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Bioremediation of Persistent Pesticides in Vegetable field Soil Environment using Microbial consortium

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Abstract

The increasing use of various pesticides in vegetable field has resulted into contamination of soil leading to toxicity in the biological diversity. Bioremediation is found to be an effective technology for treatment of pesticides polluted soil using microbial consortium. In the present study, the soil samples were collected from the vegetable field of Alampur village, Gandhinagar district of Gujarat state, having repeated application of pesticides such as chlorpyrifos and methyl parathion. Isolation of pesticides degrading bacteria was carried out and the isolated bacteria were identified as *Serratia* sp., *Bacillus* sp., *Moraxella* sp., *Photobacterium* sp. and *Ralstonia* sp. The bacterial consortium (*Bacillus* sp., *Moraxella* sp. and *Photobacterium* sp.) was developed for bioremediation of commonly used pesticides namely chlorpyrifos and methyl parathion in Surface Soil Treatment Reactor (SSTR) under simulated environmental conditions. The analysis of pesticides degradation was performed by HPLC. The results of bioremediation of pesticides contaminated soil using bacterial consortium in surface soil treatment reactor show the 62.72 % degradation of chlorpyrifos and 65.99% degradation of methyl parathion within a period of 10 days.

Key words: Pesticides, Bioremediation, surface soil treatment reactor, microbial consortium.

1. Introduction

India is an agrarian country with more than 60-70% of its population dependent on agriculture and plays an important role in its economy (Sachdeva, 2007). Among all the agricultural crops, vegetables are an important cash crop. Fresh vegetables are an essential part of a healthy diet as it is an important source of vitamins and minerals. The global share of India, in vegetable production is about 13.4%. However, vegetables can also be a source of poisonous toxic substance pesticides (Knezevic Z. and Serdar M., 2008). Earlier surveys carried out by different institutions throughout the country indicated that 50-70% of total vegetables are contaminated with pesticide residues (Karanth, 2002). The consumption of pesticides in

vegetable field is 14% of the total pesticides used in India, in which, organophosphorus (50%) is the maximum used pesticides followed by pyrethroids (19%), organochlorines (18%), carbamates (4%) and biopesticides (1%) (Dhaliwal, GS. and B. Singh, 2000).

Indian farmers use about 6000 tonnes of active ingredients to control pests in vegetables and fruits (Mohan, M. and GT. Gujar, 2003). This extensive and improper use of pesticides causes accumulation of huge amount of pesticide residues in the environment, therefore leading to a substantial environmental health hazard due to uptake and accumulation of these toxic compounds in the food chain and drinking water (Mohammed, 2009). Improper handling and unsafe spraying of the agrochemicals cause high risk of health hazards (Bag, 2000; Gupta, 2004).

According to the data available by World Health Organization, only 2-3% of these pesticides applied for mitigation of pests are utilized at target point whereas rest remains in the environment causing surface runoff, leaching and percolation into soil water environment leading toxicity to biota and human being through food chain (EPA, 2005). Increased use of pesticides can result in various health and environmental problems like pesticides poisoning in farmers and farm workers, neurological and skin disorders, cardiopulmonary, miscarriages, foetal deformities and lowering the sperm counts in applicators (Bag, 2000).

The hazardous effects of pesticides draw attention towards their removal from the environment. Although, different conventional methods such as chemical treatment, recycling, pyrolysis, incineration are able to degrade persistent pollutants hazardous to human health as well as the environment, but they are less efficient. The natural degradation of the pesticides in the environment using microbial action may leads to conversion of parent compounds into intermediates or comparatively less toxic compounds. However, the process of natural bioremediation is slow and needs to enhance the biodegradation of contaminants in the environment by the action of the potential microorganisms (Fulekar, 2005a). The adaptability of microorganisms during bioremediation releases certain enzymes, which metabolizes wide spectrum of anthropogenic chemicals (Fulekar, 2005b). Many bacteria having capacity to degrade organophosphate pesticides have been isolated from soil around the world (Zhongli *et. al*, 2001, Horne *et. al*, 2002 and Chang *et. al*, 2005). The most common Gram negative soil bacterium *Pseudomonas aeruginosa* has potential to degrade chlorpyrifos (Fulekar and Geetha, 2008).

In the present study the commonly used pesticides chlorpyrifos and methyl parathion have been taken for bioremediation under controlled environmental conditions. The bacterial consortium was developed for bioremediation of surface soil containing pesticides in surface soil treatment reactor by monitoring and maintaining environmental parameters under simulated conditions. This technique will be effective for bioremediation of pesticides in agricultural soil.

2. Materials and Methods

2.1 Soil collection

The soil samples were collected from vegetables field up to a depth of 15 cm from Alampur village, Gandhinagar district of Gujarat state. The samples collected were air dried, ground, passed through 2 mm sieve and stored in the sealed plastic bags at room temperature. The important physico-chemical parameters of the soil, viz. temperature, pH, electrical conductivity, moisture contents, water holding capacity, bulk density, hardness, chloride, alkalinity, total organic carbon, total organic matter, sulphate, nitrate, nitrite, ammonium, available phosphorus and total phosphorus were carried out using standard methods (APHA,1998).

2.2 Isolation and identification of bacterial isolates

Pour plate technique was used for the isolation of pesticides degrading bacteria in nutrient agar medium. Well grown bacterial colonies were picked and further purified by streaking. Identification of these seven different bacterial isolates were carried out by the routine bacteriological methods i.e., by the colony morphology, preliminary tests like Gram staining and biochemical analysis (Bergrey's Manual of Determinative Bacteriology, 1994).

2.3 Development of microbial consortium for bioremediation of pesticides

The microbial consortium was developed from bacterial cultures, which were compatible with each other in order to concomitantly produce all those enzymes required for the degradation of pesticides from agricultural field. For this, all isolated bacteria were grown on nutrient broth and incubated at 37°C at 120 rpm. The combination of bacterial isolates was based on permutation combination. The compatibility of the bacterial strains within the consortium was checked by increasing optical density at 600 nm by spectrophotometer (Dynamica CE, model no. DB 20).

2.4 Bioremediation of pesticides in surface soil treatment reactor

A surface soil treatment reactor (SSTR) was designed and fabricated with the dimension of 26 x 16 x 8 cm. The reactor was developed in such a way that continuous aeration was provided with the help of aerator. The soil samples collected from vegetable fields were taken in the reactor. Pre developed microbial consortium was added to the soil and mixed properly. Bioremediation conditions like moisture content, temperature were monitored and maintained in the surface soil treatment reactor. During the period of experiment of 10 days soil sampling was done on alternate day for analysis of physico-chemical parameters.

2.5 Extraction of pesticides from soil samples

Soil samples (10 g) drawn at the interval of two days were dried for pesticides extraction using 200 ml dichloromethane and acetonitrile in a soxhlet extraction assembly. The solvents dichloromethane and acetonitrile were selected according to the solubility of methyl parathion and chlorpyrifos respectively. The 200 ml soxhlet extract was concentrated with a rotary evaporator to 10 ml for HPLC analysis.

2.6 Analysis of Chlorpyrifos and Methyl parathion degradation by High Performance Liquid Chromatography (HPLC)

All reagents were of analytical or HPLC grade. Acetonitrile (CH_3CN), Dichloromethane, Chlorpyrifos and Methyl parathion were purchased from Sigma Aldrich. The water used in HPLC was from milli/Q system. The mobile phase was filtered through a whatman filter paper (90 mm, 0.45µm pore size). All data for quantification of chlorpyrifos and methyl parathion were obtained by applying the gradient elution program shown in **Table 1.** Chlorpyrifos and Methyl parathion were analysed with Agilent 1260 series LC system with UV detector at 225 nm and 273 nm respectively having flow rate 1ml/minute.

Table: 1. HPLC conditions used for analysis of Chlorpyrifos and Methyl parathion

HPLC Conditions	Chlorpyrifos	Methyl parathion
Column	C18	C18
Flow rate	1ml/min	1ml/min
Column temperature	25°C	25°C
Injection volume	10 µl	20 µl
Mobile phase	Acetonitrile : Water (70:30)	Acetonitrile:Water (90:10)
Retention time	6.9 minute	1.1 minute
Wavelength	225 nm	273 nm

3. Results and Discussion

The repeated and continuous use of pesticides in vegetable fields has posed a serious environmental problem. The aim of the present study was to establish an advanced and highly effective remediation method for the removal of persistent pesticides (chlorpyrifos and methyl parathion) using developed microbial consortium in surface soil treatment reactor. The isolated indigenous bacterial isolates were identified morphologically and biochemically and further used for the bioremediation of chlorpyrifos and methyl parathion in surface soil treatment reactor. Before bioremediation, the physico chemical characteristics of vegetable field soil were carried out as shown in **Table 2**, which indicates presence of chloride, sulphate, phosphorus, nitrogen, total organic carbon and total organic matter. The microbiological characterization of soil was also carried out, which includes isolation and identification of seven different species of bacteria as shown in **Table 3, Table 4** and **Fig. 1**. The presence of nutrients as well as bacterial consortium in soil has been found to have great influence for the bioremediation of pesticides. The bacterial consortium used for the remediation of chlorpyrifos and methyl parathion in surface soil treatment reactor has combination of bacterial species i.e *Bacillus* sp., *Moraxella* sp. and *Photobacterium* sp.

Table: 2. Physico-chemical properties of Vegetable field soil

Parameters	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Average**	**S D**
Temperature (°C)	27.5	27.4	27.4	27.5	27	27.36	0.207364
pH	7.7	7.4	7.5	7.4	7.6	7.52	0.130384
Electrical conductivity (µs cm $^{-1}$)	142	125	137	148	169	144.2	16.23884
Moisture content (%)	16.13	16.15	18.04	18.31	18.57	17.44	1.201457
Water holding capacity (%)	37.07	36.80	37.32	37.05	36.86	37.02	0.204573

Bulk density (g/ml)	1.69	1.71	1.70	1.70	1.70	1.7	0.007071
Hardness (mg $CaCO_3$/kg)	32	34	30	34	28	31.6	2.607681
Chloride (mg/kg)	130	115	165	150	115	135	22.0794
Alkalinity ($CaCO_3$ mg/L)	140	90	120	100	100	110	20.00
Sulphate (mg/kg)	3.08	3.24	3.48	3.03	3.32	3.23	0.182483
Inorganic phosphorus (mg/kg)	0.83	0.83	0.91	0.91	0.84	0.864	0.04219
Total phosphorus (mg/kg)	3.66	3.46	7.84	7.71	3.39	3.212	0.413606
Nitrate-N (mg/kg)	10.91	11.38	11.05	11.24	11.85	11.286	0.362671
Nitrite-N (mg/kg)	0.095	0.095	0.092	0.093	0.095	0.094	0.001414
Ammonium -N (mg/kg)	1.96	1.75	1.85	1.99	1.83	1.876	0.098387
Total organic carbon (%)	1.2	1.05	1.2	1.35	0.9	1.14	0.171026
Total organic matter (%)	1.64	1.70	1.65	1.69	1.68	1.672	0.025884

Table 3. Biochemical characteristics of bacteria isolated from Vegetable field

Tests	V1	V2	V3	V4	V5	V6	V7
Gram stain	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods
CFU counts (N x 10^5Cfu/g)	1.50	1.32	1.15	2.25	1.65	1.72	1.38
Motility test	-	+	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+
Oxidase test	-	-	+	+	+	+	-
Urease test	+	-	-	-	-	-	+
Indoletest	-	+	-	+	+	-	-
Starch hydrolysis	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	-	-	-	+
Methyl Red test	+	+	+	+	+	+	+
V-P test	+	+	+	+	+	+	+
O-F test	O^+/F^-	O^-/F^-	O^-/F^-	O^-/F^-	O^-/F^-	O^-/F^-	O^+/F^-
Triple Sugar Iron test	K/K	K/A	K/K	K/A	K/A	K/A	K/A
Glucose	+	+	+	+	+	-	+
Sorbinol	V	-	-	-	V	-	V
Lactose	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	-	+
Fructose	+	+	+	-	-	-	+
Dextrose	+	+	+	+	+	-	+
Galactose	-	-	-	-	-	-	+
Raffinose	-	-	-	-	+	-	-
Trehalose	+	+	+	+	+	-	+
Melibiose	-	-	-	+	+	-	-
Sucrose	+	+	+	+	+	-	+
L-Arabinose	+	-	-	-	-	-	+
Mannose	+	-	-	-	+	-	+
Inulin	+	-	-	-	-	-	+
Sodium gluconate	-	-	-	-	-	-	-
Glycerol	-	-	-	+	+	-	-
Salicin	+	-	-	+	+	-	+
Dulcitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-

Adonitol	-	-	-	-	+	-	-
Arabitol	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-
α-methyl -D glucoside	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Cellobiose	+	-	-	-	+	-	+
Melezitose	-	-	-	-	-	-	-
α-methyl -D mannoside	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
ONPG	-	-	-	-	-	-	-
Esculinhydrolysis	+	-	-	-	-	-	-
D- Arabinose	-	-	-	-	-	-	+
Citrate utilization	-	-	-	-	-	-	-
Malonate utilization	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-
Identification	*	*	*	*	*	*	*

(+ = Positive, - = Negative, V= 11-89% positive, O^+/F^-= only oxidative; O+/F+ = Oxidative and fermentative; O^-/F^- = glucose not metabolised; A/A= Glucose, lactose & sucrose fermentation; K/A = Glucose fermentation; K/ K = Non fermentative)

Table: 4. Identification of bacteria isolated from rice field soil environment

Isolates	Identification
V1	*Serratia* sp.
V2	*Bacillus* sp.
V3	*Bacillus* sp.
V4	*Moraxella* sp.
V5	*Photobacterium* sp
V5	*Photobacterium* sp.
V7	*Ralstonia* sp.

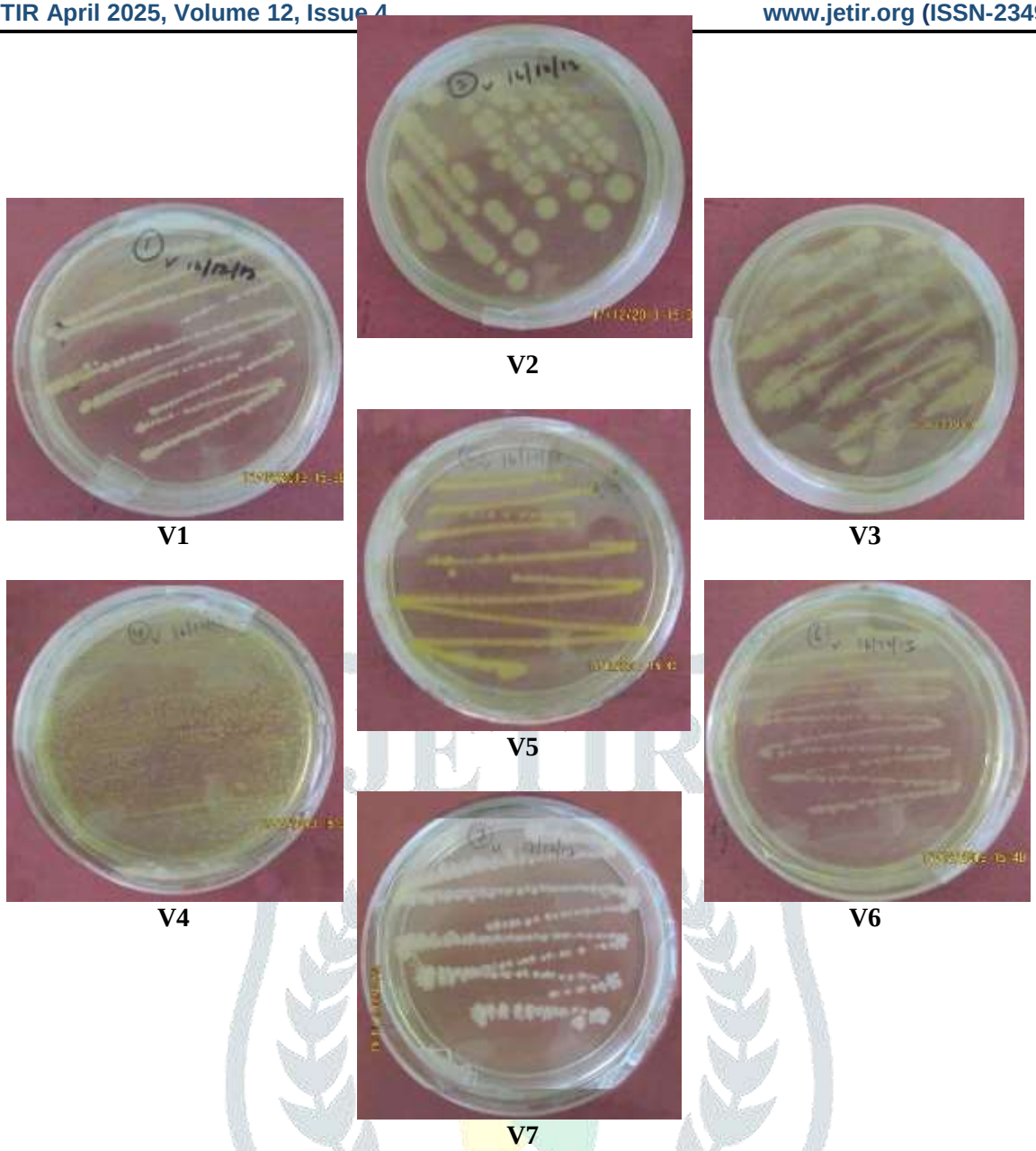


Fig.1. Bacteria isolated from Vegetable field (V1, V2, V3, V4, V5, V6 and V7 are pure culture of different isolates

3.1 Bioremediation of Chlopyrifos and Methyl parathion by isolated bacterial consortium in surface soil treatment reactor

The naturally occurring bacterial isolates capable of metabolizing pesticides were isolated from pesticides polluted vegetable field soil. The bacterial isolates identified by various biochemical analysis are *Serratia* sp., *Bacillus* sp., *Moraxella* sp., *Photobacterium* sp. and *Ralstonia* sp. A surface soil treatment reactor (SSTR) has been designed where bioremediation of chlorpyrifos and methyl parathion polluted agricultural soil was carried out using developed bacterial consortium with a combination of three bacterial species (*Bacillus* sp., *Moraxella* sp. and *Photobacterium* sp.). The environmental condition such as temperature (25-28°C) and moisture contents (60 -70%) were continuously monitored during the whole process. During the experiment, samples were collected from surface soil treatment reactor periodically at 0, 2, 4, 6, 8 and 10 days intervals of time for estimation of chlorpyrifos and methyl parathion degradation. The degradation of chlorpyrifos and methyl parathion was determined by means of High Performance Liquid Chromatography

(HPLC). The HPLC chromatograms of the respective pesticides degradation are shown in **Fig.3 and Fig. 4.** During this period up to 62.72 % degradation of chlorpyrifos and 65.99 % degradation of methyl parathion was achieved shown in **Fig. 2.**

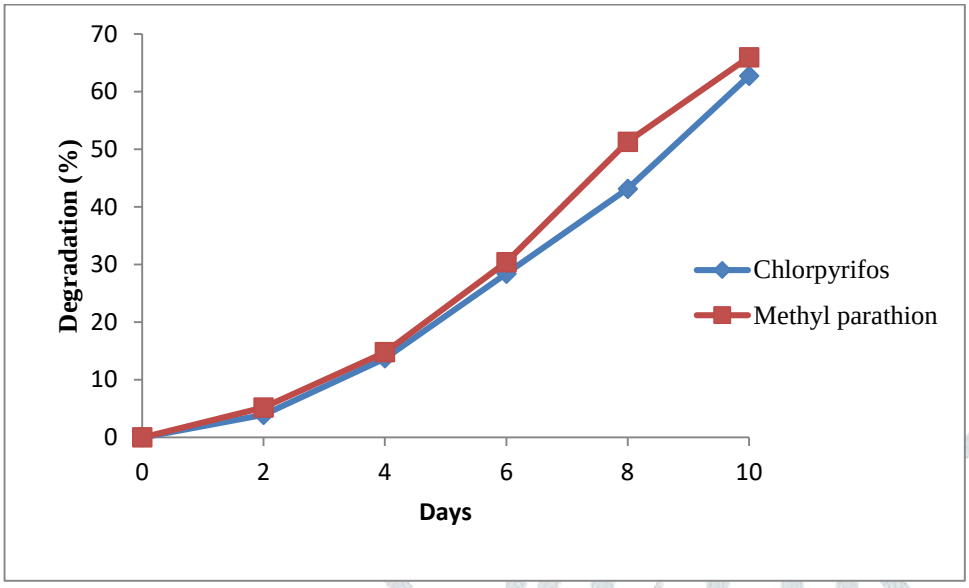
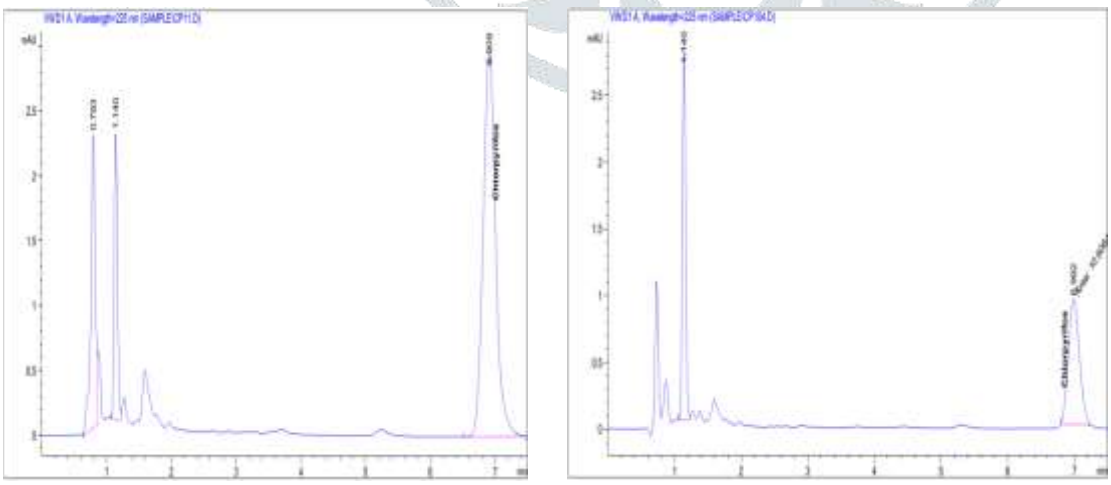
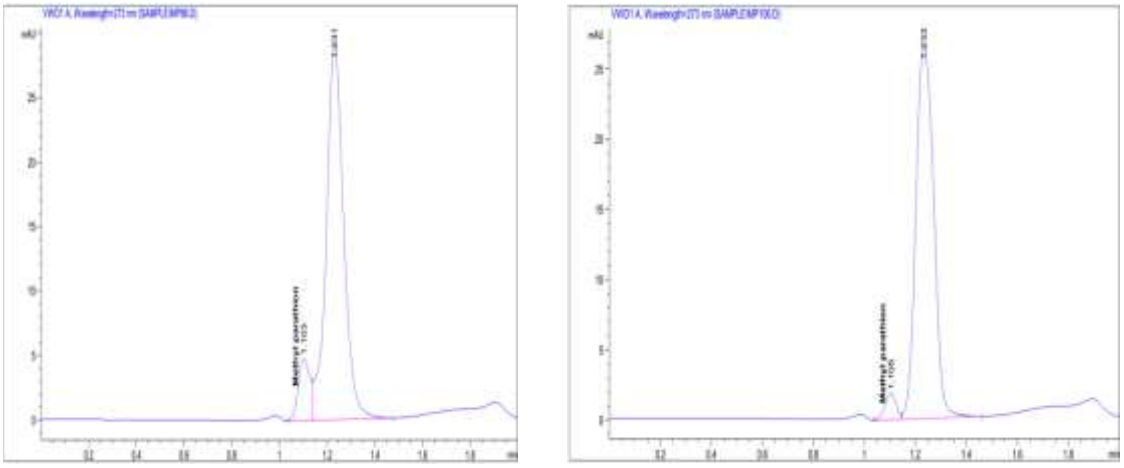


Fig. 2. Biodegradation of chlorpyrifos and methyl parathion in SSTR



A B

Fig. 3. HPLC chromatogram of Chlorpyrifos (A- Before bioremediation, B - After bioremediation)



A B

Fig. 4. HPLC chromatogram of Methyl parathion (A- Before bioremediation, B - After bioremediation)

3.3 Variation in Environmental parameters during bioremediation of pesticides using microbial consortium

Bioremediation of chlorpyrifos and methyl parathion was carried out using identified bacterial consortium of *Bacillus* sp., *Moraxella* sp. and *Photobacterium* sp. in surface soil treatment reactor under controlled environmental conditions. During this period the environmental parameters such as pH, electrical conductivity, nitrate, nitrite, ammonium, sulphate, phosphate and total organic carbon were monitored and assessed as mentioned in **Table 5**. During the process of bioremediation, it was found that pH values were gradually deceasing from 7.5 to 6.8, while the values of electrical conductivity increase from 144 µs cm $^{-1}$ to 214 µs cm $^{-1}$**.** The reason behind the reduction in pH value is the organic acids produced from intense fermentation of carbohydrates (Dibble and Bartha, 1979), while the increase in electrical conductivity was due to the aeration and moistening during remediation, which cause release of dissolved solutes and increase in electrical conductivity (Akpan *et. al*, 2013).

The sulphate value decreased from 3.16 mg/kg to 1.96 mg/kg, while phosphate value increased from 0.84 mg/kg to 1.17 mg/kg during bioremediation. The reason behind the lowering of sulphate was sulphate reducing bacteria that can obtain energy by oxidizing organic compounds or molecular hydrogen (H_2) while reducing sulfate (SO_4^{-2}) to hydrogen sulphide, which reduces sulphate by producing hydrogen sulphide gas (Ernst-Detlef and Harold, 1993), while the reason behind the increase in phosphate concentration was release of PO_4^- ion during bioremediation of organic compounds such as pesticides (Mishra *et. al*, 2001).

Table: 5. Variation in environmental parameters during bioremediation of pesticides

Parameters	**0th Day**	**2nd Day**	**4th Day**	**6th Day**	**8th Day**	**10th Day**
pH	7.5	7.3	7.1	7.0	6.9	6.8
Electrical conductivity(µs cm $^{-1}$)	144	149	156	168	191	214
Sulphate (mg/kg)	3.16	3.0	2.76	2.52	2.28	1.96
Phosphate (mg/kg)	0.84	0.90	0.95	1.02	1.07	1.17

Nitrate(mg/kg)	10.06	9.35	9.07	8.64	8.22	7.37
Nitrite(mg/kg)	0.093	0.097	0.099	0.101	0.105	0.110
Ammonium(mg/kg)	1.87	2.02	2.10	2.30	2.50	2.70
TOC (%)	1.2	1.05	0.96	0.93	0.915	0.9

In the case of nitrate, decreased value was recorded during bioremediation process, which decreased from 10.06 mg/kg to 7.37 mg/kg, while nitrite value and value of ammonium found to be increased. The value of nitrite increased from 0.093 mg/kg to 0.110 mg/kg and the ammonium value increased from 1.87 mg/kg to 2.70 mg/kg. The reason behind the lowering of nitrate-N concentration is reduction of nitrate into nitrite and finally into ammonia during bioremediation, resulting increased value of nitrite-N and ammonium-N (Francis *et. al*, 2007; Hayatsu *et. al*, 2008). Similarly, a decrease in total organic carbon value was recorded during bioremediation process, which decreased from 1.2 % to 0.9 % due to bacterial metabolic activities (Chefetz *et. al*, 1998).

Conclusion

The results presented in this study show that bacterial consortium play the key role in the degradation of chlorpyrifos and methyl parathion. The presence of a natural microbial community is a necessary and prerequisite for an effective remediation of pesticides. The use of bacterial consortia makes it possible to evaluate the natural microbial potential to degrade persistent pesticides in soil. The choice of the bioremediation strategy should be made on the basis of type and properties of pesticide, environmental matrix and the organisms present in the environment.

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