World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

ISOLATION AND IDENTIFICATION OF PESTICIDE DEGRADING BACTERIA

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Article Received on 14/07/2020 **Article Revised on 04/08/2020** **Article Accepted on 25/08/2020**

ABSTRACT

In India pesticides play an important role in agricultural. Their importance in crop yield improvement is a well known fact but at the same time their toxic nature is a threat to the environment. Due to its toxicity and persistence in the environment, there is an immediate need to eliminate them from contaminated sites by biodegradation. In this study, an enrichment culture technique was used to isolate bacterial strains from farm soil, garden soil and nursery soil for degrading high concentrations of chlorpyrifos pesticide. Five bacteria (G1, F2, F3, F4, F5) isolated and both qualitative and quantitative screening was done for isolated bacteria. Also the optimum temperature, pH and NaCl concentration was determined for them. Isolated bacteria were identified by their gram nature, biochemical characterization and according to Bergey's manual of systematic bacteriology isolated bacteria were identified as *Staphylococcus aureus* (G1), *Pseudomonas aeruginosa* (F2), *Micrococcus luteus* (F3), *Pseudomonas aeruginosa* (F5) and F4 could not be identified. Then, their antibiotic sensitivity test by disc diffusion method was studied. By miniprep method of plasmid DNA isolation it was found out that four isolates (F2, F3, F4, F5) showed presence of plasmid DNA and then plasmid curing was done using ethidium bromide as curing agent. After curing all bacteria were able to grow on nutrient agar plate supplemented with chlorpyrifos pesticide, it showed that pesticide degrading responsible gene present on bacterial chromosome. Thus, these pesticide degrading bacterial species can be used for bioremediation of contaminated soils.

KEYWORDS: Bacteria, Chlorpyrifos, Biodegradation, Plasmid curing, Bioremediation.

INTRODUCTION

Agriculture is the very essential for the Indian economy. It ensures the food security for more than 1 billion Indian population with diminishing cultivable land resource.[1] In recent years, plant protection has become one of the essential inputs for enhancing crop production. As time passes, there is a change in cropping patterns, introducing high yielding varieties, applying high doses of fertilizers, and enhanced irrigation facilities, pests have become a major part of agricultural system and therefore more pesticide amounts should be applied.[2]

Pesticides are defined as any substance or mixture of substances which are used to control destructive pests such as insects, plant disease that pose a threat to the food supply indirectly affecting our, health. In particular, pesticides are chemical substances that alter biological processes of living organisms such as pests, whether these are insects, mould or fungi, weeds. Pesticides are widely used in most areas of crop production to minimize risks by pests and thus protect crops from potential yield losses and reduction of product quality.[3] Approximately 90% of agricultural pesticide application never reaches its target organisms but instead of reaching towards the target organisms, it gets dispersed through the air, soil, and water. Therefore, they are routinely detected in air, surface and ground water, sediment. Many soil-applied pesticides are applied to soil so that's oil, vegetable, and to some extent in foods. the pesticide residues and their metabolic products accumulate in the soil at very high levels.[4]

Pesticides also harm to the human health by entering into the food chain and also interfere in the reproductive capabilities of both male and female causing infertility problems as well as delayed conceptions and multiple miscarriages in females. It also causes damage to the central nervous system (CNS). Pesticides are also carcinogenic in nature.[5] To make pollution free environment which is caused by pesticides many studies have been conducted for the bioremediation of these xenobiotic through microbial agents which is an ecofriendly technique of cleaning up the environment.[6]

Chlorpyrifos (CPF) is a broad spectrum organophosphate pesticide used extensively for domestic as well as industrial applications. The routes of exposure to CPF are inhalation, ingestion of contaminated food and by

dermal contact. The empirical formula of chlorpyrifos is $C_9H_{11}Cl_3NO_3PS$ and the chemical structure is O, O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate. Chlorpyrifos is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea, beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton field, fruit and vegetable crops as well as on lawns and ornamental plants. The primary mechanism of toxicity for chlorpyrifos is cholinesterase (ChE) inhibition. Chlorpyrifos affects the nervous system by inhibiting the breakdown of a neurotransmitter called acetylcholine (ACh). Chlorpyrifos is moderately toxic to humans; moderately to very highly toxic to birds and very highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms. Acute and chronic exposure to CPF can elicit several adverse effects like neurological effects, developmental disorders, autoimmune disorders and oxidative stress.[7]

Due to its toxicity and persistence in the environment, there is an immediate need to eliminate it from contaminated sites by biodegradation. One of the efficient, eco-friendly and cost effective method for decontamination of toxic organophosphorus pesticides from the environment is by their microbial degradation.

MATERIALS AND METHODS

Collection of soil sample

Three soil sample were collected from different areas. Sample 1 was collected from rice field, Sandap village, Dombivali, Thane, Maharashtra, India (labeled as F). Sample 2 was collected from Pathare nursery, Kalyan, Thane, Maharashtra, India (labeled as N). Sample 3 was collected from garden of B.K. Birla college, Kalyan, Thane, Maharashtra, India (labeled as G). Samples were taken from the top 15 cm of soil and kept in plastic bag at 4^0C until use.

Enrichment of pesticide degrading bacteria in mineral salt medium (MSM)

1g of each soil sample was inoculated in 30 mL sterile mineral salt medium broth supplemented with 100 µL chlorpyrifos pesticide as a carbon source. {Composition of MSM:- KH_2PO_4, 4.8; K_2HPO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; $Ca(NO_3)_2.4H_2O$, 0.04; and $Fe(SO_4)_3$, 0.001 with pH 7.0}. Conical flasks were kept at 28^0C for 7 days on rotatary condition at 100 rpm (1^{st} enrichment). Further for each sample 5 mL culture re-inoculated to the other flasks with the same medium composition asepectically from 1^{st} enrichment and further incubated at 28^0C for 7 days on rotatary condition at 100 rpm (2^{nd} enrichment).[1]

Isolation of Pesticide Degrading Bacteria

For respective soil samples, loopful cultures from the 2^{nd} enrichment flasks were streaked using 5-sided streaking technique on to sterile Nutrient Agar plate and incubated at 28^0C for 48 hours to get isolated colonies of bacteria.[1]

Qualitative Analysis of pesticide degrading bacteria

Spot inoculation of all the isolated bacteria was done on sterile nutrient agar plate which contained chlorpyrifos pesticide and incubated at 28^0C for 3-4 days to get clear zone.[8]

Quantitative Analysis of pesticide degrading bacteria

All the isolated bacteria were inoculated in minimal salt medium supplemented with chlorpyrifos pesticide in different flasks one for each bacteria. The pesticide degradation by isolates in the media was tested at regular interval for 10 days by taking optical density of cell free extract of the minimal media amended with chlorpyrifos pesticide at 300 nm on UV-spectrophotometer (V-630).[6]

Identification of pesticide degrading bacteria

The isolates were subjected to morphological and cultural studies (Gram staining, motility). Standard biochemical studies included indole test, methyl red test, Vogues- Proskauer test, citrate test, nitrate reductase test, catalase test, oxidase test, TSI slant, sugar utilization tests.

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 were used (Hi-media): Erythromycin, Tetracycline, Penicillin, Trimethroprim, Sulfasomidine, Vancomycin, Ciprofloxacin, Chloramphenicol.[7]

Effect of temperature, pH, NaCl concentration on Isolated Bacterial Growth

The optimum temperature, pH, NaCl concentrations for potential pesticide degrading bacteria were determined by inoculating pure culture in Nutrient Broth.

- For effect of temperature, the inoculated tubes were kept at 10^0C, RT, 37^0C, 55^0C.
- For effect of pH, sterile nutrient broth with pH 3, 5, 7, 9, 11 were used and tubes were kept at R. T.
- For effect of NaCl concentration 0.5%, 4.5%, 8.5%, 12.5% were used and tubes were kept at R. T.[1]

Isolation of plasmid DNA using Agarose Gel Electrophoresis

Plasmid isolation was carried out from potential pesticide degrading bacteria. Following protocol was followed:-

Pure culture of each potential isolate was grown overnight in 25 mL of sterile Luria-Bertani broth Centrifuged at 6000 rpm for 10 mins at 16^0C.Cells were suspended in 100 µL of ice cold solution 1(50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8.0); 200 µL solution 2 (0.2 N NaOH, 1% SDS); 150 µL of solution 3 (8 M Potassium acetate). The tubes were mixed properly and kept on ice for 5 minutes Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) in each tube was added. Tubes were mixed properly and centrifuged at 10,000 rpm for 10 minutes at 16^0C. Aqueous phase was taken, double volume of cold absolute alcohol and $1/10^{th}$ volume of 3 M sodium acetate was added. Tubes were

kept at R. T. for 15 minutes. Then, tubes were kept at 4^0C for 48 h for precipitation of plasmid DNA. After precipitation, tubes were centrifuged at 12000 rpm for 20 minutes at 16^0C. To pellet 500 µL of 70% ethanol was added. Tubes were mixed properly and centrifuged at 12,000 rpm for 10 minutes at R.T. Supernatant was discarded, pellet dried at R.T. Pellet was further suspended in 20 µL of TE buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris, pH 8.0).

10 µL of plasmid DNA was taken and 2 µL of gel loading buffer was added in it. Then Samples were run on 1% agarose gel electrophoresis at 60 volts for 60 minutes.

Agarose gel electrophoresis – For this 50 mL 1% agarose was prepared and 4 µL of ethidium bromide also added. The molten agarose was poured onto the gel- casting tray with comb at one end (near cathode) and was allowed to set. Then 1X TAE buffer was poured in the tank to submerge the gel. Then 10 µL of samples were loaded in the wells and electrophoresis carried out at 60 Volts for 60 minutes. Then after 60 minutes, gel was removed and examined over UV-transilluminater for observing plasmid DNA bands.[1,9]

Plasmid Curing

In order to validate whether pesticide degradation is due to presence of plasmid, curing of plasmid was carried out. Ethidium bromine (EtBr) was used as curing agent.

Plasmid curing procedure

All five cultures were grown in the presence of ethidium bromide (Curing agent) at 100 µg/mL sub lethal concentration for 24 hours at 30^0C. Cultures were plated on sterile nutrient agar plate to obtain colonies. Then prepared a master plate of each on sterile nutrient agar plate and incubated at 30^0C for 24 hours. Single colony from master plate was duplicated on to nutrient agar plate and nutrient agar plate supplemented with 500 µg/mL chlorpyrifos pesticide and incubated at 30^0C for 24 hours.[2]

RESULTS AND DISSCUSSIONS

Isolation of Pesticide degrading bacteria

Three samples were processed by the enrichment culture technique for the isolation of bacterial strains. Soil enrichment was carried out in minimal salt medium with the chlorpyrifos (100 µL), which are capable of utilizing it as a sole source of carbon and energy. Thirteen isolates were observed on sterile nutrient agar plate after the enrichment technique of about 14 days in MSM medium supplemented with chlorpyrifos pesticide (Table 1).

Table 1: Numbers of isolated pesticide degrading bacteria.

Site of soil	No. of isolates obtained
F	06
G	04
N	03

These isolates were then further screened for quantitative analysis.

Qualitative Analysis of isolated pesticide degrading bacteria

In qualitative analysis, thirteen isolates were screened. Among thirteen only few bacterial isolates showed maximum clear zone for chlorpyrifos pesticide degradation. In this from six farm soil isolates only four isolates showed maximum clear zone for chlorpyrifos pesticide degradation (labeled as F2, F3, F4, F5). From four garden soil isolates only one isolate showed maximum clear zone for chlorpyrifos pesticide degradation (labeled as G1). None of isolates from nursery soil showed clear zone for chlorpyrifos pesticide degradation.

Table 2: For qualitative analysis.

Bacterial isolates	Clear zone (mm)
G1	12 mm
F2	36 mm
F3	30 mm
F4	20 mm
F5	32 mm

In qualitative analysis, F2 showed highest capacity of pesticide degradation upto 36 mm. G1 showed lowest capacity of pesticide degradation about 12 mm (Table 2). These five isolates were further screened for quantitative analysis. According to the Iqbal and Bartakke *et al*., they were found six bacterial isolates for maximum degradation of pesticide.[8]

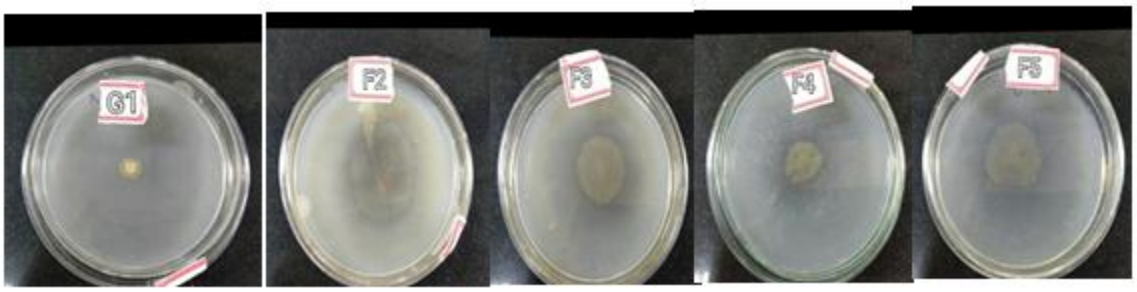

Figure 1: Clear zone of isolated pesticide degrading bacteria.

Quantitative Analysis of isolated pesticide degrading bacteria

The isolates G1, F2, F3, F4, F5 screened for quantitative estimation by using UV- Spectrometer. In quantitative analysis, F2 showed about 93.16% of pesticide degradation which is maximum as compared with other four isolates. Here, F4 showed low percentage of pesticide degradation about 57.16% (Table 3). While According to Sharma *et al*., the chlorpyrifos degrading organisms Bacillus sp. and Micrococcus sp. showed 71.6% at 0.1% v/v and 46% at 0.05 %v/v of chlorpyrifos after 10 days of incubation where as in the case of Bacillus sp. 40% and 44% at the same concentrations.[6]

Table 3: For quantitative analysis.

Bacterial isolates	Percentage of degradation
G1	74.08%
F2	93.16%
F3	62.33%
F4	57.16%
F5	71.00%

Identification of isolated pesticide degrading bacteria

The colony characteristics and biochemical tests of potential pesticides were studied. The results are summarized in the tabular format below (Table 4) (Table 5).

Table 4: Colony characters of isolated pesticide degrading bacteria.

Isolates designation	G1	F2	F3	F4	F5
Size	1 mm	2 mm	1 mm	1 mm	1 mm
Shape	Circular	Circular	Circular	Circular	Circular
Color	White	Green	Yellow	White	Green
Margin	Entire	Entire	Entire	Entire	Entire
Elevation	Flat	Flat	Convex	Flat	Flat
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Mucoid	Butyrous	Butyrous	Butyrous	Butyrous
Gram nature	Gram positive	Gram negative	Gram positive	Gram negative	Gram negative

Table 5: Biochemical tests of isolated pesticide degrading bacteria.

Biochemical tests	G1	F2	F3	F4	F5
Indole test	-	-	-	-	-
Methyl red test	-	-	-	-	-
Voges-Prouskour test	-	-	-	-	-
Citrate utilization test	+	+	+	+	+
Catalase test	+	+	+	+	+
Nitrate reductase test	+	+	+	+	+
Motility	+	+	+	+	+
Oxidase test	+	-	+	-	-
Triple sugar iron test					
Slant	Alk	Alk	Alk	Alk	Alk
Butt	Alk	Alk	Alk	Alk	Alk
Gas	+	-	-	-	-
H_2S production	+	-	-	-	-
Sugar tests					
Glucose	+	-	-	-	-
Lactose	+	-	-	-	-
Mannitol	+	+	-	-	+

Key:- + = Positive; - = Negative; A = Acid Formation; A+G = Acid + Gas formation; Aci = Acidic, Alk= Alkaline.

The colony characters, gram nature and biochemical tests were studied. The Bergey's manual was referred and isolates were found to be Staphylococcus aureus (G1), Pseudomonas aeruginosa (F2), Micrococcus luteus (F3), Pseudomonas aeruginosa (F5), F4 was non-identified.

Antibiotics sensitivity test by disc diffusion method of isolated pesticide degrading bacteria

Antibiotic sensitivity test was performed by using antibiotic disc of Erythromycin, Tetracycline, Penicillin, Trimethroprim, Sulfasomidine, Vancomycin, Ciprofloxacin, Chloramphenicol (from Hi-media). The isolates showing zone of inhibition were found to be sensitive. All five isolates were found to be resistant towards penicillin, trimetroprim, vancomycin. All five isolates were found to be sensitive towards sulfasomidine, ciprofloxacin, chloramphenicol (Table 6)

Table 6: Antibiotics sensitivity test of isolated pesticide degrading bacteria.

Antibiotics	G1	F2	F3	F4	F5
Erythromycin	S	R	R	R	R
Tetracycline	S	R	R	R	R
Penicillin	R	R	R	R	R
Trimethroprim	R	R	R	R	R
Sulfasomidine	S	S	S	S	S
Vancomycin	R	R	R	R	R
Ciprofloxacin	S	S	S	S	S
Chloramphenicol	S	S	S	S	S

Key :- R = Resistance; S = Sensitive

Effect of temperature, pH, NaCl concentration on isolated pesticide degrading bacteria

A) For Temperature

All five isolates showed ability to grow in the range of 10^0C to 37^0C. The maximum growth was showed at R.T. (Table 7). According to Naphade *et al.*, growth was seen in the temperature range of 10^0C to 37^0C with the maximum growth seen at 28 ± 2^0C [1].

Table 7: Effect of temperature.

Bacterial isolates	10^0C	R. T.	37^0C	55^0C
G1	+	+	+	-
F2	+	+	+	-
F3	+	+	+	-
F4	+	+	+	-
F5	+	+	+	-

B) For pH

Bacteial isolates G1, F2, F3, F5 showed growth in the range of pH 3 to pH 11 whereas F4 showed growth in the range of pH 5 to pH 11. For all isolates optimum pH was found to be 7.00 (Table 8). According to the Naphade *et al.*, the optimum pH found to be 7.0 [1]

Table 8: Effect of pH.

Bacterial isolates	pH3	pH5	pH7	pH9	pH11
G1	+	+	++	+	+
F2	+	+	++	+	+
F3	+	+	+	+	-
F4	+	+	+	+	-
F5	+	+	++	+	+

C) For NaCl concentration

All five isolates were grown in the range of 0.5%-12.5% NaCl concentration (Table 9). According to the Naphade *et al.* the optimum NaCl concentration for all five isolates was found to be 0.5% and EC2, EC3 and EC 5 growing upto 12.5% NaCl concentration.[1]

Table 9: Effect of NaCl concentration.

Bacterial isolates	0.5%	4.5%	8.5%	12.5%
G1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	+	+

Isolation of plasmid DNA and Agarose gel electrophoresis

Four isolates showed presence of plasmid DNA on performing miniprep method of plasmid DNA extraction. By doing agarose gel electrophoresis, isolates F2, F3, F4, F5 were showed DNA bands but G1 didn't show band (Fig. 2).

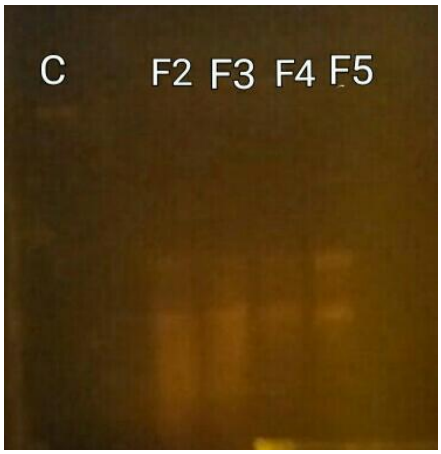

Figure 2: Plasmid DNA extraction.

Plasmid Curing

Pesticide degradative genes in microbes have been found to be located on plasmid, transposons and on chromosomes.[10] For all five isolates, all cured colonies did not lose ability to grow on media supplemented with pesticide chlorpyrifos. This indicated that genes responsible for pesticide degradation are located on bacterial chromosome. However these results do agree with many studies which found that some organophosphorus insecticides degradative genes (opaA, hocA, mpd, adpB, pdeA,PepA, Phn and pehA) are located on bacterial chromosome [2] [11] [12] [13].

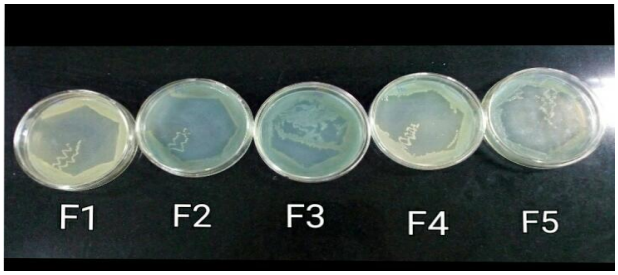

Figure 3: Growth of bacteria after plasmid curing.

CONCLUSION

In this study, Pesticide degrading bacteria were isolated from farm soil, garden soil, nursery soil and screening was done to check their capacity to degrade the chlorpyrifos pesticide. Then isolated bacteria were checked against different temperature, pH, NaCl concentration for observing their effects. Also isolated bacteria were identified by their gram nature, biochemical characterization by comparing with Bergey's manual of systematic bacteriology. Then, their antibiotic sensitivity test by disc diffusion method was studied. Extraction of plasmid DNA and plasmid curing was done to check whether pesticide degrading bacterial gene present in chromosomal or extra-chromosomal.

Five bacteria (G1, F2, F3, F4, F5) isolated by enrichment in MSM medium supplemented with chlorpyrifos pesticide after 14 days on sterile nutrient agar plate and according to Bergey's manual of systematic bacteriology isolated bacteria were identified as *Staphylococcus aureus* (G1), *Pseudomonas aeruginosa* (F2), *Micrococcus luteus* (F3), *Pseudomonas aeruginosa* (F5) and F4 was could not identified.

In qualitative analysis, F2 showed maximum degradation capacity about 36 mm, while G1 showed minimum degradation capacity about 12 mm. F3, F4 and F5 showed degradation capacity about 30 mm, 20 mm and 32 mm respectively.

In quantitative analysis, F2 showed maximum percentage of chlorpyrifos pesticide degradation upto 93.16%, while F4 showed minimum percentage of chlorpyrifos pesticide degradation upto 57.16%. G1, F3 and F5 showed percentage of chlorpyrifos pesticide degradation about 74.08%, 62.33% and 71.00%.

All five isolates were showed ability to grow at wide range of temperature, pH and NaCl concentration. All five isolates growth was seen in the temperature range of 10^0C to 37^0C and maximum growth was seen at R.T.

Bacterial isolates G1, F2, F3, F5 showed growth in the range of pH 3 to pH 11 whereas F4 showed growth in the range of pH 5 to pH 11. For all isolates optimum pH found to be 7.00. All five isolates were grown in the range of 0.5%-12.5% NaCl concentration.

By comparing with standard Kirby-bauer chart, all five isolates were found to be resistant towards penicillin, trimetroprim, vancomycin. All five isolates were found to be sensitive towards sulfasomidine, ciprofloxacin, chloramphenicol.

All five isolates, detection of plasmid DNA was done by miniprep method of plasmid DNA extraction, four isolates plasmid were showing single band on agarose gel electrophoresis and then plasmid curing was done using ethidium bromide as curing agent. After curing all bacteria were able to grow on nutrient agar plate supplemented with 500 µg/mL chlorpyrifos pesticide that showed that pesticide degrading bacterial gene present on chromosome.

Thus, bioremediation techniques are more economical than traditional methods and pollutants can be treated on site. Other than that the risk of spread of contamination reduces making the process a safe one for dealing with very harmful xenobiotics like pesticides. Bioremediation is also very useful because the fertility of the soil that had been lost or hampered due to accumulation of pesticide for a very long period of time, thus improving the capacity of the agricultural field to meet the demands of the ever growing population efficiently [14]. Thus, Bioremediation can be used to stabilize, detoxify, or reduce the toxicity of contaminated soil.

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