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Biodegradation of Methyl Parathion by *Bacillus species*

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ABSTRACT

Microbial degradation of pesticides in the soil is a natural process and one of the main mechanisms of detoxification. Several bacteria have been reported to play a vital role in pesticide degradation. Methyl parathion was purchased from Samy Agro Farm, Mannargudi, Thiruvarur District, Tamil Nadu, India. Effect of physical and chemical parameters was analyzed for effective degradation of the methyl parathion. *Bacillus subtilis* was showed maximum activity at the pH (7), temperature (35ºC), incubation periods (72hrs) and concentration of methyl parathion (15%) of methyl parathion degradation. The soil samples were collected from cultivated fields (mainly vegetable field) in mannargudi, Thiruvarur District, Tamil Nadu. The serial dilution method was followed for the isolation of bacteria. The isolated bacteria were identified as *Bacillus subtilis* and *Bacillus cereus* by cultural, Gram staining and biochemical characteristics. The identified bacteria were used for degradation of methyl parathion by broth method. After incubation, the degradation of samples was analyzed by High-performance liquid chromatography.

1. INTRODUCTION

Microbial degradation of pesticides in the soil is a natural process and one of the main mechanisms of detoxification. Several bacteria have been reported to play a vital role in pesticide degradation. The process causing the breakdown of the pesticides may contribute to the maintenance of a safe environment. It has been shown in several studies that some various types of bacteria were responsible for accelerated degradation of some pesticides and herbicides. Such microorganisms are like ubiquitous organisms found in a variety of environmental including soil and freshwater sources.

Methyl parathion is another widely used organophosphorus insecticide. It gets hydrolyzed on soil and also in flooded soil (Sharmila *et al.*, 1989). Biotransformation of methyl parathion along with other agrochemicals was studied using culture filtrates of bacteria and mixed cultures which stimulated bacterial transformation rates. Increase in cell biomass stimulated an increase in the biotransformation rates. Extensive investigation into utilization of methyl parathion, its enzymatic hydrolysis and presence of genes homologous to (organophosphate degradation) genes was very interesting.

Bacterial cultures isolated from soil were capable of utilizing methyl parathion and parathion as a sole source of carbon. While some isolates from the mixed cultures lost their ability to use the pesticides independently in serial transfers, a *Bacillus sp.* hydrolyzed the pesticides to *p*-nitrophenol but required glucose or other carbon source for growth. The crude cell extracts prepared hydrolysis in pH range of 7.5 to 9.5 and at temperature of 35 to 40ºC. Another isolate, a *Flavobacterium sp.* used *p*- nitrophenol for growth and degraded it to nitrite. The hybridization data showed that the DNAs from a *Bacillus sp.* and from the mixed culture had homology with the organophosphate degradation gene from previously reported parathion hydrolyzing bacterium, *Flavobacterium sp.* in the soil, other than bacteria microalgae and cyanobacteria also have been found to degrade methyl parathion. Bioremediation of methyl parathion by free and immobilized cells of *Bacillus sp.* isolated from soil have reported (Sreenivasulu and Aparna, 2001).

Biodegradation of organophosphorus pesticides covering aspects like microbial degradation, metabolites of degradation, enzymatic degradation and genetic basis of degradation of widely used parathion, methyl parathion, malathion, monocrotophos, dimethoate *etc.*

Bioremediation of methyl parathion by free and immobilized cells of *Bacillus sp*. isolated from soil. However, even with IPM, pesticides are often the only effective way to deal with emergency pest outbreaks. Moreover, in some situations any level of a pest is intolerable. For example, most people would consider even one in their house intolerable and most shoppers would not buy fruit or vegetables with blemishes from insects or plant diseases. As a result, farmers cannot afford to produce foods with even minor signs of pest damage, so they are forced to use pesticides.

The household demand for food till 2020 shows that, between 1995 and 2020, the demand for food grains is likely to be doubled, for vegetables more than 2.5 times and for fruits 5 times. Thus, increase in the consumption of pesticide is likely to be at least two to three times more in the years to come. Modern industry and agriculture require the use of numerous synthetic chemical compounds particularly a wide variety of pesticides. India, consumption of pesticides is increasing at the rate of 2 to 5 % per annum. To data, pesticide consumption ranges between 480-520 g / ha and this accounts for about 3% of the total pesticides used in the world.

The present study was performed to collect the soil sample from cultivated land mannargudi, Thiruvarur district, Tamil Nadu, India and to isolate and identify the bacteria and to screen the pesticides degradation of isolated soil bacteria, to analyze the biodegradation of methyl parathion in liquid medium and optimization of degradation.

2. MATERIALS AND METHODS

2.1 Chemical

Methyl parathion was purchased from Samy Agro Farm, Mannargudi, Thiruvarur District, Tamil Nadu, India.

2.2 Collection of soil sample

The soil samples were collected from cultivated fields (mainly vegetables field) in mannargudi, Thiruvarur District. The soil samples were collected from surface to 10cm depth and transported to the laboratory in airtight plastic bags and stored at 4ºC.

2.3 Analysis of physicochemical parameters of the soil (Ghosh *et al.*, 1983)

Analysis of the physicochemical parameter of the collected soil sample was done using the standard methods. The soil pH, Organic carbon, Nitrogen, Phosphorus, Zinc, copper, Iron, and manganese, were analyzed.

2.4 Isolation and Identification of Bacteria (Bailey and Scott, 1966)

The collected soil samples were subjected to serial dilution and were plated on Nutrient agar plates. Identification of these different bacterial isolates were carried out by the routine bacteriological methods as colony morphology, preliminary tests like Gram staining, Motility, Catalase and Oxidase and plating on selective media and by performing biochemical tests.

2.5 Biodegradation of Methyl parathion in liquid medium (Barcelo, 1991)

The degradation of methyl parathion in liquid media, a stock culture of each *Bacillus subtilis* and *Bacillus cereus* were grown on a nutrient medium for 48 hours to mid-log phase of growth. Methyl parathion was added to pre-sterilized 100ml Erlenmeyer flask at the concentration of 50ug/ml in acetone. After evaporation of acetone, 50 ml of mineral salts medium (Mg SO_7H_2O, 0.2g; $CaSO_4$, 0.4G; $FeSO_47H_2O$, 0.001g, and distilled water,1L, pH 6.5) were placed in 100 ml Erlenmeyer flasks and the flasks were shaken for two hours. The medium was inoculated with a suspension of the cells of *Bacillus subtilis* and *Bacillus cereus* grown on nutrient media for 48 hours. The bacterial cultures were centrifuged at 8,000 rpm for 10 minutes and the precipitate was resuspended in sterile distilled water to obtain a final density of about $1x10^8$ CFU (colony forming units) ml^{-1}. Bacterial concentration was determined by the plate counting method, in terms of CFU. Medium not inoculated with a bacterial suspension served as control. Both inoculated and uninoculated samples were incubated under intermittent shaking to provide an aerobic condition. After1,3, and 6 days, duplicate flasks from inoculated and uninoculated samples were withdrawn aseptically and analyzed for pesticide residues by HPLC after its extraction in hexane.

2.6 Optimization of biodegradation by physical and chemical parameters

2.7 Effect of temperature

The percentage of degradation was influenced by temperature. The rate of biodegradation of Methyl parathion was estimated at different temperature ranges from (25, 30, 35 and 40^0 C). The bacterial isolate was incubated at each specified temperature in Mineral Salt broth with 1% Methyl parathion for 10 days.

2.8 Effect of pH

The percentage of degradation was influenced by pH. The effect of pH was evaluated by incubating the culture at the optimum temperature with various pH (4, 6, 7 and 9) for 30 days. The pH of Mineral salt broth with Methyl parathion (1%) was maintained using 1N HCl and 1M NaOH. To maintain the pH, citrate- phosphate buffer (pH 4-6, Phosphate buffer (pH 7-8), and carbonate-bicarbonate buffer (pH 9) were used. Every 5-day interval, pH was checked. The optimum pH for maximum Methyl parathion degradation was determined.

2.9 Effect of Incubation periods

To find the optimum condition for biodegradation activity, the production media were prepared, after sterilization, 1% of inoculum was added into different flasks containing media and the flasks were incubated at various incubation periods for 48, 60 and 72 hrs.

2.10 Effect of Concentration of Methyl parathion on biodegradation

The influence of concentration of Methyl parathion was studied by varying the concentration of Methyl parathion (1, 5, 10, 15%) in Mineral salt broth at optimum temperature and pH for the same incubation period.

2.11 High performance of liquid chromatography (HPLC)

The separation and identification of compounds were made through High performance of liquid chromatography (HPLC). The extracts used for HPLC analysis were passed through a 0.45 µm filter (Millipore, MSI, Westboro, MA) before injection into a HPLC column of 150 mm length (Agilent technologies 1200 series). The mobile phase was acidified water containing 0.1% formic acid (A) and acidified acetonitrile containing 0.1% formic acid (B), eluted in gradient. The flow rate was 0.8 mL/min and the wavelengths of detection were set at

UV 300 nm, temperature at 30°C, injection volume = 20 µl and analysis time was 60 min. Reference substances is a mixture of gallic acid, vanillic acid, ascorbic acid, quercetin, caffeic acid, catechin and coumaric acid(solutions in methanol, each of them 0.5 mg/ml).

2.12 HPLC analysis

2.13 Colum specification

Reverse phase HPLC (Cyberlab, USA) analysis was carried out in a C18 column (250mmx4.6mm) version (lake forest, CA USA) equipped with ac 18 curved column. The components were eluted with an isocratic elution of acetonitrile vs water at the flow rate of 1ml / min and absorption recorded at 680nm.

2.14 Sample preparation

One ml of the samples was centrifuged (at 3000rpm for 15 minutes) and dissolved in specific solvent of HPLC grade and filtered through o.22 microfilter. The filtrate was collected and degassed using sonicator for 50 times at 4ºC.

2.15 Solvent preparation

Solvent was prepared using acetonitrile and water in radio 65:35 and degassed using sonicator for 15 times at 4ºC

2.16 Column equilibration

Column equilibration was done using 65% acetonitrile in water until zero baseline.

2.17 Sample injection

Twenty microliter of the sample was injected into the injection head using injection needle. Required time and wavelength were set and the purification profiles were seen on the screen that shows the degraded components with its retention time.

3. RESULT AND DISCUSSION

3.1 Collection of pesticides

Methyl parathion was purchased from Samy Agro farm, Mannargudi, Thiruvarur District, Tamil Nadu, India.

3.2 Collection of soil sample

In the present study, bacterial species were isolated from soil sample collected from cultivated fields (mainly vegetables field) in mannargudi, Thiruvarur Districts, Tamil Nadu.

3.3 Physicochemical parameters of the soil sample

Analysis of the physicochemical parameter of the collected soil sample using the standard methods. The soil pH 5.8, Organic Carbon 13.0, Nitrogen 0.11, Phosphorus 3.0, Zinc 4.5, Copper 0.8, Iron 4.8 and Manganese 4.5. In the present study, the methyl parathion is degradable effort of bacteria. Nutrients such as carbon, Nitrogen, Phosphorus, Zinc, Copper, Iron and Manganese and physicochemical parameters (pH, temperature) had significant effect on methyl parathion degradation. Similarly (Amel, 2008) the methyl parathion was degraded under aerobic condition. Nutrients and physicochemical parameters were significant on degradation.

3.4 Isolation and Identification of bacteria

The selected two bacterial colonies were identified by cultural, morphological and biochemical characteristics and compared with standard manuals. Based on the result, the isolated colonies were confirmed as *Bacillus subtilis* and *Bacillus cereus* respectively (Table-1).

Table 1: Biochemical characterization of isolated bacterial *spp.* from the soil

Isolated bacterial *spp*	**Indole**	**MR**	**VP**	**Citrate**	**Urease**	**Catalase**	**Oxidase**
Bacillus cereus	-	+	-	-	-	+	+
Bacillus subtilis	-	+	-	-	-	+	+

3.5 Biodegradation of methyl parathion

Bacillus subtilis and *Bacillus cereus* utilized methyl parathion in mineral salts media as the sole carbon and phosphorus sources. *Bacillus subtilis* appeared to be more effective than the *Bacillus cereus* in degrading methyl parathion. After 24 hours of incubation, methyl parathion was degraded 100 and 100% by *Bacillus subtilis* and respectively, 39.60 by *Bacillus cereus*, compared to 25 and 57.30% in uninoculated control. *Bacillus subtilis* and *Bacillus cereus* caused 100% and 91.10% dislodge of methyl parathion in mineral salts media in 3 days incubation period. The abiotic dissipation rates of methyl parathion were 40.42%. Only 67.02% and 59.45% losses of methyl parathion were recorded at the end of 6 days exposure to *Bacillus cereus* compared to 7.00% in uninoculated control. The half-life (t1/2) values for methyl parathion, were found to be undetected, and 24 days in mineral salts media inoculated by *Bacillus subtilis* and *Bacillus cereus*, compared to 4.4 and 16 days in uninoculated control. Such rapid degradation indicated the enzymes involved in the degradation of methyl parathion.

Bacillus cereus appeared to be more effective than *Bacillus subtilis* (Prazmowski) in degrading soil-applied methyl parathion, while in mineral salts media supplemented with methyl parathion, *Bacillus subtilis* was more effective than *Bacillus cereus* in degrading methyl parathion. The reason for this discrepancy in the methyl parathion degrading the ability of the two bacteria in the soil and mineral salts media is not clear. Probably *Bacillus subtilis* in competing with the indigenous microorganisms in the complex soil environment (Mallick *et al.*, 1999).

3.6 Optimization studies

3.7 Effect of temperature

The maximum biodegradation activity for *Bacillus subtilis* was recorded in (35ºC) when compared with other temperature and minimum biodegradation activity was recorded in (25ºC) (Table-3). Allan Walker reported that methyl parathion degradation by the isolate B-14 was rapid at temperatures ranging from 15 to 35ºC, and the slowed degradation was observed at 5 and 45ºC.

Table 2: Optimization of physicochemical parameters for methyl parathion degradation

Sr. No.	Optimization study	Days			
		4th	8th	12th	16th
1	Temperature (ºC)	25	30	35	40
2	PH	4	6	7	9
3	Incubation periods (hours)	48	60	68	74
4	Concentration of Methyl parathion(%)	2	0.7	10	15

3.8 Effect of pH

The optimum pH for maximum Methyl Parathion degradation was determined. The maximum biodegradation activity was recorded in pH (7), when compared with other pH range and minimum biodegradation activity was recorded in pH (6). Change in the optical density of medium during the sixteen days of treatment of methyl parathion by *Bacillus subtilis* and *Bacillus cereus* were illustrated. Allan Walker reported the isolated bacterial species were capable of degrading methyl parathion in pH range from 5.5 to 7.6.

3.9 Effect of Incubation period

The maximum biodegradation activity was recorded in 72 hours, when compared with other incubation periods and minimum biodegradation activity was recorded in 30 hours, were significant decrease in the concentration, of methyl parathion even in the uninoculated culture medium. Thus about 22% of the insecticides were lost from the uninoculated controls during30 day incubation period (Rebecca, 2002).

3.10 Effect of Concentration of methyl parathion on biodegradation

The maximum biodegradation activity was observed in (15%) of Methyl Parathion followed by the minimum biodegradation activity was observed in (10%) of Methyl Parathion.(Brajesh,2003) the changes in pH recorded after 4,8,12 and 16 days of treatment with *Bacillus sp* initially the pH was decline indication the formation of organic acid except 7.5% methyl parathion concentrations. Later pH on increase in all the concentrations except 5% and the highest pH were observed for10% methyl parathion concentration.

3.11 HPLC analysis

The absorption spectra of the samples at 300nm are presented in figures. The HPLC elution profile of the methyl parathion control showed 3 peaks with a retention time (RT) 5.831, 22.247, and 24.813, and 30 minutes. The elution profile obtained for the bacteria treated samples significantly different from the control in terms of number, the height of peaks obtained and retention time. The HPLC profile of methyl parathion treated with bacterial isolate *B.subtilis* showed 1 peaks with retention time 3.649 (figure 1 and figure 2). Methyl parathion was dissolved in 1.0ml of methanol and an aliquot (10*u*l) were analyzed by high-performance liquid chromatography (HPLC) with a UV-detector set at a wavelength of 248 nm. AC18 columns used, and the mobile phase was a mixture of methanol and water (70:30,v/v). The flow rate was 0.7 ml min^{-1}. Mean recovery values obtained for methyl parathion 90.5 in the soil sample (Mukerji 1996).

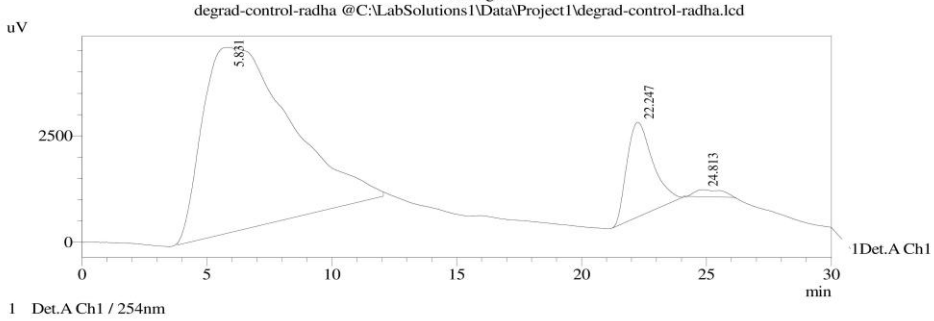
HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: degrad-control-radha
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: degrad-control-radha.lcd
Method Filename	: SJC-Law-Method.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 31-03-2017 PM 03:15:44
Data Processed	: 31-03-2017 PM 04:06:02

Chromatogram

degrad-control-radha @C:\LabSolutions1\Data\Project1\degrad-control-radha.lcd



1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.831	1063417	4365	85.980	64.621
2	22.247	160444	2224	12.972	32.927
3	24.813	12961	166	1.048	2.452
Total		1236822	6754	100.000	100.000

ANALYSED BY

APPROVED BY

HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: dye-deg-radha
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: dye-deg-radha.lcd
Method Filename	: SJC-Law-Method.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 30-03-2017 PM 02:48:03
Data Processed	: 30-03-2017 PM 03:18:05

Chromatogram

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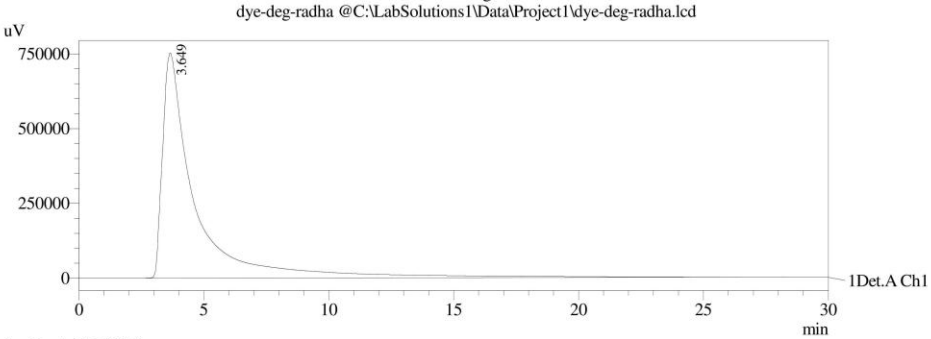

1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.649	68582976	752290	100.000	100.000
Total		68582976	752290	100.000	100.000

ANALYSED BY

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4. CONCLUSION

Bacillus sp., are ubiquitous in soil and water, of considerable scientific and technological importance and capable of utilizing a wide range of simple and complex organic compounds. They are known to be involved in the biodegradation of natural or man-made toxic chemical compounds and the present study confirms that the isolated methyl parathion degrading bacterium could be used successfully for the removal of methyl parathion from contaminated soils as the bacterial systems successfully degraded methyl parathion in soil and liquid media. It is well known that bioremediation is an attractive alternative to the classic treatment of methyl parathion pollution by taking advantage of microbial metabolism.

5. ACKNOWLEDGEMENT

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