Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **3 (1):** 109-114 (2015)

Research Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Isolation of Pesticide Tolerating Bacteria from Cultivated Soil in Kerala andthe Study of the Role of Plasmid in Pesticide Tolerance

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ABSTRACT

Pesticide degradation is the process by which pesticide is transformed into a benign substance that is environmentally compatible with the site to which it was applied. In the present work, twelve pesticide resistant bacterial isolates capable of tolerating four pesticides such as chlorpyrifos, malathion, chlordane and chlorothalonil were obtained by spot assay enrichment technique from field soil. Among them Klebsiellasps (K1, K2 and K4), Pseudomonas sps(P3) and Bacillus sps(B4) showed more resistance and were used for further studies. We found that among those organisms, the three Klebsiellasps were the most effective pesticide tolerating organisms and also found that the pesticide tolerating property was plasmid origin.

Keywords *– Pesticides, spot assay, Klebsiellasps, plasmid.*

INTRODUCTION

Kerala is an agricultural state. Various kinds of pesticides are commonly applied to agricultural fields to increase crop production and pest control. The use of pesticide in Kerala has increased many times during the last 20 years. Although the use of pesticides is considered beneficial in augmenting crop yields, excessive and indiscriminate use can lead to microbial imbalance, environmental pollution and health hazards[1]. It is necessary to develop a rapid and efficient disposal process to eliminate orminimize the concentrations of pesticides in the environment. A variety of physical and chemicalmethods are available to treat the soils contaminated with hazardous materials but many of them do not actually destroy the hazardous compounds butare bound in a modified matrix or transferred from one phase to another[2].

Microbial degradation process to detoxify pesticide contaminants can be effectively used to overcome the pollution problems. Many pesticides have proven resistant to microbial biodegradation and therefore persist in the environments in which they are found[3].

The pesticides used in this study are of four types such as chlorpyrifos, malathion, chlordane and chlorothalonil. Here clorpyrifos and malathion are organophosphate pesticides and chlordane and chlorothalonil are organochlorine pesticides. The aim of this work was to identify the potential microbial strains able to tolerate and utilize pesticides from the contaminated soil. In the present study isolation, characterization and identification of native strains of bacteria that are capable of tolerating the four pesticides was done. It also aimed to investigate the role of plasmids in pesticide tolerance.The results of thepresent study suggest that the use of potential microorganisms in the treatment system cansuccessfully overcome many of the disadvantages associated with the conventional methodused for the degradation of pesticides.

MATERIALS AND METHODS

The soil samples were collected from different field area of central Kerala. For the isolation of chlorothaloniltolerating bacteria, the soil sample was collected from paddy field at Alagappanagar (soil sample 1), for the isolation of chlordane degrading bacteria the soil sample was collected from paddy

field at Vendor (soil sample 2) and for the isolation of malathion and chlorpyrifos degrading bacteria, the soil sample was collected from vegetable cultivating field at Alagappanagar (soil sample 3 and 4). These fields had been already sprayed with the pesticides for the past few years. Soils were collected from the 2-5mm surface and processed to remove lumps and debris. The soil samples were transferred to sterile polythene bags and readily brought to the laboratory for analysis.

For the isolation of pesticide tolerating bacteria, 1gm of each soil sample was added to fourdifferent 150 ml conical flasks containing30 ml of sterile liquid Mineral Salt Medium (MSM) with 10 ppmofchlorpyrifos, chlorothalonil, malathion and chlordane respectively. All the above flasks were incubated at 28^0C for seven days on static conditions for two successive enrichment processes. From every flask a loopful of culture was streaked on sterile Nutrient agar (NA) plate, MacConkey agar (MA) plates and Eosin-Methelyne Blue agar (EMB) (all Hi-Media, India) plates and the plates were incubated at 28^0C for 24 hours to get isolated colonies of bacteria. The well isolated,pure colonies weremaintained as stock cultureson sterile nutrient agar slants. The colonies were identified based on the morphology and biochemical reactions.

The effect of various pesticides on the growth of bacterial isolates (twelve isolates) was performed using spot assay[4].The pesticide tolerating activity was determined by cross checking the organisms with varying concentrations of pesticides (10, 20, 40. 60, 80 and 100 ppm) on sterile Nutrient agar plates and incubated at 37^0C for 24 hours. By this method highly pesticide resistant bacterial isolates were determined.

Presence of plasmid was confirmed by extracting them from the four most pesticide tolerating bacterial isolatessuch as *Klebsiellasps* (K1*), Klebsiellasps* (K2), *Klebsiellasps* (K4) *and Pseudomonas sps* (P3)[5]. Curing of plasmid was done[6] for proving the role of plasmid in pesticide tolerance. Ethedium bromide at different concentration (75µg/ml, 100µg/ml and 125µg/ml) was used as curing agent. Plasmid curing was confirmed and the role of plasmid in pesticide tolerance was also done.

RESULT AND DISCUSSION

Morphologically distinguishable twelve bacterial colonies were observed. (Table 1, 2 and 3).

Table 1. Cultural characteristics of isolated microorganisms

Soil sample	Isolate No	Cultural characteristic		Isolated Organism
		On Nutrient Agar (NA)	On MacConkyAgar (MA)	
Sample No 1 with chlorothalonil	1	Golden yellow coloured, round, shiny smooth, opaque, convex colonies on nutrient agar.	No growth on MacConkey agar.	*Staphylococcus sps*
	2	Round, creamy white, moist, smooth, opaque colonies on nutrient agar.	Lactose fermenting pink coloured pin pointed round regular colonies on Mac Conkey agar.	*Escherichia coli*
	3	Large, mucoid, raised colonies on nutrient agar.	Large, mucoid, lactose fermenting pink coloured colonies on MacConky agar.	*Klebsiellasps*
Sample No 2 with chlordane	4	Small, light green coloured, flat colonies on nutrient agar.	Non lactose fermenting colourless colonies on MacConkey agar.	*Pseudomonassps*
	5	Large, white coloured smooth, irregular colonies on nutrient agar.	Non lactose fermenting colourless colonies on MacConkey agar.	*Bacillus sps*
	6	Golden yellow coloured, round, shiny, smooth, opaque, convex colonies on nutrient agar.	No growth on MacConkey agar.	*Staphylococcus sps*
	7	Large, mucoid, raised colonies on nutrient agar.	Large, mucoid, lactose fermenting pink coloured colonies on Mac Conkey agar.	*Klebsiellasps*

Sample No 3 with malathione	8	Large, white coloured smooth, irregular colonies on nutrient agar.	Non lactose fermenting colourless colonies on MacConkey agar.	*Bacillus sps*
	9	Round, creamy white, moist, smooth, opaque colonies on nutrient agar.	Lactose fermenting pink coloured pin pointed round regular colonies on Mac Conkey agar.	*Escherichia coli*
	10	Small, light green coloured flat colonies on nutrient agar.	Non lactose fermenting colourless colonies on MacConkey agar.	*Pseudomonassps*
Sample No 4 with chlorpyrifos	11	Large, white coloured smooth, irregular colonies on nutrient agar.	Non lactose fermenting colourless colonies on MacConkey agar.	*Bacillus sps*
	12	Large, mucoid, raised colonies on nutrient agar.	Large, mucoid, lactose fermenting pink coloured colonies on MacConkey agar.	*Klebsiellasps*

Table 2 - Motility and staining properties of isolated microorganisms

Isolate No	Gram	Capsule	Endospore	Granule	Motility
1	+	-	-	-	+
2	-	-	-	-	+
3	-	+	-	-	-
4	-	-	-	-	+
5	+	-	+	-	+
6	+	-	-	-	+
7	-	+	-	-	-
8	+	-	+	-	+
9	-	-	-	-	+
10	-	-	-	-	+
11	+	-	+	-	+
12	-	+	-	-	-

+ positive reaction, - negative reaction

Table 3- Biochemical reactions of isolated microorganisms

Isolate No	Indole	Methyl	Vogues Proskauer	Citrate	TSI	Mannitol	Urease	Nitrate reduction	Glucose	Lactose	Sucrose	Maltose	Catalase	Oxidase
1	-	-	+	-	A/A	-	+	-	+	+	+	+	+	-
2	+	+	-	-	A/A	+	-	+	+	+	+	+	+	-
3	-	-	+	+	A/A	+	+	+	+	+	+	+	+	-
4	-	-	-	+	K/K	+	+	+	+	-	-	-	+	+
5	-	-	+	+	K/K	-	-	+	+	-	-	-	+	-
6	+	+	-	-	A/A	+	-	+	+	+	+	+	+	-
7	-	-	+	+	A/A	+	-	+	+	+	+	+	+	-
8	-	-	+	+	K/K	-	-	+	+	-	-	-	+	-
9	+	+	-	-	A/A	+	-	+	+	+	+	+	+	-
10	-	-	-	+	K/K	+	+	+	+	-	-	-	+	+
11	-	-	+	+	K/K	-	-	+	+	+	-	-	+	-
12	-	-	+	+	A/A	+	-	+	+	+	+	+	+	-

TSI - Triple sugarironagartest,K/K- Alkaline slant and alkaline butt, A/A- Acid slant and acid butt, + positive reaction, - negative reaction

After spot assay it was found that *Klebsiellasps.*(K1, K2 and K4) were able to tolerate chlorothalonil, chlordane and Malathion with 100 ppm concentration, but *Pseudomonassps* (P3) showed tolerance of Malathionupto 80 ppm and *Bacillus sps* (B4) was also able to tolerate chlorpyrifos, upto 80 ppm (Table 4). The growth of bacteria in the pesticide containing medium demonstrated that the pesticide molecule had been used as a carbon source by them. But growth observed at a particular concentration pointed out that the excessive amount of pesticide has inhibitory effect on bacteria[7].

Table 4.Effect of various pesticides on bacterial isolates

Pesticide	Concentration (ppm)	Organisms											
		S1	**K1**	**E1**	**P2**	**B2**	**S2**	**K2**	**B3**	**E3**	**P3**	**B4**	**K4**
Chlorothalonil	10	+	+	+	+	+	+	+	+	+	+	+	+
	20	+	+	+	+	-	+	+	-	+	+	-	+
	40	-	+	-	+	-	-	+	-	-	+	-	+
	60	-	+	-	-	-	-	+	-	-	-	-	+
	80	-	+	-	-	-	-	+	-	-	-	-	+
	100	-	+	-	-	-	-	+	-	-	-	-	+
Chlordane	10	+	+	+	-	+	+	+	+	+	-	+	+
	20	+	+	+	-	+	+	+	+	+	-	-	+
	40	-	+	-	-	+	-	+	-	+	-	-	+
	60	-	+	-	-	+	-	+	-	+	-	-	+
	80	-	+	-	-	-	-	+	-	-	-	-	+
	100	-	+	-	-	-	-	+	-	-	-	-	+
Malathion	10	+	+	+	+	+	+	+	+	+	+	+	+
	20	-	+	-	+	-	-	+	-	+	+	-	+
	40	-	+	-	+	-	-	+	-	-	+	-	+
	60	-	+	-	+	-	-	+	-	-	+	-	+
	80	-	+	-	-	-	-	+	-	-	+	-	+
	100	-	+	-	-	-	-	+	-	-	-	-	+
Chlorpyrifos	10	+	+	+	-	+	+	+	+	+	-	+	+
	20	+	+	+	-	+	+	+	+	+	-	+	+
	40	-	+	-	-	+	-	+	+	-	-	+	+
	60	-	-	-	-	+	-	-	+	-	-	+	-
	80	-	-	-	-	-	-	-	-	-	-	+	-
	100	-	-	-	-	-	-	-	-	-	-	-	-

Amongst the five highly pesticide tolerating isolates, presence of plasmid DNA was detected in *Klebsiellasps* (K1, K2, and K4) and *Pseudomonas sps*.(Plate 1).These results strongly suggested that genes responsible for the ability to metabolize clorothalonil, clordane and malathione might be plasmid mediated. Previously similar results were obtained by some andthey found out the role of plasmid in the degradation of organic compounds[7].

The presence of plasmid having pesticide tolerating activity was confirmed by curing of plasmid. Cured cells were achieved by treating with various concentration of ethidium bromide (Table 5).

Table 5 – plasmid curing capacity of four more pesticide resistant isolated microorganisms

Isolates	Concentration of Ethidium Bromide (µg/ml)	curing of plasmids
Klebsiellasps (K1)	75	+
	100	+
	125	-
Klebsiellasps(K2)	75	+
	100	+
	125	-

Klebsiellasps(K4)	75	+
	100	+
	125	-
Pseudomonas sps(P3)	75	+
	100	-
	125	-

\+ Presence of growth

It was found that, in *Klebsiellasps* (K1, K2 and K4) plasmid curing was achieved with 125 µg/ml of ethidium bromide. In *Pseudomonas sps* (P3) cured cells were achieved with 100µg/ml of ethidium bromide. The cured colonies again rechecked for showing the presence of plasmid and pesticide tolerance property (Plate 2). But they lost the pesticide resistance property and were not able to grow on media containing pesticide. This loss of the plasmid can be correlated with the concomitant loss of pesticide resistance[6].

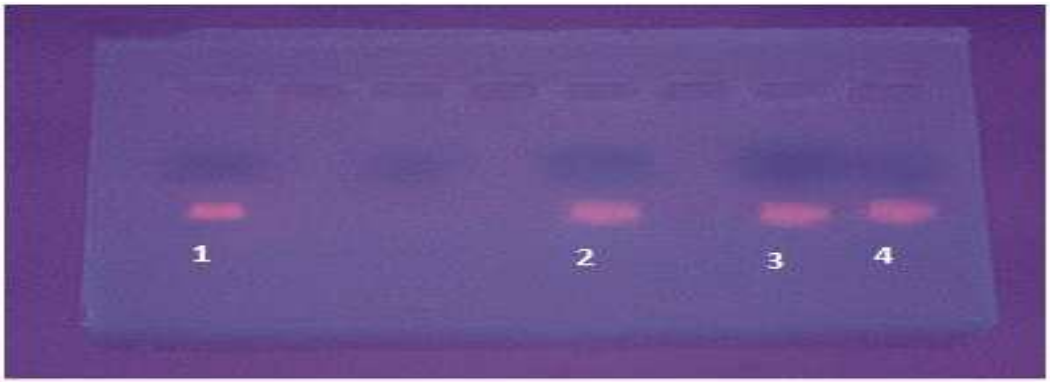

Plate 1. Extraction of plasmid [Lane1: *Pseudomonas sps* (P3),
Lane 2, 3 & 4: *Klebsiellasps* (K1, K2 & K4)]

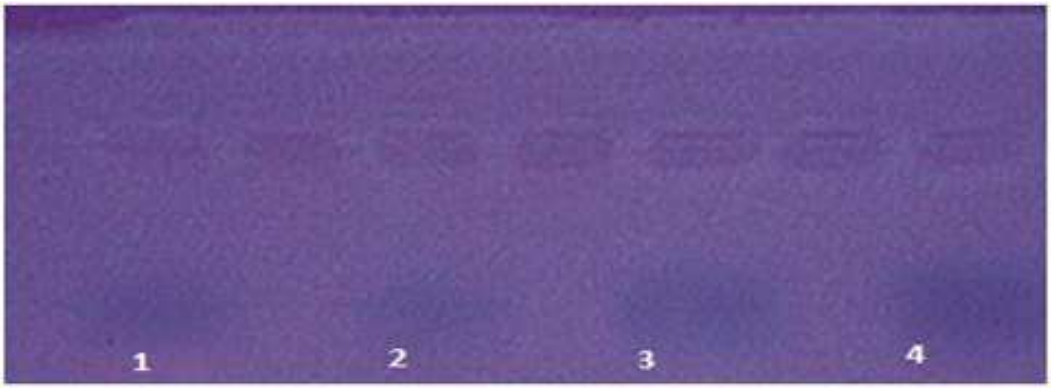

Plate 2. After curing of plasmid[Lane1: *Pseudomonas sps* (P3),
Lane 2, 3 & 4: *Klebsiellasps* (K1, K2 & K4)]

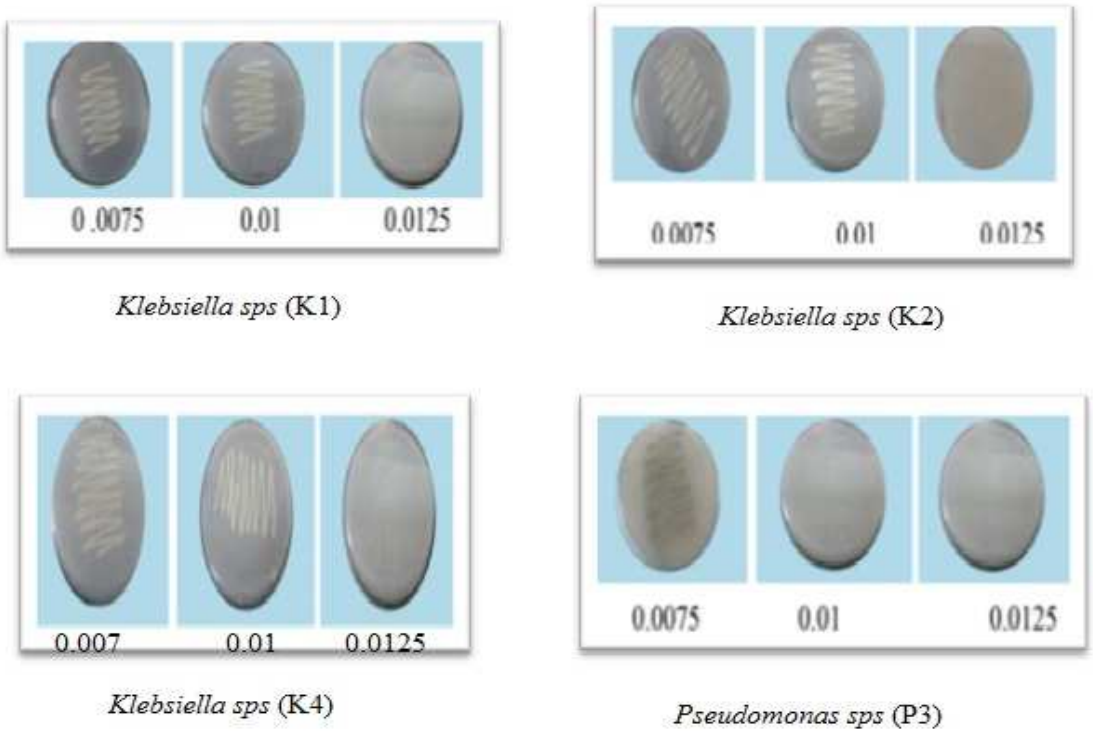

Plate 3- Curing of plasmid using Ethidiumbromide(Con. in µg/ml)

CONCLUSION

The results of the present study suggest that the bacterial isolates are able to grow in medium in the presence of added pesticides and may therefore be used for bioremediation of pesticide contaminated soil. The presence of plasmid DNA was detected in four isolates indicating their role in resisting toxic effect of pesticides and confirmed that the pesticide tolerance property is plasmid mediated.

Our results indicated that these bacterial isolates could be a good choice for the bioremediation of pesticides contaminated water and soil.

REFERENCES

1. Köhler, H.R. and Triebskorn, R.WildlifeEcotoxicology of Pesticides: Can we track effects tothe population level and beyond? Science., **341**: 759-765(2013)
2. Murugesan, A. G. Jeyasanthi, T. and Maheswari, S. Isolation and characterization of cypermethrinutilizingbacteria from Brinjal cultivated soil. *Afr. J. Microbiol. Res.*, **4(1)**: 010-013 (2010)
3. KaviKarunya,S. and Saranraj, P. Toxic Effects of Pesticide Pollution and its Biological Control by Microorganisms: A Review. *Appl.J. Hygiene.,* **3(1):** 01-10(2014)
4. Sayali, R.Naphade, A. Annika, A.MeetaBhot, Jossy Varghese and Naresh Chandra. Isolation, characterization and identification of pesticide tolerating bacteria from garden soil. *Euro. J. Exp. Bio.*, **2(5)**: 1943-1951 (2012)
5. Ajaz, M. Jabeen,N.AkhtarS.and Rasool,S.A. Chlorpyrifos resistant bacteria from Pakistani soil: Isolation, identification, resistance profileand growth kinetics. *Pak J Bot.*, **37(2)**: 381-388 (2005)
6. Zaman, M. A. Pasha, M. H. and Akher, M. Z. Plasmid curing of *Escherichia coli* cellswith Ethidium Bromide, SodiumDodecyl Sulfate and Acridine Orange. *Bangladesh J.Microbiol,*. **27**: 28-31 (2010)
7. AsgharIshaq, Junaid Ahmed Khan and Nuzhat Ahmed. Biodegradation of pesticide α- Cyano, 3-Phenoxybenzyl-2,2 Dimethyl 3 (2,2,Dichlorovenyl) by *Pseudomonas aeruginosa* species. *Pakistan J. Agric.Res.,* **15**: 16 – 27 (1994)