A Study of Bioremediation of Pesticides In vitro Using Different Bacterial Species Isolated from Agricultural Soil, Chhattisgarh, India

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Abstract-*India is primarily an agricultural country and the demand of organophosphate pesticides (OPs) is increasing because of the increasing population. Farmers use these pesticides to protect and improve crop production because they are not costly and are broad spectrum. Indiscriminate use of these pesticides has led to contamination of soil and ground water and owing to its xenobiotic nature, its disposal in biosphere poses tremendous problem and its presence is a serious threat to environment and indeed to human life. A large number of microbial enzymes are known to have the ability to degrade harmful organophosphorus compounds that are present in some pesticides and nerve agents. Microorganisms have been reported to mediate pesticide degradation. The use of biological agents to overcome the deleterious effects by removal of xenobiotic compound is termed as "Bioremediation". The present study was carried out to isolate and characterize organophosphorus pesticide degrading bacteria from soil of some selected agro ecosystems of Janjgir-Champa district, Chhattisgarh with a view of Bioremediation.*

Keywords-Bioremediation of Pesticides, Agricultural Soil, Organophosphate Pesticides, Malathion, Quinalphos.

I. INTRODUCTION

India is primarily an agriculture based country along with a well-established agrochemical industry. The demand of organophosphate pesticides (OPs) is on rise in accordance to an alarming population rise in India, projected to cross 1.3 billion by 2020 (Kanekar et al., 2004).

Organophosphate pesticides such as Malathion and Quinalphos come handy and used extensively as agriculture and domestic pesticides. It is a contact and ingestion insecticide, having P=S bond and due to its low persistence in the environment its utility is indispensable in agriculture. Indiscriminate use of these pesticides has led to contamination of soil and ground water (Ahmed et al., 2006). Owing to its xenobiotic nature, its disposal in biosphere poses tremendous problem and its presence is a serious threat to environment and indeed to human life (Hashmi et al., 2002 and Ritmann et al., 1988).

From various reports available (Theriot C.M. and Gruden, 2011), a large number of microbial enzymes are known to have the ability to degrade harmful organophosphorus compounds that are present in some pesticides and nerve agents. Thus, bio detoxification of the xenobiotic compounds can be carried out by use of biological agent i.e., microbes (Sharmila et al., 1989). Microorganisms have been reported to mediate in both soil-bound pesticide formation and pesticide degradation (Gevao et al., 2000). For dissociating pesticides, sorption properties of the molecule can be modified by a pH adjustment (Ou et al., 1983; Spadotto and Hornsby, 2003). The use of biological agents to overcome the deleterious effects by removal of xenobiotic compound is termed as "Bioremediation".

II. RESEARCH METHODOLOGY

1. Soil Source:

The soil was obtained from some selected pesticide contaminated agricultural fields near Janjgir-Champa District, Chhattisgarh.

2. Soil Sampling Procedure:

For the purpose of this study samples were collected from some selected agricultural fields of Nawagarh region of Janjgir-Champa District, Chhattisgarh. Two areas each from a vegetable farm and a paddy field was selected. Three soil samples at 0-15 cm depth were collected randomly from each area of the fields to prepare a single composite sample of each area of the fields.

In order to collect soil samples (0-15 cm depth) grasses, mosses, litter and other plant residues were removed from soil surface. Collection of soil samples was done by using a trowel. In each case, a triangular block was cut with the help of the trowel. Soils were collected in plastic bags, which were sealed and labelled properly. Three soil samples from a rooting depth of 15 cm were collected randomly from each sampling area and each sample composite was labelled as V1 - Vegetable Farm Composite Sample 1; V2 - Vegetable

Farm Composite Sample 2; R1 - Rice Field Composite Sample 1 and R2 - Rice Field Composite Sample 2.

3. Soil Sample Preparation:

Preparation of soil samples is based on the ISO 11464 method (Soil Quality Pre-Treatment of Samples for Physicochemical and Biological Analysis).

Collected samples were brought to the laboratory for analysis. Before analysis, the samples were spread out thinly on a piece of hard paper for drying in air in a shade. The big lumps were broken down, and visible plant roots, pebbles and other undesirable matters were removed. After the soil become completely dry, and after homogenization, a portion of each sample was passed through a 2-mm mesh screen and preserved in clean sealed polythene bags and stored in sealed polythene boxes to avoid air contamination at 4°C before microbial and biochemical analysis.

4. Pesticides Used:

Pesticides used in this present study are:

i) Malathion (50% E.C.) and,

ii) Quinalphos (25% E.C.)

5. Isolation of Malathion Degrading Bacteria:

Pour plate technique was used for the isolation of pesticide degrading bacteria in Nutrient agar. For isolation and selection of Malathion degrading bacteria, microbial colonies were isolated from collected soil samples.

Standard analytical grade solution of Malathion (50% E.C.) was purchased from the local market. 5 grams of each soil sample except control sample was mixed in 100 ml distilled water in 6 different conical flasks and kept at 100 rpm for 24 hours at 37°C.

A Selective Mineral Salt Agar (MSA) Medium was prepared containing the following composition:

Ingredients	In Gram/100 ml
Malathion (E.C. 50%)	0.5
KH_2PO_4	0.1
$MgSO_4$	0.02
NH_4NO_3	0.5
Agar	1.5
Distilled Water	100 ml

Table 1. Composition of Mineral Salt Agar (MSA) Medium

To this solution 15µl of a mineral solution (MS) containing the following composition was added:

Table 2. Composition of Mineral Solution (MS)

Ingredients	In Gram/Litre
$FeSO_4$	10
$CaCl_2$	10
$CuNO_3$	0.5
$MnCl_2$	0.4
Distilled Water	1000 ml

After adjusting the pH at 7.3 ± 0.1 at 25ºC, the medium was heated with frequent agitation and boiled for one minute to completely dissolve the medium. Then the medium was Autoclaved at 121°C for 15 minutes and subsequently, cooled to room temperature. The cooled MSA was then poured into sterile petri dishes on a level, horizontal surface to give uniform depth. Then allowed the plates to cool to room temperature and stored the plates at 2-8ºC until use. From each processed samples plating was done on plates with MSA media by evenly spreading by the use of sterile cotton-swabs by streaking method.

The inoculated plates were subsequently incubated at 37◦C for 48 hrs. Colonies obtained were further cultured in nutrient agar medium (NAM) containing the following composition:

Ingredients	In Gram/100 ml
Yeast extract	0.3
Peptone	0.5
Glucose	0.5
K_2HPO_4	0.3
$MgSO_4$.7H2O	0.07
Malathion (E.C. 50%)	0.5
Agar	1.5
Distilled Water	100 ml

Table 3. Composition of Nutrient Agar Medium (NAM)

Serial transfer of microorganisms was made by streaking and inoculating to nutrient agar medium containing Malathion.

Selection of pure culture is done by repeating sub-culturing for 4-6 times. The isolated strains were maintained on nutrient agar plates and stored at 4°C.

6. Isolation of Quinalphos Degrading Bacteria:

The bacterial cultures capable of degrading Quinalphos were isolated from collected soil samples using enrichment technique, with some concentration of Quinalphos. Standard analytical grade solution of Quinalphos (25% E.C.) was purchased from the local market. 1 gram of each soil sample was inoculated into 6 different 100 ml Erlenmeyer flask containing 100 ml of mineral salt medium (MSM) supplemented with 0.5 ml concentration of Quinalphos. The composition of Mineral Salts Medium (MSM) is given below:

Ingredients	In Gram/100 ml
$NaNO_3$	0.3
$MgSO_4$	0.05
KCl	0.05
K_2HPO_4	0.1
KH_2PO_4	0.05
$FeSO_4$	0.001
Yeast Extract	0.05
Glucose	1.0
Quinalphos	0.5
Distilled Water	100 ml

Table 4. Composition of Mineral Salts Medium (MSM)

The flasks were incubated on a rotary shaker at 150 cycles per minute for 7 days at room supplemented with Quinalphos (5 grams) and incubated at room temperature for 24-48 hours. Nutrient agar media was prepared by adding 7 grams of agar and 5 grams quinalphos in 250 ml water. Individual colonies of bacteria which temperature (25-30°C). At daily intervals, one loop full of enrichment culture from the flask was streaked on nutrient agar plates varied in shape and colour were picked up and were sub-cultured onto nutrient agar plates containing same concentration of Quinalphos until pure culture was isolated. The isolated strain was maintained at 4°C.

7. Microscopic Study of Bacterial Cultures:

The bacterial isolates were studied for their various microscopic characters such as:

i) Colony Morphology: Study of colony morphology includes colour, size, margin, elevation etc.

ii) Gram's Staining: Gram's staining of the cultures was performed by the standard method.

iii) Motility Test: Motility test was performed by hanging drop method.

8. Identification of Bacterial Isolates:

Identification of bacterial isolates were carried out by the routine bacteriological methods i.e., by the colony morphology, preliminary tests like Gram staining, Motility test, etc. Gram staining reaction was performed to evaluate type of strain.

III. RESULT

A. Isolation of Pesticides Degrading Bacterial Strain:

A total of four bacterial cultures were isolated based on colony morphology. Two different colonies were observed on nutrient agar medium enriched with MALATHION from soil samples of Vegetable Farm Composite and Rice Field Composite. The isolated strains were designated as MS1 and MS2.

i) MS1 - Pesticides Degrading Bacterial Isolate from Rice Field 1 Composite Sample.

ii) MS2 - Pesticides Degrading Bacterial Isolate from Vegetable Farm 1 Composite Sample.

Two different colonies were observed on nutrient agar medium enriched with QUINALPHOS from soil samples of Vegetable Farm Composite and Rice field Composite and were designated as QS1 and QS2.

i) QS1 - Pesticides Degrading Bacterial Isolate from Rice Field 2 Composite Sample.

ii) QS2 - Pesticides Degrading Bacterial Isolate from Vegetable Farm 1 Composite Sample.

B. Microscopic Study of the Bacterial Strains:

i) Colony Morphology: The colony morphology of the four bacterial isolates was recorded in the Table 5.

Sl. No.	Strain label	Colony colour	Size	Shape
1	MS1	Yellowish Grey	0.5 mm	Round, Granular
2	MS2	White	1.5 mm	Round, Smooth
3	QS1	Light Yellow	0.5 mm	Flat and Irregular
4	QS2	Off White	1.0 mm	Irregular Shaped

Table 5. Morphology and Characteristics of Isolated Bacterial Strains

ii) Gram's Staining: The result of the Gram's reactions was recorded in the Table 6.

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Sl. No	Strain label	Gram Character
1	MS1	Positive, Rod
2	MS2	Positive, Coccus
3	QS1	Negative, Rod
4	QS2	Positive, Rod

Table 6. Gram Characters of the Isolated Bacterial Strains

iii) Motility Test: The result of the motility test is recorded in the Table 7.

Sl. No	Strain label	Motility
1	MS1	Motile
2	MS2	Non-motile
3	QS1	Unipolar motility
4	QS2	Motile

Table 7. Motility Test of the Isolated Bacterial Strains

iv) Identification of the Bacterial Isolates: Based on the morphological studies and preliminary tests like Gram staining, Motility test of the isolated colonies, the observed colonies may be identified as follows:

Sl. No	Strain label	Identified Species
1	MS1	*Bacillus species*
2	MS2	*Staphylococcus species*
3	QS1	*Pseudomonas species*
4	QS2	*Bacillus species*

Table 8. Identification of the Bacterial Isolates

IV. DISCUSSIONS

Pesticides constitute the key control strategy for pest management and have been making significant contribution towards improving crop yields. Currently, among the various groups of pesticides that are being used world over, organophosphates form a major and most widely used group, accounting for more than 36 per cent of the total world market (Kanekar et al., 2004). Quinalphos, monocrotophos, chlorpyriphos, malathion, parathion are some of the widely used organophosphorus pesticides.

The widespread use of these pesticides over the years has resulted in problems caused by their interaction with the biological systems in the environment (Serrano et al., 1997). Considering the toxic effect of these pesticides, it is essential to remove these chemo pollutants from the environment. Biological removal of chemo-pollutants becomes the method of choice, since microorganisms can use a variety of xenobiotic compounds including pesticides for their growth and mineralize and detoxify them.

With extensive use of pesticides, environment hazards has led to several problems such as deterioration of soil quality, leaching, acidification, denitrification, air pollution, and reduced biodiversity, disrupting the ecosystem. To protect the environment, best remedy is to use the ecofriendly microbes to reduce the contamination.

V. CONCLUSIONS

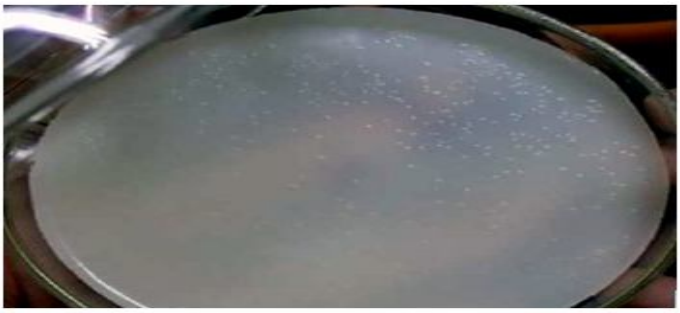
The focus of the present study was to isolate and characterize the most efficient microorganisms capable of degrading malathion and quinalphos pesticides. Some bacterial species isolated from the agricultural soil, namely Bacillus species, Staphylococcus species, Pseudomonas species and Bacillus species which were labelled as MS1, MS2, QS1 and QS2, respectively, showed the ability to degrade malathion and quinalphos pesticides. The results obtained suggest that the bacterial species, being isolated, can be used to decontaminate the area polluted by these organophosphorus pesticides by the process of Bioremediation.

Thus, this study concludes that the isolated bacterial species were capable of degrading the organophosphorus pesticides and therefore, had potential of Bioremediation of these pesticides. Further research into the molecular biology of these species can result into the development of 'Superbugs' that can degrade these pesticides more effectively and efficiently.

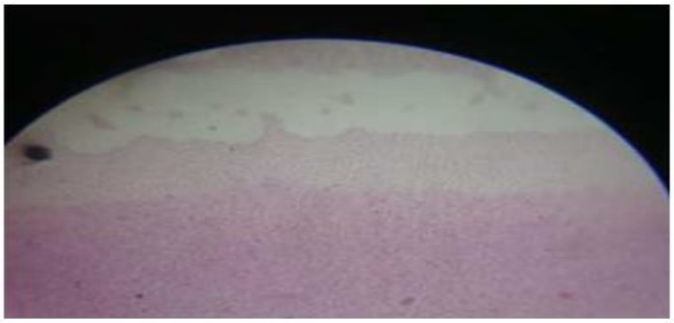
Photograph 1: Pure Culture of Bacterial Strain Isolated from Rice Field 1 Composite Sample (MS1)

Photograph 3: Pure Culture of Bacterial Strain Isolated from Rice Field 2 Composite Sample (QS1)

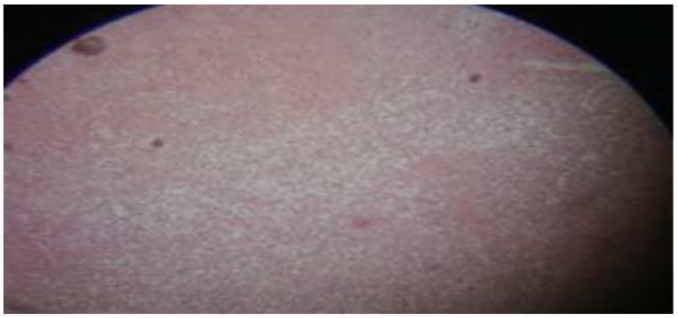
Photograph 2: Pure Culture of Bacterial Strain Isolated from Vegetable Farm 1 Composite Sample (MS2)



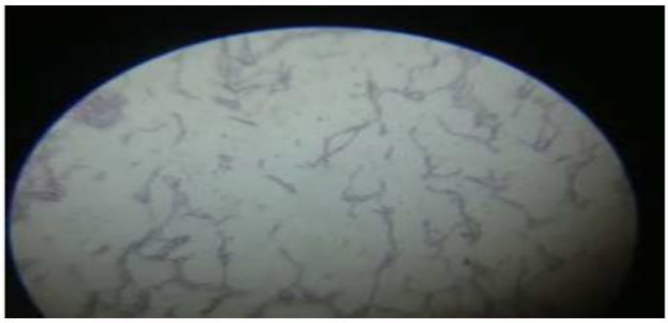
Photograph 4: Pure Culture of Bacterial Strain Isolated from Vegetable Farm 1 Composite Sample (QS2)



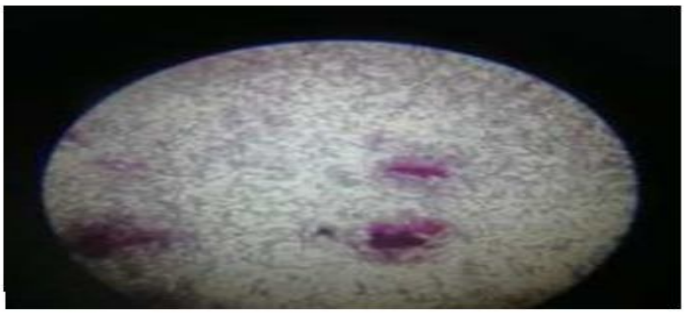
Photograph 5: Microscopic View of Bacterial Strain Isolated from Rice Field 1 Composite Sample (MS1)



Photograph 7: Microscopic View of Bacterial Strain Isolated from Rice Field 2 Composite Sample (QS1)



Photograph 6: Microscopic View of Bacterial Strain Isolated from Vegetable Farm 1 Composite Sample (MS2)



Photograph 8: Microscopic View of Bacterial Strain Isolated from Vegetable Farm 1 Composite Sample (QS2)

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