present investigation of four different colonies were observed on nutrient agar medium entriched with cypermethrin. One of the largest, most rapidly growing colonies of bacterial isolates were selected for growth kinetic study. The ability of isolated organisms to utilize and degrade cypermethrin were evaluated (Table 4).

Effect of pesticide - Planting method

The effect of cypermethrin were analyzed in

Vigna radiata for their growth. Biodegradation also studied by pot culture experiment.

Morphometric Parameters

From the experimental plants morphometric parameter germinating ability, shoot and root length were calculated at 14 th day. From the table 6 maximum germinating ability (98%) were noted in Treatment I and IV. At the same time minimum germinating ability (38%) also recorded in treatment II, V and VI. But little bit germinating ability was increased in treatment III.Over all morphometric parameter were enhanced in Treatment I and Treatment IV . Minimum growth response was occurred in Treatment II, V and VI.Shoot and root length was also calculated in all the plants. Highest shoot and root length were recorded in treatment I and IV. Lowest values were identified in treatment II, V and VI (Tabl 5).

Biochemical Parameters

Biochemical parameter such as chlorophyll content and total protein was analyzed in the all treated plants. Maximum chlorophyll content was recorded in treatment I and VI for chlorophyll (0.81 ± 0.05), (0.38 ± 0.02) followed by Protein (0.27 ± 0.01), (0.16 ± 0.01) and carotenoid content was recorded in Treatment II and III (0.39 ± 0.05) , (0.38± 0.04).Similar results were also recorded in total protein and at the same time chlorophyll content, total protein and carotenoid total content was increased in *S.aureus, E.coli, B.subtilis and P.aeruginosa* treated plants. Among the organism effectively increase in pesticide treated plants. In control pot (Treatment I) maximum seed germination was above 98% and it was reduced to 38% in Cypermethrin. Cypermethrin is a potent pesticide, and also found to have toxic effects on the seed germination of *Vigna radiata*. As the concentration was increased in the pot, significant reduction in germination was also observed (Table 6).

These study similar to the finding [14], the physico chemical properties of the soil samples namely pH, Temperature, Moisture content, Phosphate, Magnesium and Chloride were analyzed by standard methods. This study report similar to[15], contaminated soil by enrichment technique. Enrichment studies to increase the derivative properties of the mixed cultures were conducted by growing the predominant bacterial isolates. This study was correlated with [16], the isolated organisms were observed by measuring of absorbance at 600 nm in *Pseudomonas aeruginosa, E.coli, Bacillus subtilis,* and *Staphylococcus aureus*

The present study similar findings to the biodegradation of bacterium were isolated from a pesticide contaminated soil by enrichment technique with the sole carbon source and energy. Morphological, physiological and biochemical characterization of the bacterium identified as *Pseudomonas species.* Biodegradation of waste water containing cypermethrin at different concentrations like 20, 50 and 100mg/I by *Pseudomonas sp,* has maximum reduction of COD at 86.73 and 25% respectively. The cypermethrin analyzed by HPLC indicated that the isolated bacterium can be used to clean the contaminated pesticide waste water in the environment [17]. These findings similar to results of the present investigation, different bacterial genera showed different resistant capacities to various doses of the commercial insecticide, cypermethrin.

Table.1 Soil analysis

S.No	Soil Analysis	Pesticide degrading soil
1	pH	7.7
2	Temperature ^{o}C	27^{o}C
3	Moisture content	88%
4	Phosphate(mg)	31.6±0.02
5	Chloride (mg)	18.0±0.7
6	Magnesium (mg)	11.5±0.5

Values are Mean ± Standard deviation

Table.2 UV-Vis Spectrophotometer Analysis of Cypermethrin Degrading Organisms

S. No	Organisms	OD value at 600 nm
1.	*E.coli*	0.56 ± 0.36
2.	*S.aureus*	1.26 ± 0.03
3.	*P.aeruginosa*	0.62 ± 0.30
4.	*B.subtilis*	1.28 ± 0.6

Values are Mean ± Standard deviation

Table.3 Total heterophic bacterial population and cypermethrin resistance at (0.01 and 0.1%) concentration in contaminated soil

S. No	Organisms	Dilution	Total Viable counts (CFU/g)	Cypermethrin resistance bacterial counts (CFU/g)		
				Cypermethrin concentration		
				0.1%	0.5%	1.0%
1	*E.coli*	10^{-3}	5.80 ±0.30	3.53 ±0.40	2.53 ±0.3	1.10 ±0.20
		10^{-4}	4.36 ±0.50	2.53 ±1.40	2.32 ±0.2	0.80 ±0.10
		10^{-5}	3.96 ±0.45	1.40 ±0.30	1.35 ±0.6	0.66 ±0.57
		10^{-6}	3.43 ±0.45	1.30 ±0.20	1.39 ±0.03	0.56 ±0.57
2	*S.aureus*	10^{-3}	4.60 ±0.28	3.43 ±0.35	2.50 ±0.8	1.09 ±0.15
		10^{-4}	4.36 ±0.40	2.43 ±0.35	2.48 ±1.9	0.60 ±0.03
		10^{-5}	3.82 ±0.35	1.20 ±0.20	2.39 ±0.9	0.59±0.002
		10^{-6}	3.26 ±0.25	1.30 ±0.30	1.20 ±0.02	0.40 ±0.05
3	*P.aeruginosa*	10^{-3}	5.60 ±0.40	3.33 ±0.30	3.53 ±0.30	1.08±0.10
		10^{-4}	4.60 ±0.30	2.53 ±0.40	2.42 ±0.40	0.60 ±0.08
		10^{-5}	3.96 ±0.50	1.30 ±0.30	1.30 ±0.30	0.50 ±0.05
		10^{-6}	3.68±0.45	1.20 ±0.20	1.20 ±0.20	0.40 ±0.57
4	*B.subtilis*	10^{-3}	5.68 ±0.50	3.63 ±0.50	2.63 ±0.4	1.09 ±0.10
		10^{-4}	4.36 ±0.45	2.63 ±0.50	2.43 ±0.5	0.90 ±0.57
		10^{-5}	3.96 ±0.55	1.50 ±0.40	1.40 ±0.4	0.80 ±0.47
		10^{-6}	3.33 ±0.35	1.40 ±0.30	1.40 ±0.3	0.70 ±0.47

Values are Mean ± Standard deviation

Table.4 Cypermethrin resistance pattern of growth kinetic study

Bacteria	Cypermethrin Concentration		
	0.01%	0.5%	1.0%
E.coli	+	+	-
P.aeruginosa	+	-	-
B.subtilis	+	+	-
S.aureus	+	+	+

Table.5 Effect of Cypermethrin pesticide on *Vigna radiata*

S.No	Morphometric parameter	Treatment					
		I	II	III	IV	V	VI
1.	Germinating ability (%)	98	38	54	82	40	68
2.	Shoot length (cm)	7.2 ± 0.5	2.1 ±0.3	3.3 ±0.5	5 ± 0.5	3.5 ± 0.6	6± 0.7
3.	Root length (cm)	5.2 ± 0.3	1.2 ± 0.4	2.2 ± 0.2	3.4 ± 0.15	2.5 ± 0.34	3.9 ± 0.5

Treated values are represented as Mean ± Standard deviation

1. Treatment – I (Stenile soil + Seeds)
2. Treatment – II (Stenile soil + cypermethrin + Seeds)
3. Treatment – III (Sterile soil ± Cypermethrin + *B.subtilis* + seeds)
4. Treatment – IV (Sterile soil + Cypermethrin + *P.aeruginosa* +Seed)
5. Treatment – V (Sterile soil + Cypermethrin +*S.aureus* +Seed)
6. Treatment – VI (Sterile soil + Cypermethrin + *E.coli* +Seed)

Table.6 Biochemical contents of pesticide Treated *Vigna radiata* leaf sample

S.No	Morphometric parameter	Treatment					
		I	II	III	IV	V	VI
1.	Chlorophyll(mg/g $fw^{-1)}$	0.81±0.05	0.29±0.03	0.25±0.03	0.51±0.01	0.21±0.02	0.38±0.02
2.	Protein (mg/g)	0.27±0.01	0.9±0.02	0.12±0.03	0.12±0.03	0.15±0.04	0.16±0.01
3.	Carotenoid(mg/g)	0.25±0.02	0.38±0.04	0.39±0.05	0.007±0.02	1.25±0.03	0.17±0.03

Treated values are represented as Mean ± Standard deviation

1. Treatment – I (Stenile soil + Seeds)
2. Treatment – II (Stenile soil + cypermethrin + Seeds)
3. Treatment – III (Sterile soil + Cypermethrin + *B.subtilis* + seeds)
4. Treatment – IV (Sterile soil + Cypermethrin + *P.aeruginosa* +Seed)
5. Treatment – V (Sterile soil + Cypermethrin +*S.aureus* +Seed)
6. Treatment – VI (Sterile soil + Cypermethrin + *E.coli* +Seed)

The growth kinetics for the bacteria was high in cypermethrin concentration. Cypermethrin showed a higher number of counts at low concentration whereas at high concentration the number of organisms decreased or very slightly increased but no inhibition in the growth was observed when completed with the control tests [18].

The availability of these nutrients to plants helps in the formation of chlorophyll in the leaves. Increased chlorophyll 'a', 'b' and carotenoids content in green leaves with foliar application of organic solution has also been observed by [19] in rice.

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