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SCREENING OF PGPR TRAITS AND MONOCROTOPHOS PESTICIDE DEGRADATION PROPERTIES OF ACTINOBACTERIAL ISOLATES

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Keywords:

Actinobacterial isolates, PGPR traits, Monocrotophos pesticides, Bioremediation, Rhizoremediation, Reduction in soil toxicity, Recombinant cells, Least toxic pesticide derivatives

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ABSTRACT: Bioremediation of organic compounds and heavy metals has been recognized to be a successful and effective technique in rhizoremediation of soil toxicity using the beneficial microbes which produce different metabolites and enzymes to degrade the toxic compounds/ pesticides available in the soil. In the present study, actinobacterial isolates were explored and identified for bioremediation of soil toxicity available in the soil. The actinobacterial isolates were utilized for the promising PGPR traits and ability to degrade the monocrotophos pesticide residues available in the soil. The "*opd"* gene of interest responsible for monocrotophos pesticide degradation trait was isolated and cloned in vector DNA to produce recombinant DNA. The recombinant DNA was transformed in *E. coli* cells to produce multiple copies in E. coli cells resulting in recombinant (transformed) and non-recombinant (non-transformed) colonies. The transformed *E. coli* cells were inoculated in nutrient broth having pesticide concentration. The transformed cells degraded the pesticide, and the HPLC method determined the derivatives produced. The formulations based on actinobacterial isolates were tested, and field applications were done to determine the reduced soil toxicity, if any, observed.

INTRODUCTION: Bioremediation is also used to convert hazardous substances into less toxic components. Microorganisms have a vital role in the breakdown and mineralization of these contaminants. Bioremediation as sustainable technology is significant in examining and reducing manmade chemicals released into the environment. Bioremediation is the microorganisms used to attain the function of bioremediation. Bioremediation technology has been commonly categorized as *ex-situ* and *in-situ* bioremediation.

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In situ bioremediation comprises the treatment and elimination of the contaminated material at the site, whereas, in *ex-situ*, the contaminated are treated somewhere else. bioventing, bioleaching, biostimulation, land farming, composting, bioaugmentation, rhizofilteration, and phytoremediation are a few examples of bioremediation technologies [1-5]. Biodegradation and bioremediation are similar methods up to an extent since both of these approaches employ microorganisms for the alteration or breakdown of pesticides.

The only difference between these two is that biodegradation is a natural process whereas bioremediation is considered technology. Various aspects are limiting factors for pesticide degrading microorganisms, such as pH, temperature, nutrients, water potential and a number of

metabolites or pesticide content in soil [6-8]. Actinobacterial isolates produce non-antibiotic molecules which exhibit bioactivities, such as immuno-suppressors, phytotoxins, bio-pesticides, Nano-particles, probiotics, enzyme inhibitors and different enzymes and proteins that are involved in the degradation of complex polymers and biomolecules [9-12], that single isolates can mineralize a limited number of these xenobiotic pesticides. Still, consortia of bacteria are mostly required for complete degradation of the process. Members of this group of gram-positive bacteria have been found to degrade pesticides with widely different chemical structures, including organochlorines, *s*-triazines, triazinones, carbamates, organophosphates, organ phosphonates, acetanilides and sulfonylureas. Single isolates can mineralize a limited number of these xenobiotic pesticides, but consortia of bacteria are often required for complete degradation [13-15].

MATERIALS AND METHODS:

Screening of Isolates for PGPR Traits: The actinobacterial isolates were screened for PGPR traits [16-20].

A. IAA Production: Indole acetic acid (IAA) production was quantitatively estimated by Salkowski method. The actinobacterial cultures were grown on Luria broth liquid medium at 36±2 °C. Fifty milliliter of Luria Bertani (LB) broth containing 0.1% DL tryptophan were inoculated with 500 µl of 48 h old actinobacterial cultures and incubated in refrigerated incubator shaker at 30±0.1°C at 180 rpm for 48 h in dark. Fully grown bacterial cultures were centrifuged at 10,000 rpm for 10 minutes at 4°C. Estimation of IAA production in the supernatants was done using a colorimetric assay. One milliliter (1 ml) of supernatant was mixed with 100 ml of 10 mM orthophosphoric acid and 2 ml of the Salkowski reagent (1 ml of 0.5 M FeCl3 in 50 ml of 35% HCIO4) at 28±2 ^{o}C for 30 minutes. The development of pink colour in test tubes at the end of the incubation indicated IAA production. The pink colour absorbance measured the quantification of IAA at 530 nm after 30 minutes in UV/VIS spectrophotometer. The results were tabulated as Higher IAA producers (+++); Medium IAA producers (++), and Lowest IAA producers (+).

B. Phosphate Solubilization: A loop full of isolated pure fresh actinobacterial cultures was streaked on the Centre of agar plates modified with Pikovskaya agar with insoluble tricalcium phosphate (TCP) and incubated for 120 h at 28±2°C 18. The halo zone around the bacterial colonies indicated positive phosphate solubilization ability (Pikovskaya, 1948). The results were tabulated as Higher phosphate solubilizers (+++); Medium phosphate solubilizers (++), and Lowest phosphate solubilizers (+).

C. Siderophores and HCN Production: Qualitative estimation of siderophore production by the actinobacteria isolates was determined by adopting the modified chrome azurol sulphonate (CAS) assay method. Production of siderophore was determined by developing an orange halo zone around the actinobacterial colonies. In addition, all the bacterial isolates were screened for HCN production. The Colour change of the filter paper from deep yellow to reddish-brown colour indicated the production of HCN. The results were tabulated as Higher Siderophores and HCN producers (+++); Medium Siderophores and HCN producers (++) and Lower Siderophores and HCN producers (+).

D. Catalase Activity: Actinobacterial cultures were grown in a nutrient agar medium for 48 h at 28°C. The 48-hour-old bacterial colonies were added with 2-3 drops of hydrogen peroxide (3%) on a clean glass slide and mixed using a sterile toothpick. Oxygen evolution as effervescence indicated catalase activity (Rorth and Jensen, 1967). The results were tabulated as Higher Catalase producers (+++); Medium Catalase producers (++), and Lower Catalase producers (+).

Screening of Actinobacterial Isolates for Monocrotophos Pesticides Degradation:

Primary Screening:

Determination of Zone of Clearance of Pesticide Degradation: The screening of actinobacterial isolates for monocrotophos pesticide degradation was performed as per the method described. The growth of isolated actinobacterial cultures were inoculated separately in 100 ml Mineral Salt Media (MSM) enriched with an addition of 25 ppm MCP pesticide. These samples were incubated on a rotary shaker (150 rpm) at 30°C for 7 days. The

growth curve for the actinobacterial isolates was determined via optical density/absorbance using UV- spectrophotometer at various time intervals. After which one, the cultures were frequently transferred every 3-4 days or until increased turbidity was evidenced.

After 3-4 times of repeated sub-culturing, 0.1 ml culture broth was pipette and introduced in wells punctured within pesticide agar plates. Cultures were incubated at 30°C for 5-8 days. Pesticide degrading isolates were then screened, which developed a clear zone around the wells determining clearance [21].

Secondary Screening:

Determination of Pesticide Degradation by Culture Streak Technique: The pesticide degradation was observed on pesticide-enriched agar by streaking the actinobacterial cultures; the pesticide degradation ability of the actinobacterial isolates was determined as a clearance zone.

Expression of Genes of Promising Isolates of Actinobacteria in *E. coli* Cells: *Escherichia coli* was utilized as one of the organisms of choice to produce recombinant proteins. Its use as a cell factory is well-established and it has become the most popular expression platform. For this reason, there are many molecular tools and protocols for the high-level production of multiple functional proteins. In the present investigation, the genes for monocrotophos pesticide (MCP) degradation were isolated from promising actinobacterial isolates, fused with vector DNA and transformed into E. coli cells. The recombinant *E. coli* cells were further utilized for the biodegradation of monocrotophos pesticides (MCP). The enzymes for degradation of monocrotophos pesticides were identified as phosphodiesterase (PTE) or organophosphorus hydrolase (OPH) specifically encoded by the opd gene. This gene has been Expression in genes of promising isolates of Actinobacteria in *E. coli* cells.

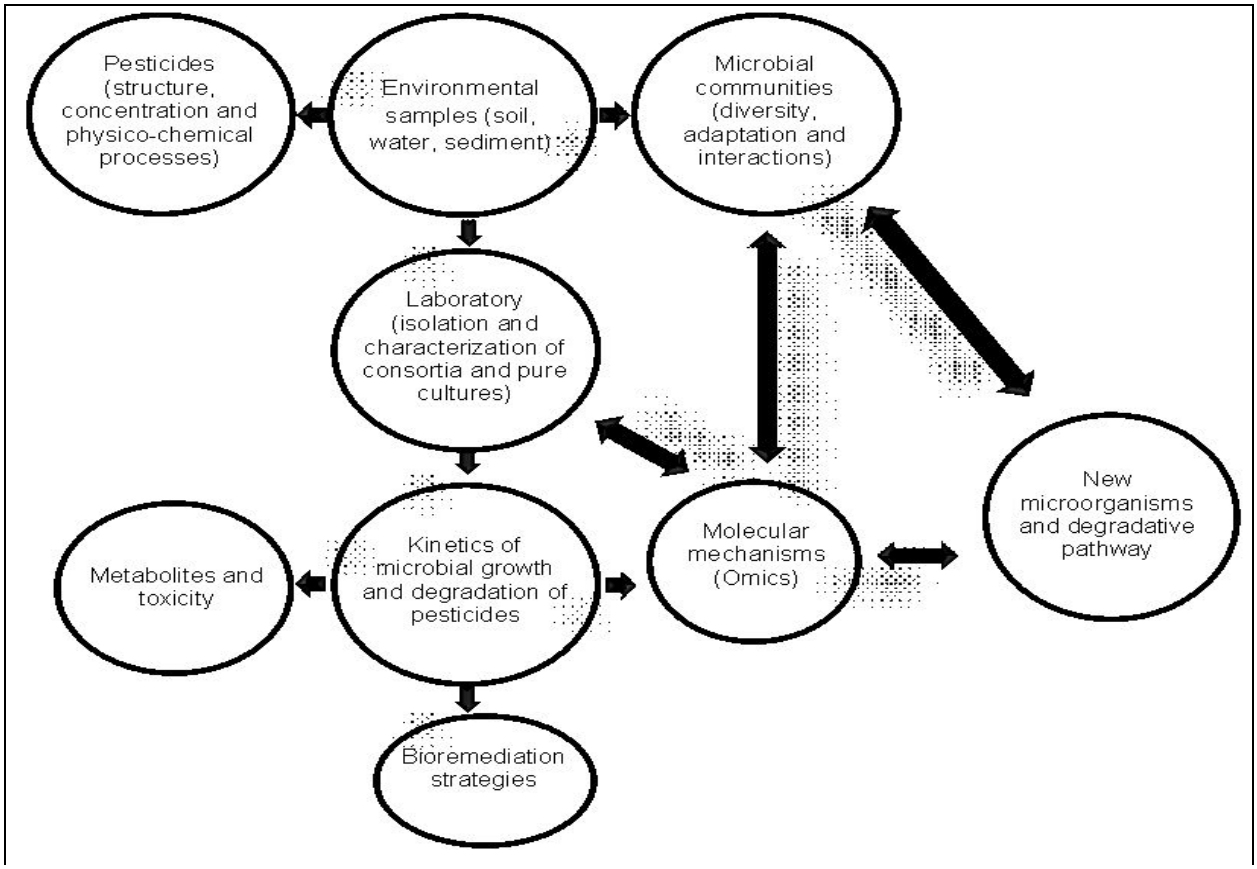


IMAGE 1: REPRESENTATION OF THE RELATIONSHIPS BETWEEN PESTICIDES, MICROBIAL COMMUNITIES AND THE DISCOVERY OF NEW BIODEGRADATION PROCESSES OMICS = HIGH THROUGHPUT-BASED CHARACTERIZATION OF BIOMOLECULES CHARACTERISTIC OF BIOPROCESSES; DNA, GENOMICS; MRNA, TRANSCRIPTOMICS; PROTEIN, PROTEOMICS; METABOLITES, METABOLOMICS

Biodegradation of Monocrotophos Pesticides: The concentrations of monocrotophos pesticide (MCP) at 10 µg/ml were utilized in nutrient broth in different sets, followed by sterilization and inoculation of the E. coli cells and incubation of the broth for 48 hours duration. The degradation of MCP pesticide in the broth culture was determined using TLC and HPLC, as described by Chao *et al.,* 2009. In brief, 4 mL of filtrate was extracted with ethyl acetate from a 5-mL aliquot of culture supernatant. All experimental MCP concentrations were determined at 276 nm by HPLC (PerkinElmer 200 series, CT, USA). The solution filtrate (10 µL) was separated and added with methanol (1:1). Ten, it went across 0.45- µm PVDF filter and injected into the HPLC column (C18, 250×4.6 mm, 5µm, Phenomenex, CA, USA). The 10 µL filtered sample was injected into the HPLC column, and the 0.5% acetic acid and methanol (1:4 v/v) were used as eluent at 1 mL/min flow rate.

Formulations of Actinobacterial Strains: The formulations were prepared as per the following modified methods [22].

1. Alginate-Kaolin Based Granular Formulation (AL-KAO Granular Formulation): Sodium alginate solution was prepared by dissolving 20 g of dry sodium alginate in a minimum volume (10 ml) of sterile distilled water. This mixture was poured into 1 L of swirling, warm, distilled water and allowed to mix on the stirrer for 30 min until a homogeneous suspension was obtained. The alginate solution was sterilized for 15 min at 121 °C and 101 kPa. Twenty grams of the potent dried Actinobacterial isolate propagules were mixed thoroughly with 20 g of previously sterilized kaolin (aluminum silicate), and the mixture was added in small portions (2 g) into 1 L of swirling, sterile distilled water supplemented with 4 drops of Tween 20. The alginate-kaolin-actinobacterial mixture was allowed to swirl in the stirrer until ready for mixing with the sodium alginate solution. A droplet-forming device was constructed by attaching a 1-L reagent bottle with a spout at the bottom to a T-valve outlet system. The entire device was sterilized for 15 min at 121 °C and 101 kPa before use. The sodium alginate solution and kaolin mixture were added to the reagent bottle in 1:1 ratio and stirred continuously. At the same time, the suspension was allowed to drip through an Eppendorf pipette tip, attached to the T- 10 valve, into a sterile solution of 0.1 M $CaCl_2$. The resulting alginate-kaolin beads were then allowed to stand in a fresh 0.1 M $CaCl_2$ solution for 30 min, filtered through a sterile cheese cloth, and washed at least three times with sterile distilled water. The beads were lyophilized at -70 °C, and their dry weight was recorded. This lyophilized granular formulation of mycobacterium (hereafter referred to as beads) was stored in the dark at 4 °C.

2. Flour-Kaolin Based Granular Formulation (FLO-KAO Granular Formulation): The flour and kaolin material were sterilized separately for 15 min at 121 °C and 101 kPa. Upon cooling, 200g of flour, 4 g of kaolin, and 20 g of the inoculum were mixed thoroughly and sieved through a sterile 250 µm pore screen to obtain a homogeneous mixture of the three components. To this mixture, 180 ml sterile distilled water supplemented with 2 drops of Tween 20 was added slowly and mixed thoroughly until a dough was formed. The flakes were lyophilized at -70°C. The lyophilized formulations in liquid hereafter referred to as granules) were stored in the dark at 4°C.

3. Calcium carbonate-CMC Based Powder Formulation (CC-CMC Powder Formulation): The calcium carbonate and CMC material was sterilized separately for 15 min at 121 °C and 101 kPa. Upon cooling, CMC was mixed with 20 g of the inoculum thoroughly and sieved through a sterile 250 µm pore screen to obtain a homogeneous mixture. For this mixture (10 Kg) produced with CMC, the quantity of calcium carbonate was mixed in 90 Kg concentration. The lyophilized formulations in liquid, referred to as powder, were stored in the dark at 4 °C. The material was further utilized to check the shelf life of isolates and the application of powder in the bioremediation of soil toxicity.

RESULTS AND DISCUSSION: As per the studies performed, 120 soil samples were collected from different field areas of the Uttarakhand region (Tehri-Garhwal, Chamoli, Srinagar, Uttarkashi, and Haridwar) having dominant usage of monocrotophos pesticides. Amongst these samples, a total of 280 microbes was isolated; out of which 24 isolates of Actinobacteria (8.57%) were isolated. The actinobacteria isolates were screened on

specific agar media and characterized by morphological colony appearance and staining procedures. The actinobacteria isolates were categorized based on a) type of pigment production and colony and color**.** These actinobacterial isolates were further screened for their identification by molecular. The results revealed the strains of the genera *viz*. Micromonospora (65%), Actinomycetes (25%) and Streptomyces (10%) (Data not shown). The isolates of each of the mentioned genera were screened for PGPR assays **Table 1.**

TABLE 1: PGPR ASSAYS OF ACTINOBACTERIAL ISOLATES

S. no.	Strain No.	PGPR Assays			
		IAA production	Phosphate solubilization	Siderophore and HCN production	Catalase activity
1	ASUK03	+++	+++	++	++
2	ASUK07	+++	+++	++	++
3	ASUK254	+++	+++	++	++
4	ASUK145	+++	+++	+++	+++
5	ASUK67	+++	+++	+++	+++
6	ASUK86	++	++	+	++
7	ASUK46	+++	+++	+++	++
8	ASUK34	+++	+++	+++	+++
9	ASUK23	+	+++	-	++
10	ASUK60	+++	+++	-	++
11	ASUK79	+++	+++	+++	+++
12	ASUK224	++	+++	+++	+++
13	ASUK185	+++	+++	+++	+++
14	ASUK145	+++	+++	+++	+++
15	ASUK76	+++	+++	+++	+++
16	ASUK216	+++	+++	+++	+++
17	ASUK237	++	+++	+++	+++
18	ASUK259	+++	+++	+++	+++
19	ASUK263	+++	+++	+++	+++
20	ASUK283	++	+++	+++	+++
21	ASUK292	+++	+++	+++	+++
22	ASUK308	+++	+++	+++	+++
23	ASUK315	++	+++	+++	+++
24	ASUK423	+++	+++	+++	+++

*+++, Dominant producer, ++, Medium producedr, +, Lowest Producer, –, Negligible producer

TABLE 2: PERCENTAGE DEGRADATION OF ACTINOBACTERIAL ISOLATES

S. no.	Strain code	Percent degradation rate of monocrotophos pesticide
1	Micromonospora	55.0
2	Actinomycetes	45.0
3	Streptomyces	42.0

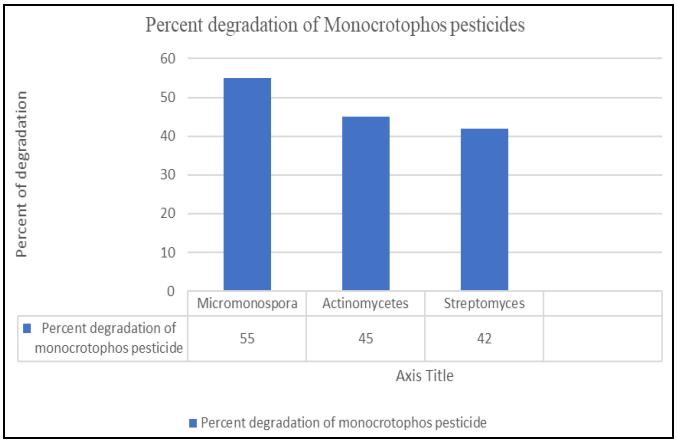


FIG. 1: PERCENT DEGRADATION RATE OF ACTINOBACTERIAL ISOLATES

The degradation rate (%) of the actinobacterial isolates was calculated based on no. of strains of the respective genera which showed a zone of clearance. The results are shown in **Table 2** and **Fig. 1, Fig. 2** and **Fig. 3.** The isolated *opd* gene of interest was isolated and cloned in vector DNA to produce recombinant DNA **Fig. 4A, Fig. 4B.** The recombinant DNA was transformed in *E. coli* cells to produce multiple copies in *E. coli* cells resulting in recombinant (transformed) and non-recombinant (non-transformed) colonies. The transformed *E. coli* cells were inoculated in nutrient broth having pesticide concentration.

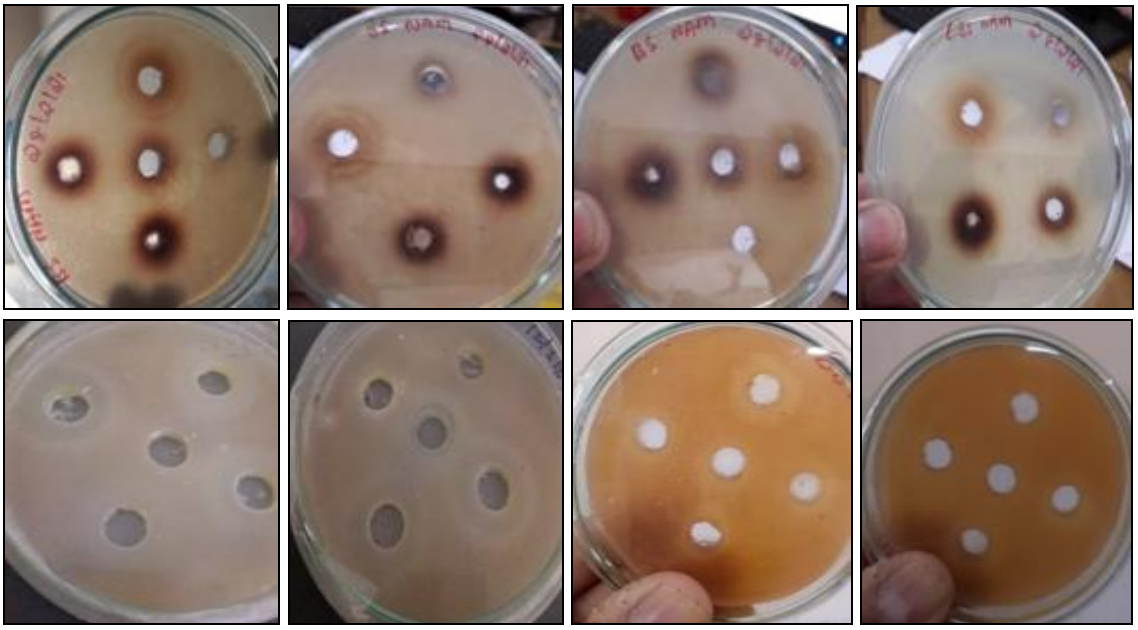


FIG. 2: PRIMARY SCREENING: DETERMINATION OF ZONE OF CLEARANCE OF PESTICIDE DEGRADATION

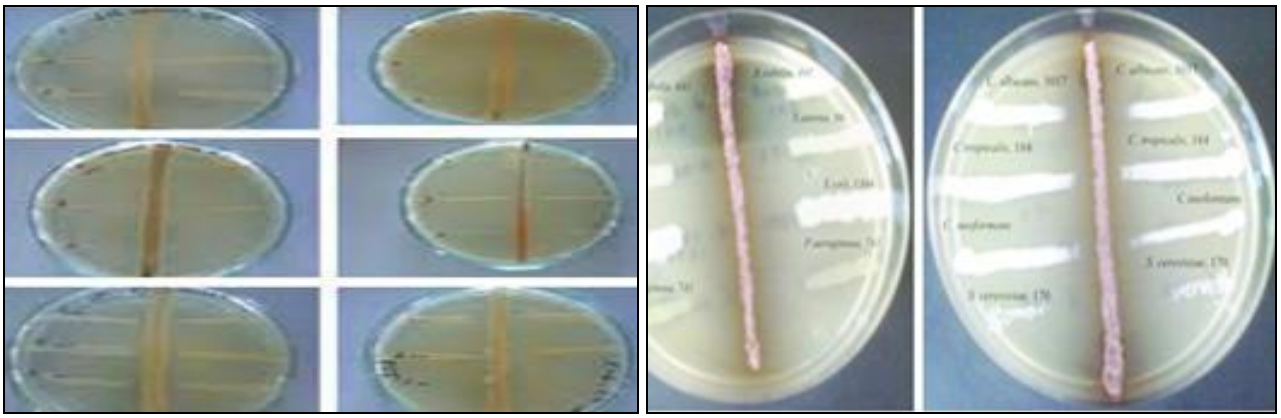


FIG. 3: SECONDARY SCREENING- DETERMINATION OF PESTICIDE DEGRADATION BY CULTURE STREAK TECHNIQUE

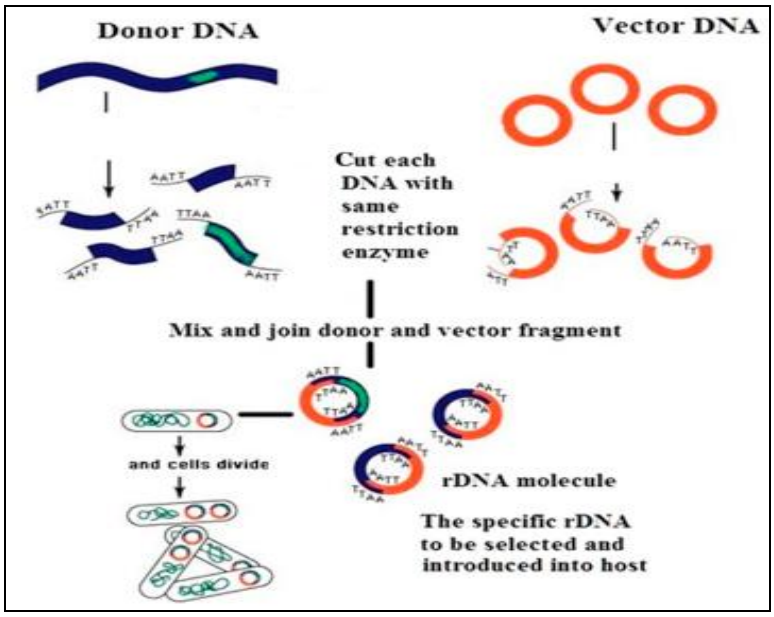


FIG. 4(A): PREPARATION OF RECOMBINANT DNA AND TRANSFORMATION IN E. COLI CELLS

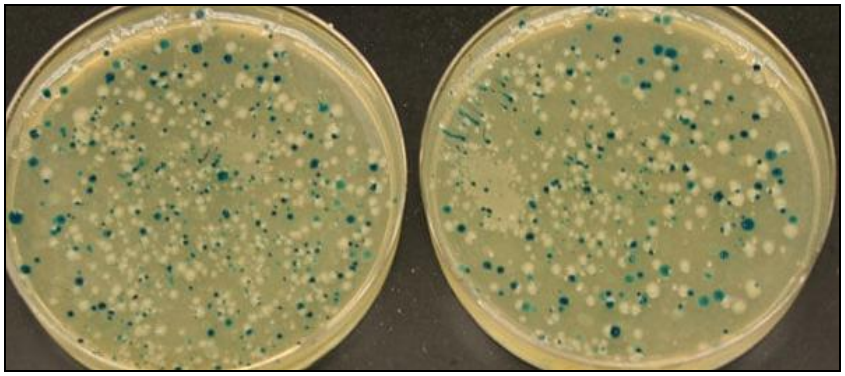
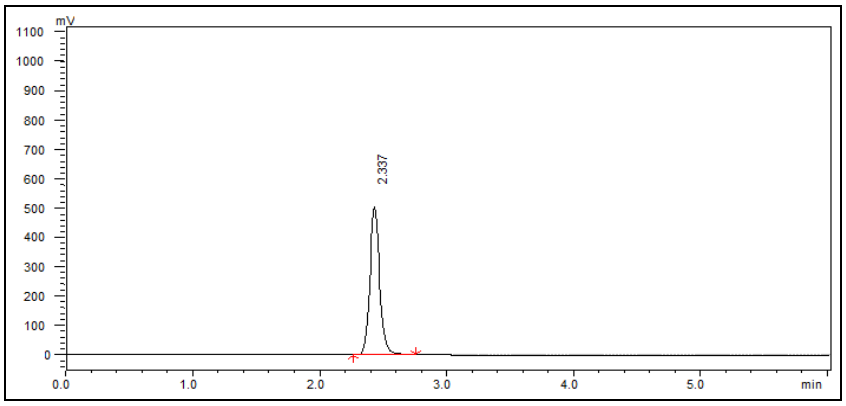


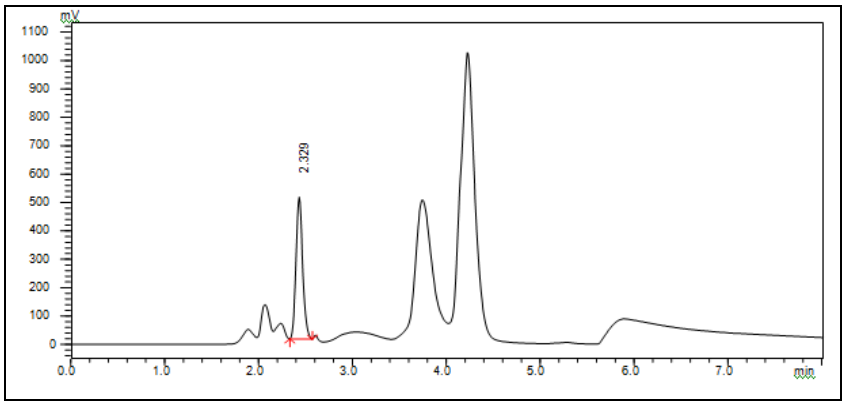
FIG. 4(B): SCREENING OF RECOMBINANT AND NON- RECOMBINANT COLONIES (BLUE COLONIES WERE REGARDED AS NON-RECOMBINANT COLONIES WHILE WHITE COLONIES WERE REPRESENTED AS RECOMBINANT COLONIES)



DETECTOR A CH1 276NM

Peak #	Name	Ret. Time	Area	Height	Area %	Height %
1	RT2.337	2.337	2732519	505147	100.0000	100.0000
Total			2732519	505147	100.0000	100.0000

FIG. 5(A): HPLC CHROMATOGRAM OF ORIGINAL MCP AVAILABLE IN NUTRIENT BROTH



DETECTOR A CH1 276NM

Peak #	Name	Ret. Time	Area	Height	Area %	Height %
1	RT2.329	2.329	2452373	500142	100.0000	100.0000
Total			2452373	500142	100.0000	100.0000

FIG. 5(B): HPLC CHROMATOGRAM OF DEGRADATIVE MCP RESIDUES AVAILABLE IN NUTRIENT BROTH AFTER BIODEGRADATION

The transformed cells degraded the pesticide and derivatives produced were determined by HPLC method **Fig. 5A, Fig. 5B.** Three formulations of actinobacterial strain *viz.,* Alginate-Kaolin Based Granular Formulation (AL-KAO granular formulation), Flour-Kaolin Based Granular Formulation (FLO-KAO granular formulation), Calcium carbonate-CMC Based Powder Formulation (CC-CMC powder formulation) were prepared and utilized in field trials to study the

reduction in soil toxicity. The results revealed that, when applied to monocrotophos enriched soil, FLO-KAO granular formulation revealed 62% degradation in toxicity **Table 3, Fig. 6.**

TABLE 3: FIELD APPLICATION TRIALS OF FORMULATIONS

Formulation treated sets/Control	Average readings of best 5 plots (25 crops per plot) after 2 months				
	Crops number per plot	Average height of crops (cm)	Average leaves number in crops	Average root length of crops (cm)	Percent reduction in soil toxicity
Control (Liquid actinobacterial inoculum)	25	30.0	10.0	15.0	30.0
AL-KAO granular formulation	25	35.0	15.0	22.0	54.0
FLO-KAO granular formulation	25	42.0	25.0	25.0	62.0
CC-CMC powder formulation	25	37.0	18.0	22.0	58.0

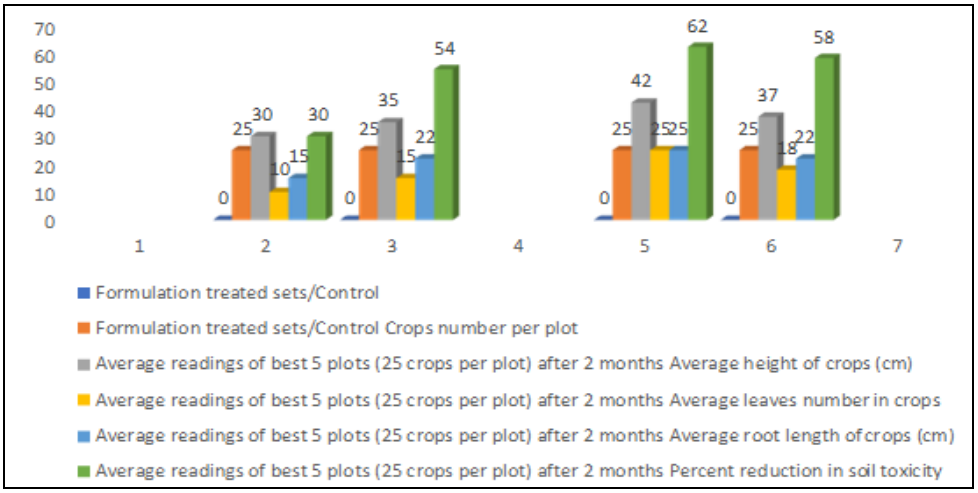


FIG. 6: GRAPHICAL REPRESENTATION OF FIELD APPLICATION TRIALS OF FORMULATIONS

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