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European Journal of Experimental Biology, 2012, 2 (5):1943-1951

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Isolation, characterization and identification of pesticide tolerating bacteria from garden soil

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ABSTRACT

Pesticides are the substances intended for preventing, destroying, repelling any pest. Due to bulk handling or accidental release, they are accumulated in soil which leads to occasional entry into ecosystems that show lethal effects on living systems. In order to overcome the problem of commonly found pesticides namely Endosulfan, Chlorpyrifos and Cypermethrin. An enrichment culture technique was used to isolate bacterial strains from garden soil tolerating high concentrations of the selected pesticides. Five pure bacterial cultures, named EC1, EC2, EC3, EC4 and EC5 were isolated and subsequently characterized by 16S rRNA gene sequencing and biochemical tests. The optimum temperature, pH and NaCl concentration was determined for them. Growth curve experiments showed that the bacterial isolates were able to grow in medium containing the individual pesticide as the carbon source. Tolerance to high levels of metal salts (Co^{2+}, Ni^{2+}, Cr^{3+}, Cu^{2+} and Mn^{2+}) and multiple antibiotic resistance was seen in all five bacterial isolates indicating a positive correlation between pesticide degradation and tolerance to metals and antibiotics. By miniprep method of plasmid DNA isolation it was found out that 3 isolates (EC3, EC4 and EC5) showed presence of plasmid DNA indicating that the resistant trait observed was plasmid borne. Further by transformation of the plasmid DNA from EC3, EC4 and EC5 to Escherichia coli DH5α strain, it was found that the transformed strain acquired the ability to grow in presence of higher concentrations of pesticides. Thus such findings may be useful in designing a multi resistant bacterium that can be used to return the altered environment to its original condition.

Keywords: Enrichment, pesticides, heavy metals, multiple antibiotic resistance.

INTRODUCTION

Agriculture is the lynchpin of the Indian economy. Ensuring food security for more than 1 bn Indian population with diminishing cultivable land resource necessitates use of high yielding variety of seeds, balanced use of fertilizers and judicious use of quality pesticides. Pesticides are the chemical substances that kill pests like fungi, insects, worms, and nematodes etc. which cause damage to field crops. The excessive use of pesticides leads to an accumulation of a huge amount of pesticide residues in the food chain and drinking water environment that further leads to a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds. Bioremediation constitutes an attractive alternative to physico-chemical methods of remediation, as it is less expensive and can selectively achieve complete destruction of organic pollutants [2]. In bioremediation, microbes that can degrade the pesticides *in situ* are used. For a successful bioremediation technique an efficient bacterial strain that can degrade largest pollutant to minimum level is required.

The current study involves use of three most widely used pesticides namely Endosulfan, Chlorpyrifos and Cypermethrin. Endosulfan [IUPAC name: 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6,9-methano-2, 3, 4-benzo-dioxathiepine-3-oxide CAS Number: **1**15-29-7, an organochlorine pesticide, is a broad spectrum contact insecticide widely used in pest control of crops including cereals, fruit, oil seeds, potato, tea and vegetables. Technically Endosulfan is a 7:3 mixture of stereoisomers, designated as α and β which are conformational isomers arising from the pyramidal stereochemistry of sulfur. Endosulfan is extremely toxic to fishes and other aquatic organisms and demonstrates a range of chronic effects, including genotoxicity, reproductive and developmental effects. Endosulfan is persistent in the environment and biomagnifies in terrestrial food chains.

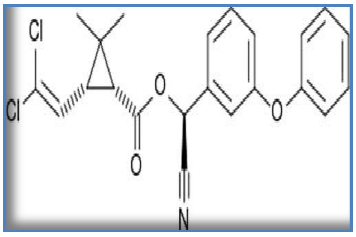
Chlorpyrifos is a broad-spectrum, chlorinated organophosphate (OP) insecticide, acaricide and nematicide displaying insecticidal activity against a wide range of insect and arthropod pests. Chlorpyrifos is the common name for the chemical 0,0-diethyl 0-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate. The Chemical Abstracts Service CAS number is 2921-88-2. Chlorpyrifos is usually soil applied whereas in the application of endosulfan to tomato plants one part of the insecticide reaches the target, while the other is deposited on the soil. Therefore, both pesticides are found in the soil, where they are subjected to different processes that will determine the fate of these agrochemicals [4,5,16] and endosulfan in soil [10,12].

Cypermethrin (IUPAC name: alpha cyano – 3 - phenoxybenzyl - 3- (2, 2-dichloro-vinyl) - 2, 2-dimethylcyclopropane – carboxylate; Molecular formula: $C_{22}H_{19}Cl_2NO_3$ CAS Number: 52315-07-8] the third pesticide used in current study occurs as a mixture of both the *cis* and *trans* isomers. The *cis/trans* ratio in technical grade cypermethrin is 1:1. It is an example of synthetic pyrethroid that is used to control many pests including lepidopterous pests of cotton, fruit and vegetable crops in commercial agricultural applications as well as in consumer products for domestic purpose.

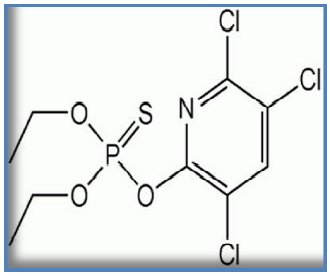
India is regarded as being the world's largest producer and user with more than 60 endosulfan manufacturers and formulators, mostly the latter. Production began in 1996 and by 2004 India had become the leading producer of endosulfan, with three companies— Coromandel Fertilisers Ltd, Excel Crop Care, and Hindustan Insecticides Ltd. Another pesticide that is considered for studies –Chlorpyrifos is fourth highest consumed pesticide in India.

Microorganisms demonstrate considerable capacity for the metabolism of these pesticides. Although they are capable of catalyzing similar metabolic reactions as mammals and plants, they possess the unique ability to completely mineralize many aliphatic, aromatic, and heterocyclic compounds [14]. Hence, isolation of indigenous bacteria capable of metabolizing pesticides has received considerable attention thus providing an environmentally friendly method of *in situ* detoxification [18].

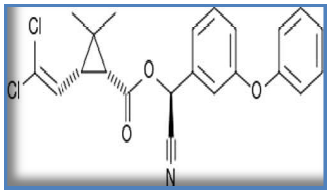
Endosulfan



Chlorpyrifos



Cypermethrin



MATERIALS AND METHODS

Pesticides used:

The commercial grade pesticides namely Endosulfan (35% E.C.Bayer-Thiodan), Chlorpyrifos (21.5% E.C.Godrej-Chlorvip) and Cypermethrin (10% E.C. Godrej- Cypervip) were obtained from Pathare Nursery**,** kalyan, Dist. Thane, Maharashtra. The commercial grade pesticides were used throughout the experimental work as it may closely resemble the active compound that the microbes are likely to be exposed to in soil environment.

Soil:

The soil used for the enrichment and isolation of pesticide tolerating bacteria was obtained from the local garden area of Kalyan.

Media:
1gm of soil sample was put into a 150ml flask containing 30ml of sterile liquid Mineral Salt Medium (MSM) with 10ppm of chlorpyrifos was used for isolating chlorpyrifos –degrading bacteria. The MSM has the following composition in (g/L): KH_2PO_4, 4.8; K_2HPO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4.7H_2O$. 0.2; Ca $(NO3)_2.4H_2O$, 0.04; and $Fe(SO_4)_3$, 0.001 with pH 7.0 [5]. The endosulfan - degrading bacteria were isolated by using Sterile nutrient culture medium (FTW) and non-sulfur nutrient culture medium (NSM) supplemented with 10ppm of endosulfan. The selected FTW medium comprised of (in g/L): K_2HPO_4, 0.255; KH_2PO_4, 0.255; $(NH_4)_2SO_4$, 0.255; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.005 and $FeCl_2.4H_2O$, 0.005 blended with 1 ml of trace elements solution. The Focht trace element solution contained (in mg/L): 169 $MgSO_4.H_2O$, 288; $ZnSO_4.7H_2O$, 250; $CuSO_4.5H_2O$, 26; $NiSO_4.6H_2O$, 28; $CoSO_4$ and $Na_2.MoO_4.2H_2O$, 24. For isolating cypermethrin degrading bacteria, the soil sample was added in Sterile Minimal Medium with 10ppm of cypermethrin incorporated into it. The medium has the composition of (in g/L): K_2HPO_4, 10.5; KH_2PO_4, 4.5; $NH_4SO_4.2H_2O$, 1.0; $MgCl_2.7H_2O$, 1ml from autoclaved stock of 200gm/ml, Vitamin B1 (Thiamin), 0.5ml from 1% stock and Cypermethrin, 10ppm. All the above flasks were incubated at 28±2 ^{0}C for 7days on static conditions (1st enrichment). From every flask, 5ml was re-inoculated to the flask with same medium composition aseptically and further incubated at 28± 2 ^{0}C for 7days on static conditions (2nd enrichment).Then from every flask a loopful of culture was streaked on Sterile Nutrient agar plate and the plates were incubated 28± 2 ^{0}C for 48hours to get isolated colonies of bacteria. The well isolated colonies were grown on sterile nutrient agar slants as pure cultures and maintained at 10^0 C as stock cultures. The colony characters were identified based on the colony morphology and staining characters.

Identification of bacterial isolates:
The isolates were subjected to morphological, cultural and biochemical studies which included Gram staining, Motility by Hanging Drop technique, Special staining – Lipid granule (Burdon's), Metachromatic Granule (Albert's), Endospore (Schaffer and fulton's) and Capsule (Manewal's) staining. Standard Biochemical tests included Indole, Methyl red, Vogues Prousker and Citrate Test, TSI slant, Nitrate Reductase, Urease and 1% sugar solutions of Sucrose, Glucose, Lactose, Xylose, maltose and Mannitol with Andrade's indicator. The total of 5 bacterial isolates were identified by 16$ rRNA sequencing at NCCS, Pune.

Effect of environment on bacterial growth:
The optimum temperature, *pH* and NaCl concentration for every bacterial isolate was determined by inoculating the pure cultures in Nutrient broth. For effect of temperature, the inoculated tubes were incubated at 10^0 C, R.T. (28±2^0 C), 37^0 C and 55^{0}C for 24hours and checked for growth in the form of turbidity. The uninoculated sterile nutrient broth tube was kept as negative control. For effect of pH, sterile nutrient broth with pH 3.0, 5.0, 7.0, 9.0 and 11.0 were used whereas for effect of NaCl, sterile nutrient broth with 0.5, 3.5.6.5, 9.5, 12.5 and 15.5% NaCl was used. All sets were performed in triplicates. The effect of aeration was studied on all 05 bacterial isolates by growth curve method (in presence and absence of every pesticide) at static and shaker conditions (120rpm).

Antibiotic sensitivity Test by Disc Diffusion method-
All the bacterial isolates were tested for their sensitivity to different antibiotics by means of Kirby –Bauer Disc diffusion method [10]. The following antibiotics (all from Hi-media, India) were used: Gentamycin (G), Ampicillin (A), Vancomycin (Va), Sulfasomidine (Sf), Chloramphenicol(C), Tetracycline (T), Streptomycin (S), Penicillin (P), Erythromycin(E), Ciprofloxacin (Cf), Aztreonam (AT), Mecillinam (MEC), Trimethoprim (TR), Doxycycline hydrochloride (Do) and Carbenicillin(CB).

Effect of metal salts and pesticides on growth of isolates:
For metal resistance profile, overnight grown cultures of bacterial isolates were inoculated in Sterile Nutrient broth with different concentrations (%) of metal salts and the tubes were incubated at R.T. for 24hours. One positive and one negative control were also run parallel. Growth in the form of turbidity was recorded and Minimum Inhibitory concentration (MIC) was determined [8,23]. Metal salts used in this study were Cobalt Chloride, Nickel Sulphate, Potassium Chromate, Copper Sulphate and Manganese Chloride. Similarly, the MIC of individual pesticide was determined by performing spot Assay on Sterile Nutrient agar plate containing different concentrations of individual pesticide, the plates were incubated at R.T. for 24hours and growth was observed

Isolation of plasmid DNA and Agarose Gel Electrophoresis:
Presence of plasmid DNA was confirmed by isolating plasmids from the pesticide tolerating bacteria by Miniprep method [9]. Pure cultures of all 05 isolates were grown overnight in 25 ml of Sterile Luria-Bertani broth (Hi-Media) and cell pellet was harvested by centrifugation at 6000rpm/10minutes/16^0 C. The cells were suspended in 100 µL of ice cold Solution-I (50mM glucose,25mM Tris-Cl,10mM EDTA , pH 8.0); 200 µL of Solution –II (0.2 N NaOH,1%SDS) and150 µL of Solution-III (8M Potassium Acetate). After proper mixing the tubes were kept on ice for 5 minutes ensuring complete suspension of the cell pellet. Equal volume of PCI (Phenol+ Chloroform+ Isoamyl

alcohol in the ratio 25:24:1) was added, mixed and centrifuged at 10,000rpm/10minutes/16^0 C. After centrifugation, the aqueous phase (that contains plasmid DNA) was separated from organic phenol phase. Double volume of ice cold absolute alcohol and 1/10th of the total volume of 3M sodium acetate was added in this aqueous phase and kept at R.T. for 15minutes. For the precipitation of plasmid DNA, the tubes were kept at 4^0 C for 48 hours. After precipitation step, the tubes were centrifuged at 12,000rpm/20 minutes/16^0 C. The supernatant was discarded and 500 µL of 70% ethanol was added, mixed properly and centrifuged at 12,000rpm/10minutes/R.T. The supernatant containing ethanol was discarded and the pellet was dried at R.T. to allow complete evaporation of ethanol residue from the pellet, the pellet was further suspended in 20 µL of TE buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris, pH 8.0) and was further analyzed by agarose gel electrophoresis or stored at -20°C until ready for use. Two most commonly used restriction endonulceases Hind III and EcoRI were used for digestion analysis of the extracted plasmid DNA samples as per the following protocol (Banglore GeNei Kit). λ /*MuI* molecular weight marker was also run parallel on agarose gel electrophoresis.

Plasmid DNA	20 µL
2X Assay Buffer	25 µL
EcoRI/ HindIII	3 µL

The reaction mixture is incubated at 37^{0}C for 90minutes

↓

Add 5 µL of gel Loading Buffer

↓

Samples are run on 1% agarose gel by electrophoresis at 60volts for 60minutes

Agarose gel electrophoresis: These plasmids extracted were characterized by agarose gel electrophoresis according to the standard procedure [1,20]. For this 50 mL of 1% agarose was prepared by melting it in boiling water bath and 1µL of Ethidium bromide (10mg/ml) was added when temperature dropped to 45^{0}C. The molten agarose was poured after assembling the gel-casting tray with comb at one end (near cathode) and allowed to set. 1 x TAE buffer was poured in the tank (Genei- Banglore) to sub-merge the gel. 15 µL of the sample (mixed with Gel Loading Buffer) was loaded in the wells and electrophoresis was carried out at 60 volts for 60-90 min. Thereafter, gel was removed and examined over UV transilluminator (Technosource –Gloworm-2) for observing the plasmid DNA bands. The stained DNA bands were observed and the fluorescent profile was photographed by gel documentation system (Bio-Imaging Systems- Gel capture software)

Transformation:

Isolated plasmids carrying the degrading gene (s) from the 03 isolates, were strongly suggested to be responsible for tolerance to higher concentrations of the pesticides used (Endosulfan, chlorpyrifos and Cypermethrin). Therefore, the plasmid DNA was transferred to *E. coli* DH5α strain by transformation ($CaCl_2$-Heat Shock method). 100 µL of the plasmids were transferred into *E. coli* DH5α competent cells. After transformation the cells were plated on sterile Luria Bertani (LB) agar plates containing 8,000ppm of Endosulfan , 20,000ppm of Chlorpyrifos and 2000ppm of Cypermethrin and allowed to grow for 18-24 hrs at 37ºC. Cell Control and DNA Control plates were performed parallel to confirm the transformation [12,16].

RESULTS AND DISCUSSION

Identification and taxonomic characterization of pesticide tolerating bacteria

Five morphologically distinguishable bacterial colonies were observed on Nutrient agar plate. Morphological, cultural and biochemical studies were carried out (Table 1A). The isolates were identified according to Bergey's Manual of Systematic Bacteriology (Vol I and II) and further characterized by partial sequencing of the 16$ rRNA gene (Table 1B.)[22].

Effect of environment on bacterial growth:

All five isolates showed ability to grow at wide range of temperature, pH and NaCl concentration. Growth was seen in the temperature range of 10^{0}C to 37^{0}C with the maximum growth seen at 28±2^{0}C. Isolate number EC1 and EC5 showed growth in the pH range of 5.0 to 11.0 whereas isolate number EC2 and EC4 showed growth in range of pH 3.0 to 11.0 with the optimum pH found to be 7.0 The optimum NaCl concentration for all five isolates was found to be 0.5% with EC2, EC3 and EC 5 growing upto 12.5% NaCl concentration. On the basis of these optimum environmental parameters, generation time of the five isolates was determined by performing the growth curve

experiment at 37^0C static and shaker conditions (in presence and absence of individual pesticide) and it was found that at shaker condition with more aeration, the isolates were able to grow faster (Table 2) .

Table -1-A Biochemical Results

No	I	MR	VP	Citrate	TSI				Glu	Xyl	Lac	Malt	Man	Sucr
					Butt	Slant	H_2S	Gas						
EC1.	-	+	-	-	Yellow	Yellow	-	-	-	-	-	-	-	-
EC2	+	-	+	+	Yellow	Yellow	-	-		-	-	A	-	A
EC3	+	+	-	+	Pink	Yellow	-	+	-	-	-	-	-	-
EC4	+	+	-	-	Yellow	Yellow	-	+	-	-	-	-	-	-
EC5	-	-	-	+	Pink	Pink	-	-	A	-	-	-	A	-

Key: I- Indole, MR- Methyl Red,VP- Voges Prouskauer, TSI- Triple Sugar Ion
+ : Positive, - : negative, A : Acid , A+ G : Acid and gas production

Table 1-B Special Staining and Identification

Isolate No	Gram staining	Capsule	Endospore	Meta. granule	Lipid granule	Identification
EC1.	Gram negative	+	-	+	-	*Pseudomonas psychrophila* (T); E-3 AB041885
EC2	Gram negative	-	+	+	+	*Devosia yakushimensis* Yak96B(T) AB361068
EC3	Gram negative	+	+	+	+	Paracoccus chinensis KS-11(T) EU660389
EC4	Gram negative	-	+	+	+	*Planococcus rifietoensis* (T); M8 AJ493659
EC5	Gram negative	-	-	+	+	*Pseudomonas aeruginosa* PAL106 DQ464061

Key: + : Positive - : Negative

Table 2: Generation time of isolates

Isolate number	Generation time (Minutes)			
	NB	NB+ES	NB+CP	NB+CM
EC1	65.87min	220min	126min	210.63mins
EC2	65.74min	187.5min	143.47min	179.06min
EC3	86.95min	140min	107.44min	137.22min
EC4	60.57min	88.167min	109.30min	191.96min
EC5	66min	81.91min	119.68min	105.31min

Key: NB: Nutrient Broth, ES: Endosulfan, CP: Chlorpyrifos, CM: Cypermethrin

Table 3 : Antibiotic Sensitivity Pattern

Isolate No.	P	E	G	Sf	C	Va	A	S	T	Cf	Mec	At	Do	Tr	Cb
EC1	R	S	S	R	S	S	R	S	S	S	S	S	S	R	R
EC2	R	R	R	S	S	R	R	S	R	S	S	S	R	S	I
EC3	R	I	I	R	S	S	R	S	R	S	S	R	I	R	S
EC4	R	I	S	S	S	S	R	S	R	I	R	S	R	R	S
EC5	R	R	I	R	R	R	R	R	R	S	S	S	R	R	I

Key: R - Resistant S - Sensitive I - Intermediate

Table 4 : Metal Tolerance

Isolate Number	Cobalt Chloride(ppm)	Nickel sulphate(ppm)	Potassium chromate(ppm)	Copper sulphate(ppm)	Manganese Chloride(ppm)
EC1	500	1000	10,000	1000	6000
EC2	500	1000	10,000	1000	6000
EC3	1000	1000	10,000	1000	6000
EC4	1000	1000	10,000	1000	6000
EC5	3000	5,000	10,000	1000	6000

Table 5 : Pesticide Tolerance

Colony Number	Endosulfan (ppm)	Cypermethrin (ppm)	Chlorpyrifos (ppm)
EC1	400	8,000	10,000
EC2	19,000	9,000	15,000
EC3	19,000	15,000	6,000
EC4	19,000	8,000	15,000
EC5	19,000	12,000	15,000

Figure 1: Percentage of antibiotic resistance

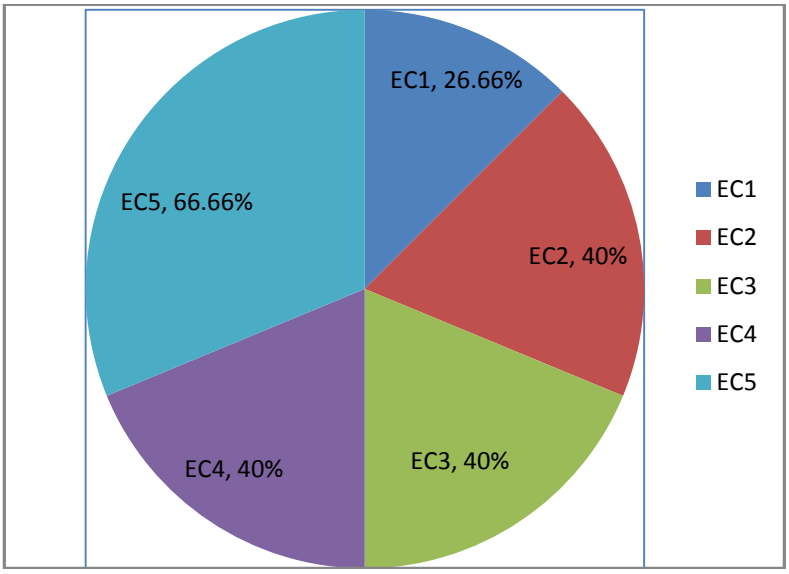


Figure 2: Plasmid Extraction

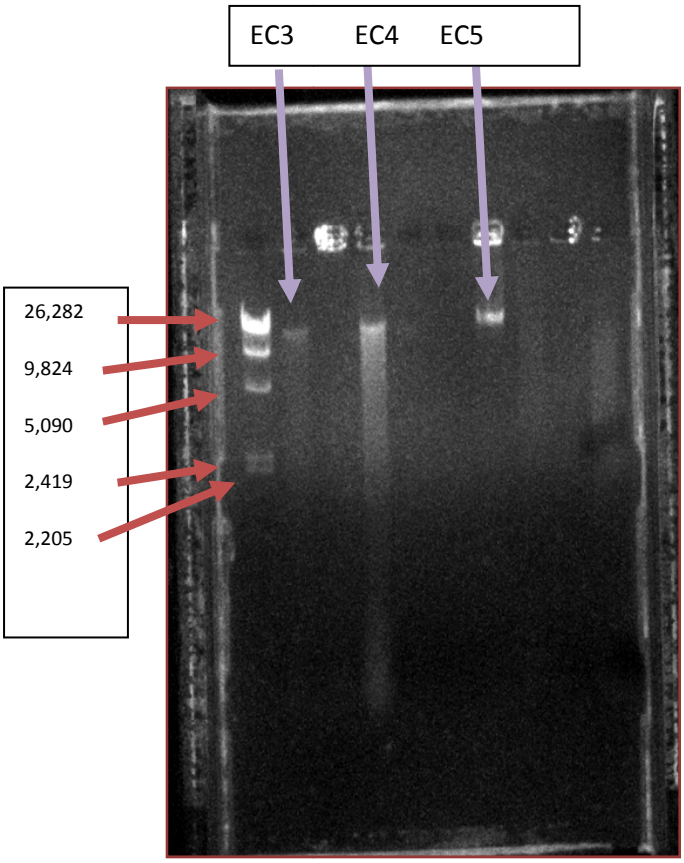


Figure 3A): Growth of transformants on LB+ 8kppm Es

Figure 3B) : Growth of transformants on LB+20k ppm Cp

Figure 3C): Growth of transformants on LB+ 2k ppm Cm

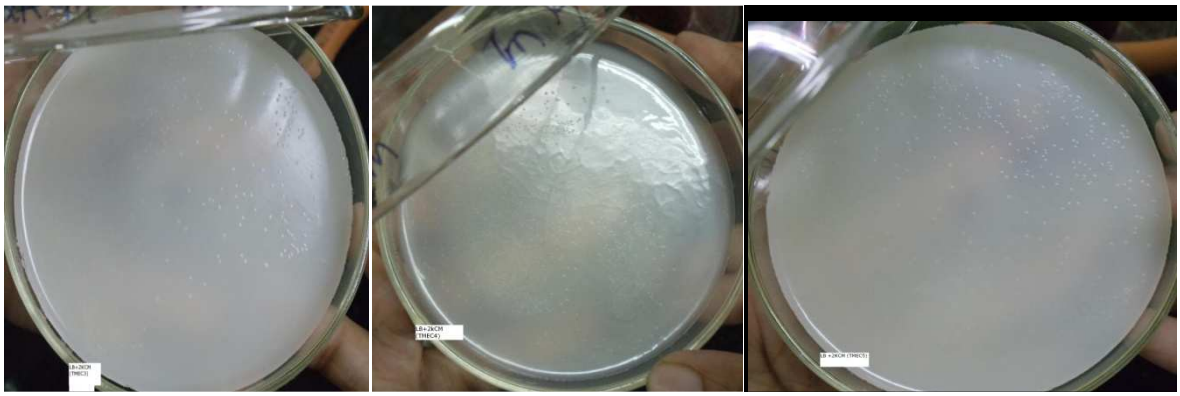


Table 6: Transformation

MEDIUM	CELL CONTROL (only competent cells)	DNA CONTROL (only plasmid DNA)		TRANSFORMATION MIXTURE (competent cells + plasmid DNA)	
LB+8kppm ES	-	EC3	-	TM3	+
	-	EC4	-	TM4	+
	-	EC5	-	TM5	+
LB+20kppm CP	-	EC3	-	TM3	+
	-	EC4	-	TM4	-
	-	EC5	-	TM5	-
LB+2k ppm CM	-	EC3	-	TM3	+
	-	EC4	-	TM4	+
	-	EC5	-	TM5	+

Key: + : Growth seen as cfu, - : No growth (No cfu)

Antibiotic sensitivity/ metal tolerance/ Pesticide tolerance:
For antibiotic resistance/ susceptibility profiling, the disc diffusion method was used. The zone of inhibition was measured in millimeter and the resistance and sensitivity of isolated bacteria towards antibiotics used was determined. It was found that all five isolates being gram negative in nature were found to be resistant to penicillin and Ciprofloxacin. The percentage of resistance towards the 15 antibiotics used was found to be lowest in EC1 (26.66%) and highest in EC5 (66.66%) whereas the remaining three isolates showed moderate resistance (40%) (Figure 1 table3).

For metal resistance profile, Minimum Inhibitory Concentration (MIC) was determined. In the varying concentrations of metal salts, the growth of the bacterial isolates was observed in the form of turbidity (compared with positive and negative control).table 4. The ability of bacterial isolates to grow in presence of pesticide was seen by observing the growth on the plates. It was observed that Chlorpyrifos was easily tolerable pesticide for EC1, EC2 and EC3 whereas EC4 and EC5 were growing faster in Endosulfan. Amongst all five isolates, EC5 was capable of tolerating highest concentrations of the pesticides when compared with the remaining four isolates (Table 5).

Isolation of plasmid DNA and Agarose Gel Electrophoresis:
Amongst the five isolates, presence of plasmid DNA was detected in EC3, EC4 and EC5 on performing Miniprep method of plasmid DNA extraction. The other two isolates EC1 and EC2 were lacking plasmid DNA indicating that the property of these isolates to tolerate high concentrations of heavy metal salts, pesticides and antibiotics was not plasmid borne. On treatment with restriction endonucleases - EcoRI and HindIII, it was seen that the plasmids from EC3, EC4 and EC5 showed single band indicating no recognition sequence (restriction site) of these two enzymes. On comparing with the λ /*MuI* molecular weight marker, molecular weight of EC3, EC4 and EC5 plasmids was found to be approximately between 9,824D and 26,282 D (figure 2).

Transformation:
The extracted plasmid DNA from EC3, EC4 and EC5 were further used for transforming the competent cells of *E. coli* DH5α strain.The transformation mixture was plated on LB agar plates containing 8,000ppm of Endosulfan, 20,000ppm of Chlorpyrifos and 2000ppm of Cypermethrin (Table 6). It was found out that competent cells of *E. coli* DH5α were successfully transformed with the plasmid DNAs isolated from pesticide tolerating bacteria and thus acquired a new extra-chromosomal property of tolerating higher concentrations of toxic chemicals (Figure 3A, 3B and 3C).

CONCLUSION

Results obtained in this work indicate the role of bacteria in tolerating high concentrations of toxic chemicals that included pesticides, heavy metal salts and antibiotics. The isolates were found to be gram negative coccobacilli with 37^0C shaker conditions as the optimum condition for their growth. The isolates also showed growth at wide range of pH and salt concentration. Further, by Miniprep method and Agarose Gel electrophoresis, presence of plasmid DNA was detected in three isolates indicating their role in resisting toxic effects of chemicals. All three plasmid DNA samples showed resistance to digestion by restriction endonucleases - HindIII and EcoRI suggesting to be an important survival mechanism of bacteria against certain stress situations. Further the tolerance property was successfully transferred to *E. coli* DH5α cells by transformation process. These findings of the study suggest a possible tool of constructing a bacterial strain that could be useful in bioremediation of pesticides and heavy metal salts.

Acknowledgments
The authors gratefully acknowledge NCCS, Pune for identification of isolates by 16$ rRNA sequencing.

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