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Role of chromosomal genes in bioremedial potential by soil bacteria

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ABSTRACT

The application of pesticides for pest control is a general practice in India. Since pesticides are very toxic by design, they have the potential to adversely impact the health of our ecosystem. However, there are few soil bacteria which can tolerate high concentrations of pesticides that may ultimately lead to degradation of pesticides. The main objective of the present study involved the isolation and identification of bacteria from garden soil, which can tolerate higher concentrations of pesticides namely Endosulfan,Chlorpyrifos and Cypermethrin. An enrichment culture technique was used to isolate 10 bacterial strains named EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9 and EC10, which were subsequently characterized by 16S rRNA gene sequencing and biochemical tests. The optimum temperature, pH and NaCl concentration was determined for all the isolates and it was found that EC8 was showing growth at wide range of these parameters. Growth curve experiments showed that the bacterial isolates were able to grow in a medium containing higher concentration of individual pesticide. Tolerance to high levels of metal salts (Co^{2+}, Ni^{2+}, Cr^{3+}, Cu^{2+} and Mn^{2+}) and multiple antibiotic resistances was seen in many of the bacterial isolates, which may indicate a positive correlation between pesticide degradation and tolerance to metals and antibiotics. Such findings may be useful in designing in-situ or on-site hazardous waste bioremediation process for field application.

Keywords: Endosulfan, Chlorpyrifos, Cypermethrin, EC strains, Bioremediation, Growth curve, MIC, Antibiotic resistance

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INTRODUCTION

The Environmental Protection Agency (EPA) defines a pesticide as "any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest". They are the class of natural or synthetic chemicals, which kills or controls growth of pests. These pesticides are used globally and extensively in order to control pests like rodents, insects, nematodes etc. that can have devastating effects on crop yield. On the basis of chemical properties, pesticides are broadly classified as organochlorines, organophosphorus, carbamate, organosulfur and pyrethroids (natural and synthetic). Several reports suggest that contamination of soil by these pesticides, due to their bulk handling or accidental release, may lead to occasional entry into aquatic and terrestrial ecosystems [17]. These contaminations show lethal effects on living systems, which include birth defects, hypertension, learning disabilities, irreversible chromosomal damage to human DNA, increased chances of heart attack etc. [4].

As these pesticides cause extensive damage to non-target organisms, studies regarding their degradation have received attention from soil microbiologists. Microorganisms demonstrate 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 [7]. Hence,

isolation of indigenous bacteria capable of metabolizing pesticides has received considerable attention thereby providing an environment friendly method of *in situ* detoxification [11].

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: 115-29-7], an organochlorine pesticide, is a broad spectrum contact insecticide widely used in pest control of crops including cereals, fruits, oil seeds, potato, tea and vegetables. 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. CAS number is 2921-88-2. Chlorpyrifos is usually applied to soil 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 [2,3,11] and endosulfan in soil [7,8].Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system and the respiratory system [20,21].

Cypermethrin [IUPAC name: alpha cyano – 3 - phenoxybenzyl - 3- (2, 2-dichloro-vinyl) - 2, 2-dimethylcyclopropane – carboxylate; 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.

These pesticides and insecticides, to which pests and insects are resistant, are not degraded in the environment by routine processes. These undegradable compounds however are degradable by bacterial activity [15]. Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemicals [10]. Rates of pesticide degradation in a soil are a function of multiple factors including population densities and activity of pesticide degrading microorganisms, pesticide bioavailability and soil parameters such as pH, soil water content and temperature [13].

MATERIALS AND METHODS

Material 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, M.S. The commercial grade pesticides were used throughout the experimental work.The media components and the biochemicals were obtained from HI-MEDIA, INDIA. All other chemicals used were of analytical grade.

Soil:

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

Media:

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.1gm of soil sample was put into a 150ml flask containing 30ml of sterile liquid Mineral Salt Medium (MSM) with 10ppm of chlorpyrifos and was used for isolating chlorpyrifos –degrading bacteria. For isolating cypermethrin-degrading bacteria, the soil sample was added in sterile minimal medium with 10ppm of cypermethrin incorporated into it. All the above flasks were incubated at 28±2 ^{0}C for 7 days 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 7 days 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 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 and 1% sugar solutions of Sucrose, Glucose, Lactose, Xylose, Maltose and Mannitol with Andrade's indicator. Total of 10 bacterial isolates were identified by 16S 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 55^0C for 24 hours 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 10 bacterial isolates by growth curve method (in presence and absence of every pesticide) at static and shaker conditions (120 rpm).

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. 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. (28 ± 2^0C) for 24 hours. One positive and one negative control were also run parallel. Growth in the form of turbidity was recorded and Minimum Inhibitory Concentration (MIC) [5, 19] was determined. Heavy 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. (28 ± 2^0C) for 24 hours and growth was observed.

Plasmid analysis:
Absence of plasmid DNA was detected in cells grown in liquid media (under selective conditions given by presence of antibiotic) by Miniprep method [6,12] and was further analyzed by Agarose Gel Electrophoresis.

Agarose Gel Electrophoresis:
The extracted DNA samples (the genomic DNA pellet obtained during plasmid DNA extraction) from all 10 isolates were confirmed to be genomic DNA by Agarose Gel Electrophoresis according to the standard procedure [1, 16]. Electrophoresis was carried out at 60 volts for 60 minutes. Thereafter, gel (Pre-stained with Ethidium bromide) was removed and examined over UV transilluminator (Technosource –Gloworm-2) for observing the genomic DNA bands. The orange fluorescent DNA bands were observed in the wells and their band pattern was photographed by Gel Documentation System (Bio-Imaging Systems- Gel capture software).

RESULTS AND DISCUSSION

Identification and taxonomic characterization of pesticide tolerating bacteria
10 morphologically distinguishable bacterial colonies were observed on Nutrient agar plate. The morphological, cultural and biochemical studies of these isolates were performed. Further these isolates were identified according to

Bergey's Manual of Systematic Bacteriology (Vol I and II) and characterized by partial sequencing of the 16S rRNA gene [18] (Table 1 and Table 2.).

Table 1: Identification based on 16S rRNA Sequencing

Isolate No	Identification
EC1.	*Stenotrophomonas maltophilia K279a* NC_010943.1
EC2	*Xanthomonas oryzae pv. oryzicola BLS256* NC_017267.1
EC3	*Marivirga tractuosa DSM 4126* NC _014759.1
EC4	*Lysinibacillus sphaericus C3-4 1*NC_010382.1
EC5	*Bacillus cereus FRI-35* NC_018491.1
EC6	*Pelagibacterium halotolerans B2* NC_016078.1
EC7	*Streptococcus infantarius subsp. infantarius CJ18* NC_016826.1
EC8	*Brevibacillus choshinensis strain DSM 8552* NR_040980.1
EC9	*Staphylococcus haemolyticus JCSC1435* NC_007168.1
EC10	*Bacillus pumilus SAFR-032* NC_009848.1

Table 2: Standard Biochemical results

Isolate No	I	MR	VP	Cit.	TSI				Glu	Xyl	Lact	Malt	Man	Sucr
					Butt	Slant	H_2S	Gas						
EC1	+	+	-	+	Yellow	Pink	-	+	-	-	-	-	-	-
EC2	+	+		+	Yellow	Pink	-	+	A+G	-	-	A+G	A+G	A+G
EC3	+	+	-	+	Yellow	Yellow	-	-	-	-	-	-	-	-
EC4	-	+	-	-	Reddish yellow	Yellow	-	-	A	-	-	A	-	A
EC5	-	+	-	-	Yellow	Yellow	-	-	A	A	-	A	-	A
EC6	-	-	+	-	Yellow	Yellow	-	-	A	A	-	A	-	A
EC7	-	-	+	-	Yellow	Yellow	-	-	A	-	-	A	-	A
EC8	-	+	-	+	Yellow	yellow	-	-	A+G	-	A	A	A	A
EC9	-	-	+	+	Pink	Pink	-	-	-	-	A	-	-	-
EC10	-	+	-	-	Yellow	Pink	+	-	A	-	-	-	-	-

I- Indole, MR- Methyl Red, VP- Voges Prouskauer, Cit- Citrate, TSI- Triple Sugar Ion
Glu-Glucose, Xyl-Xylose,Lact-Lactose,malt-maltose, Man-Mannitol, Sucr-Sucrose
+ : Positive, - : negative, A : Acid , A+ G : Acid and gas production

Table 3a: Effect of pH on bacterial growth

Isolate Number	pH				
	3.0	**5.0**	**7.0**	**9.0**	**11.0**
EC1	-	-	+	+	-
EC2	-	+	+	+	+
EC3	-	+	+	+	+
EC4	-	-	+	+	+
EC5	-	-	+	+	-
EC6	-	+	+	+	+
EC7	-	-	+	+	+
EC8	+	+	+	+	+
EC9	-	-	+	+	+
EC10	-	+	+	+	-

+ : Growth seen (Turbidity) - : No growth (No turbidity)

Table 3b: Effect of temperature on bacterial growth

Isolate Number	Temperature			
	10^0C	**R.T.**	**37^0C**	**55^0C**
EC1	-	+	+	-
EC2	+	+	+	+
EC3	+	+	+	+
EC4	-	+	+	+
EC5	-	+	+	-
EC6	-	+	+	+
EC7	+	+	+	+
EC8	+	+	+	+
EC9	+	+	+	+
EC10	+	+	+	-

+ : Growth seen (Turbidity) - : No growth (No turbidity)

Table 3c : Effect of NaCl on bacterial growth

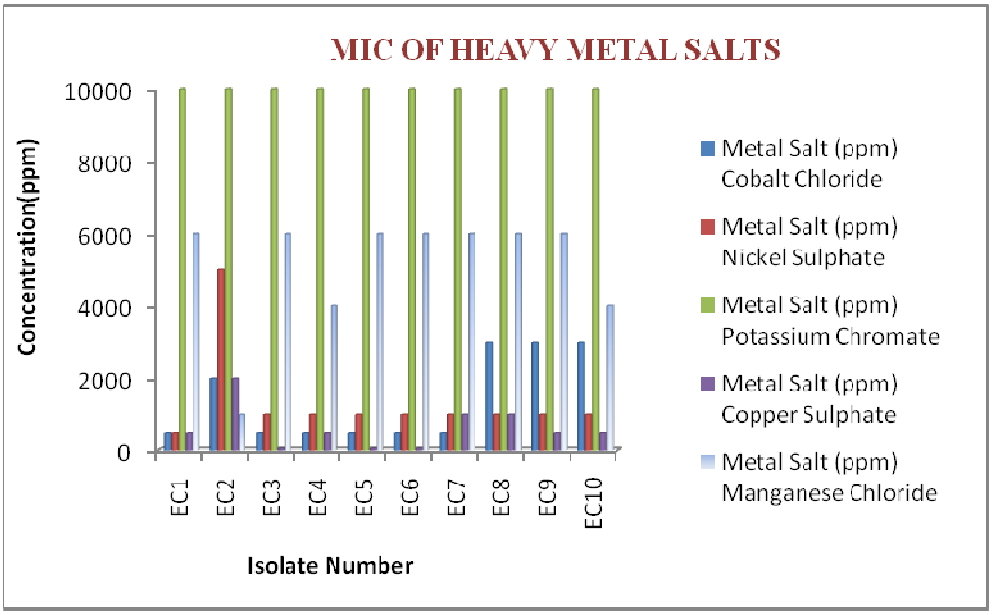
Isolate Number	NaCl in %					
	0.5	3.5	6.5	9.5	12.5	15.5
EC1	+	+	-	-	-	-
EC2	+	+	+	+	-	-
EC3	+	+	-	-	-	-
EC4	+	+	+	-	-	-
EC5	+	+	-	-	-	-
EC6	+	+	-	-	-	-
EC7	+	+	-	-	-	-
EC8	+	+	+	+	+	-
EC9	+	+	-	-	-	-
EC10	+	+	+	+	-	-

+ : Growth seen (Turbidity) - : No growth (No turbidity)

Effect of environment on bacterial growth:

All 10 isolates showed ability to grow at wide range of temperature, pH and NaCl concentration. (Table 3a, 3b and 3c). Growth was seen in the temperature range of 10^0C to 37^0C. The maximum growth was seen at 28 ± 2^0C. Isolate number EC2, EC3 and EC6 showed growth in the pH range of 5.0 to 11.0 whereas isolate number EC8 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 and EC10 growing upto 9.5% NaCl concentration and EC8 tolerating maximum NaCl concentration upto 12.5%. On the basis of these optimum environmental parameters, generation time of the ten 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.

Figure 1: MIC of metal salts (ppm)



Antibiotic sensitivity/ metal tolerance/ Pesticide tolerance:

For antibiotic resistance/ susceptibility profiling, the Kirby-Bauer disc diffusion method was used. The zone of inhibition was measured in millimeter and the resistance and sensitivity of isolated bacteria was determined. It was found that all ten isolates being Gram negative in nature were found to be resistant to penicillin. Amongst the ten isolates, EC1 was found to be resistant to 08 antibiotics from the total 15 antibiotics used (Table 4).

Table 4 : Antibiotic Sensitivity Testing

	Antibiotic														
Isolate No.	**P**	**E**	**G**	**Sf**	**C**	**Va**	**A**	**S**	**T**	**Cf**	**Mec**	**At**	**Do**	**Tr**	**Cb**
EC1 8	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**I**	**R**	**S**	**S**	**I**	**S**	**R**	**S**
EC2 6	**R**	**R**	**S**	**S**	**S**	**R**	**R**	**S**	**R**	**R**	**S**	**I**	**S**	**S**	**S**
EC3 2	**R**	**I**	**S**	**S**	**I**	**S**	**S**	**S**	**S**	**S**	**S**	**I**	**S**	**R**	**I**
EC4 5	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**I**	**S**	**R**	**S**
EC5 3	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**I**	**S**	**R**	**R**
EC6 3	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**I**	**R**	**R**	**I**
EC7 5	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**	**S**	**S**	**I**	**S**	**R**	**I**
EC8 3	**R**	**S**	**I**	**R**	**S**	**S**	**S**	**R**	**I**	**S**	**S**	**I**	**S**	**S**	**S**
EC9 4	**R**	**S**	**R**	**S**	**S**	**S**	**R**	**I**	**S**	**S**	**S**	**I**	**R**	**S**	**S**
EC10 5	**R**	**I**	**I**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**S**	**S**	**S**

R - Resistant S - Sensitive I - Intermediate

Figure 2: MIC of pesticides (ppm)

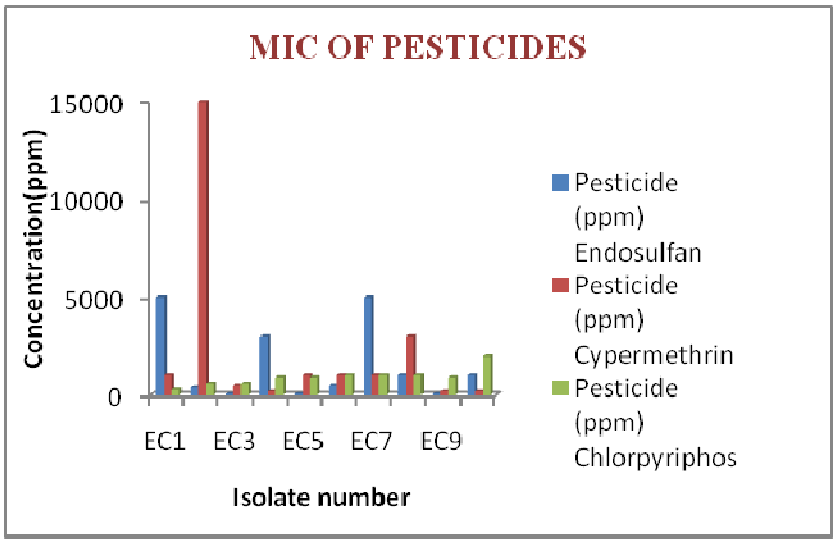
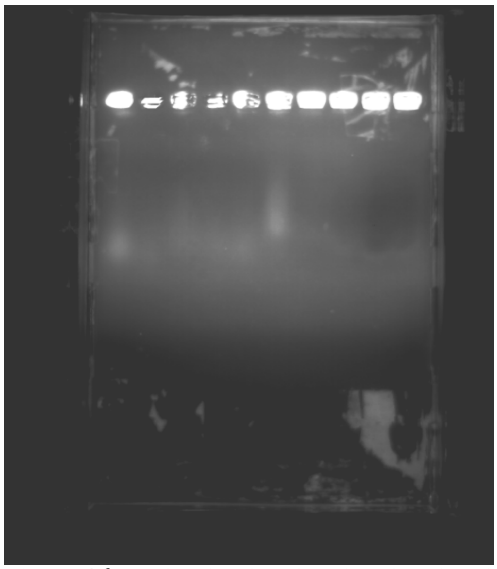


Figure 3: Genomic DNA



Lane 1 to lane 10 : gDNA from EC1,EC2 EC3, EC4, EC5, EC6, EC7, EC8, EC9, EC10.

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 on comparing with positive and negative control (Figure 1). The ability of bacterial isolates to grow in presence of pesticide was seen by observing the growth on the plates. It was observed that 5000 ppm of Endosulfan was tolerated by EC1 and EC7 whereas as high as 15,000 ppm of Cypermethrin was tolerated by EC2. Chlorpyrifos was easily tolerable pesticide for EC10 showing growth upto 2000ppm Chlorpyrifos (Figure 2).

Isolation of plasmid DNA and Agarose Gel Electrophoresis:
Presence of plasmid DNA was not detected in any of the 10 isolates on performing Miniprep method of plasmid DNA extraction instead all 10 isolates showed genomic DNA bands in the wells of agarose gel (Figure 3) suggesting role of chromosomal DNA in pesticide tolerance. The role of chromosomal genes in bioremediation can also be due to an episomal condition where the plasmid has integrated in the chromosomal DNA.

CONCLUSION

Results obtained in this work indicate the role of bacteria in tolerating high concentrations of toxic chemicals like pesticides, heavy metal salts and antibiotics. The isolates were found to be Gram negative coccobacilli having 37^0C shaker condition 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, no plasmid DNA was isolated from any of the ten isolates suggesting role of chromosomal DNA genes in the bioremedial potential of all isolates. These findings of the study suggest a possible tool of isolating indigenous bacteria from soil with ability to tolerate extreme physical and chemical conditions that could be useful in bioremediation of pesticides and heavy metal salts.

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