# BIODEGRADATION OF METHYL PARATHION IN CONTAMINATED AGRICULTURAL SOIL USING NITROGEN FIXING BACTERIAL SPECIES

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***Abstract*:** Chemical pesticides mainly organophosphorus pesticides are widely used for crop protection and to increase productivity. The excessive use has led to its bioaccumulation in the environment leading to generation of various health hazards. One such organophosphorus pesticide is Methyl Parathion. The objective of this study is the biodegradation of this hazardous methyl parathion through nitrogen fixing bacteria. In this investigation the degradation of methyl parathion was studied by thin layer chromatography. The potential degraders were *Azotobacter spp., Bradyrhizobium spp., Mezorhizobium spp., Rhizobacterium spp. and Azospirillium spp.,* etc. Bioremediation plays a key role in order to combat from this bioaccumulation using microorganism involving the preliminary step that is study of biodegradation of methyl parathion through nitrogen fixing bacteria. This study revealed that *Azotobacter spp. and Rhizobium spp.* could biodegrade harmful methyl parathion. Further studies could be carried out by using molecular techniques to formulate the suitable innoculum for the development of effective bioremediation strategy to decontaminate harmful methyl parathion.

***Key Words:*** **Biodegradation, bioremediation, methyl parathion, nitrogen fixing, organophosphorus pesticide.**

## I. INTRODUCTION

Pesticides are being used extensively which has resulted in the disturbance of natural biological system. These pesticides lead to a variety of degradative, transport, and adsorption/desorption processes in soil due to which alteration in physiological and biochemical properties of soil occurs. **(Gulhane *et al*., 2015)**

Methyl parathion (*O,O-dimethyl O-4 nitrophenylphosphorothioate*) is one such organophosphorus pesticide which is classified as category-1 insecticide and widely used in agriculture. It has been reported that it causes lethal damages by oral, inhalation or dermal exposure. It's potential of genotoxicity, oncogenicity, reproductive toxicity, developmental toxicity, neurotoxicity and immune toxicity **(Tiwari B *et al*., 2017)**. This pesticide acts as a neurotransmitter inhibitor, thus inhibiting acetylcholinesterase activity; however, it is metabolically activated by cytochrome P450 to produce oxons and inhibit acetylcholinesterase enzyme **(Sultatos LG *et al.,* 2006)**. Hence, pesticide biodegradation involving strategies of bioremediation can be attained to combat bioaccumulation. It has been found that microorganisms possess genetically preserved character that facilitates the pesticide degradation, solving problems of soil contamination. Methyl parathion consists of phosphodiester bond (P–O alkyl and aryl bonds) which can be effectively hydrolysed by microorganisms as a source of their limiting nutrients such as carbon(C) and/or phosphorus (P) **(Cui *et al*., 2001; Singh and Walker, 2006; Botero *et al*., 2012; Kapoor and Rajagopal, 2011; Gao *et al*., 2012; Lu *et al*., 2013).**

Nitrogen fixing bacteria play an essential role in enhancing the soil fertility by fixing nitrogen and increasing the productivity of the crop efficiently. *Azotobacter* spp. is used as a biofertilizer for the cultivation of most agricultural crops as it has been found that they posses and produce different types of secondary metabolites such as vitamins (Riboflavin), amino acids (Thiamine), plant growth hormones (Nicotin, IAA and gibberellins), antifungal compounds and siderophores. These growth promoting substances such as IAA, GA, nicotinic acid etc., have direct influence on plants and induces plant gowth. Biodegradation studies of pesticide through *Azotobacter spp.* have shown the enzyme activity due to the presence of pesticide hydrolyzing gene in them.

Another efficacious microorganism *Rhizobium japonicum* exhibit nitrogenase activity in free-living culture or in symbiosis. (**Pankhurst,1977)** have isolated rifampicin-resistant mutants of both fast- and slow-growing strains of *Rhizobium* which were found to nodulate their respective host plants **(Pain, 1979).** The degradation of methyl parathion through rhizobia reduced the nitro group of methyl parathion to an amino group in the formation of aminoparathion and metabolized half of the p-nitrophenol. Another characteristic feature which has been reported is the presence of phosphatase enzyme in these bacteria that aid in biodegradation through utilization of the complex phosphorus through their metabolic processes as an energy source, rendering the contaminants harmless or less toxic products (**Ortiz-Hern´andez*et al*., 2001; Bhadbhade *et al*., 2002; Sogorb and Vilanova, 2002; Zuo *et al*., 2015)**.

The complete degradation of methyl parathion carried out by microorganisms leads to its conversion into *p*-nitrophenol (PNP) **(Abd-Alla MH, 1994).** Also various microorganisms which have been reported and studied include species of

*Azotobacter, Achromobacter, Acinobacter, Arthrobacter* and *Rhizobium*. These rhizospheric microorganisms have shown 80-100% pesticide (methyl parathion) degradation rate **(Kumar *et al.*, 2017; Chennappa *et al*., 2018)**.

The main objective of this study was to isolate the potential nitrogen fixing bacterial strains from methyl parathion contaminated agricultural soil and examine their degradation activity against methyl parathion. The degradation activity was studied through Thin Layer Chromatography.

## II. MATERIALS AND METHODS

### 2.1 Collection of soil sample

For sampling different agricultural fields were chosen having history of repeated methyl parathion application and rhizospheric soil was collected. The soil samples were collected up to a depth of 10 cm. The collected samples were air dried, passed through 2 mm sieve and stored in the sealed plastic bags at room temperature for further experimentation. Rj(S) TAL 102 (*Bradyrhizobium japonicum*) and Ab.c01 (*Azotobacter chrooccocum*) were collected from MP State Agro, Bhopal.

### 2.2 Pesticide used

Commercial grade methyl parathion pesticide (2%D.P.) was used throughout the experiment. The pesticide was purchased from the local pesticide supplier in Bhopal district of Madhya Pradesh.

### 2.3 Isolation and Purification of Methyl Parathion Resistant Bacteria

Enrichment culture technique was used in order to isolate 10 morphologically distinct bacteria. Mineral Salt Agar medium was prepared having different concentrations ranging from 100mg/L to 1000 mg/L of methyl parathion mixed with and checked for the growth of bacteria. A single isolated colony of the pesticide resisting bacteria was picked up with the help of sterilized wire loop and was inoculated in 100mL nutrient broth and it was incubated at 37$^{o}$C for the characterization of isolates.

### 2.4 Screening of Nitrogen Fixing Bacteria

The ten methyl parathion resistant bacteria were tested for their nitrogen fixing ability by growing them on Yeast Extract Mannitol Medium. The bacterial species which showed the appearance of gummy colony were further grown in Congo Red Yeast Extract Mannitol Agar Medium and their biochemical characterization was done.

### 2.5 Screening of Potential Pesticide Degrading Bacteria

Nitrogen fixing bacterial isolates which showed their growth at higher concentration (1000mg/l) were re-streaked on Mineral Salt Agar medium (MSM) containing 20 mg/l of methyl parathion for confirmation of pesticide degradation. Then MSM plates were incubated for 2-3 days and pesticide degradation was checked through the growth of bacterial strains on MSM plates and they were reported as pesticide degrading bacterial isolates.

### 2.6 Characterization and Identification of Bacterial Isolates

The biochemical tests and gram's staining were performed for physical, morphological characterization and identification of bacterial isolates. Morphological characters viz. size, shape, surface, opacity, texture, elevation and pigmentation were determined by visual observation as well as by using light trans-illuminator and microscopy.

### 2.7Analysis of Methyl Parathion Degradation By Thin Layer Chromatography (TLC)

#### 2.7.1 Pesticide extraction for chromatographic analysis

The bacterial isolates were inoculated into 100ml of MSM broth having 200mg/l of methyl parathion. After 72 hours of incubation, turbidity was seen in MSM broth, indicating pesticide degradation. From the above suspension 10ml of MSM broth was taken in eppendorf and centrifuged at 4000rpm for 20 min. After centrifugation 5ml of supernatant was collected into another tube having 5ml of diethyl ether into it. For 10 min the tube was shaken and it was allowed to settle for 30 min. Evaporation of solvent was done at room temperature to obtain the residue and dissolving in 2 ml of ethanol, it was further taken for TLC **(Curini M *et al*; 1980).**

#### 2.7.2 Plate Preparation

Aqueous suspension of silica gel and $CaSO_4$was prepared in the ratio of 4:4 and using spreader it was poured onto TLC plate.

#### 2.7.3 Plate Development

Solvents, n-hexane and acetone in the ratio of 9:1 were prepared and saturation of TLC chamber was done. One of the TLC plate was labelled as control and it was loaded with 20µl of sample (pesticide + MSM without culture). Another plate was labelled as reference having application of 200mg/l pesticide dissolved in 5ml of ethanol and 20µl was loaded on TLC plate. Similarly the different MSM isolates with pesticide were applied into the TLC plates. The plates were air dried and they all were placed in chamber containing solvent **(Kumavat G *et al;* 1980).**

#### 2.7.4 Visualizing Agent

For visualization 2% of silver nitrate was dissolved in acetone and water in ratio of 3:1 and then it was sprayed onto all the TLC plates. The plates were checked for colour development **(Curini M *et al*; 1980).**

## III. RESULTS AND DISCUSSIONS

**Table 3.1 Growth of Bacterial Isolates in Yeast Extract Mannitol Agar Medium**

| S.No | Bacterial Isolates | Growth In YEMA Medium |
|---|---|---|
| 1 | Rj(S) TAL 102 | + |
| 2 | Rj(S) 02 | + |
| 3 | Rj(S) 05 | + |
| 4 | Ac(S) 01 | + |
| 5 | Ac(S) 02 | + |
| 6 | Ac(S) 03 | + |
| 7 | Ac(S) 04 | + |
| 8 | Ac(S) 05 | + |
| 9 | Ab.c 01 | + |
| 10 | Ab.c02 | + |

**Table 3.2: Growth of Bacterial Isolates Resisting Methyl Parathion Containing Medium**

| **S.No.** | **Bacterial Isolates** | **Concentration of Pesticide in mg/l** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **100** | **200** | **300** | **400** | **500** | **600** | **700** | **800** | **900** | **1000** |
| 1 | Rj(S) TAL 102 | + | + | + | + | + | + | + | + | + | + |
| 2 | Ac(S) 01 | + | + | + | + | + | + | + | + | + | + |
| 3 | Ac(S) 04 | + | + | + | + | + | + | + | + | + | + |
| 4 | Ab.c 01 | + | + | + | + | + | + | + | + | + | + |
| 5 | Rj(S) 02 | + | + | + | + | + | + | + | + | + | + |

**(Note: Results are the mean of three replicas)**

**Table 3.3: Physical and Morphological Test of Methyl Parathion Resisting Bacterial Isolates**

| **Bacterial Isolates** | **Parameters** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Size** | **Margin** | **Elevation** | **Surface** | **Opacity** | **Pigmentation** | **Form** |
| Rj(S) TAL 102 | Medium | Entire | Raised | Shiny | Translucent | White gummy | Irregular |

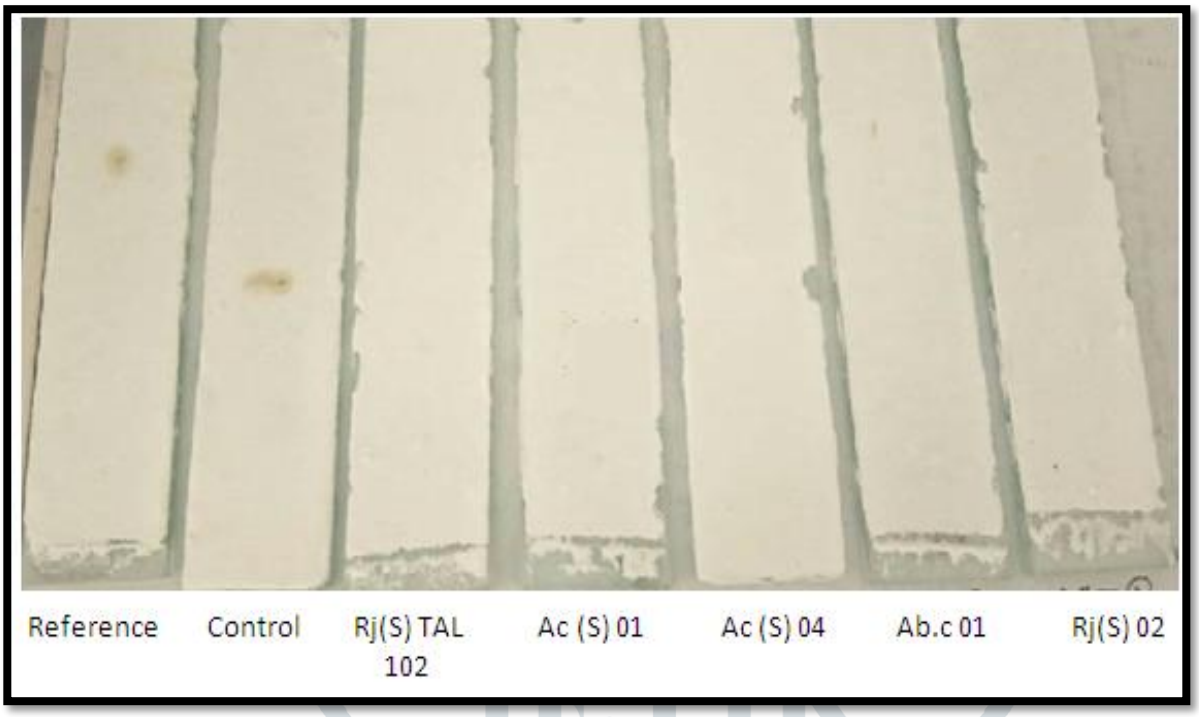
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ac(S) 01 | Medium | Entire | Raised | Shiny | Translucent | White gummy | Irregular |
| Ac(S) 04 | Medium | Entire | Raised | Shiny | Translucent | White gummy | Irregular |
| Ab.c 01 | Medium | Entire | Moderately raised | Shiny | Translucent | White gummy | Irregular |
| Rj(S) 02 | Medium | Entire | Moderately raised | Shiny | Translucent | White gummy | Irregular |

**Table 3.4: Biochemical Tests and Identification of Bacterial Isolates**

| S. No. | Biochemical Test | Bacterial Isolates | | | | |
|---|---|---|---|---|---|---|
| | | **Rj(S) TAL 102** | **Ac(S) 01** | **Ac(S) 04** | **Ab.c 01** | **Rj(S) 02** |
| **1.** | Indole production Test | + | ++ | ++ | ++ | + |
| **2.** | Methyl Red Test | + | + | + | + | + |
| **3.** | VogesProskauer Test | + | - | - | - | + |
| **4.** | Citrate Utilization Test | + | + | + | + | + |
| **5.** | Starch Hydrolysis Test | - | - | - | - | - |
| **6.** | MacConkey Test | + | + | + | + | + |
| **7.** | Gelatin Hydrolysis Test | - | - | - | - | - |
| **8.** | Caesin Hydrolysis Test | - | - | - | - | - |
| **9.** | Urease Hydrolysis Test | + | + | + | + | - |
| **10.** | Glucose Peptone Agar Medium Test | - | - | - | - | - |
| **11.** | Gram's Staining | - | - | - | - | - |
| **12.** | Shape | Rod | Rod | Rod | Rod | Rod |
| **13.** | Identification | *Rhizobium spp.* | *Azotobacter spp.* | *Azotobacter spp.* | *Azotobacter spp.* | *Rhizobium spp* |

Note: (+) positive reaction; (-) negative reaction

**Fig.1 Biodegradation of Methyl Parathion by the Isolates studied through TLC**




Total 10 bacterial isolates were used for the experimentation, out of which 5 isolates were nitrogen fixers and nitrogen was estimated by Kjheldahls method. The potency of these isolates to resist methyl parathion was determined by their growth in mineral salt agar medium (MSM). Identification of the isolates was done on the basis of their morphological and biochemical characterization as depicted in the Table 3.3 and 3.4. These isolates were Rj(S) TAL 102**,** Ac(S)01, Ac(S) 04, Ab.c 01and Rj(S)02 in which Rj(S) TAL 102 and Ab.c01 were collected from MP State Agro, Bhopal and already identified.

Thin layer chromatography (TLC) was performed to detect the biodegradation of pesticide using MSM culture extracts of these isolates. The TLC chromatographic plates of the isolates Rj(S) TAL 102(*Rhizobium spp.*)**,** Ac(S)01 (*Azotobacter spp.)*, Ac(S) 04 (*Azotobacter spp.*), Ab.c 01 (*Azotobacter spp.*) and Rj(S)02 (*Rhizobium spp*) showed no colour development (Figure. 1) which depicted biodegradation of methyl parathion in MSM broth after 72 hours of incubation; whereas plates labelled as reference and control showed spot development because the sample extracts were not inoculated by any of the isolates and degradation of pesticide did not take place. Thus, those bacterial isolates with no spot development were identified as potent degraders.

Therefore, it can be concluded that due to presence of enzymes such as phoshodiesterase and phoshotriesterase; must have played a key role in metabolizing methyl parathion actively and showed no spot development **(M.H. Abd-Alla , 1994).**

Further, the techniques of molecular biology and biotechnology could be exploited to formulate inoculum exhibiting both features that is; growth promotion of plants as well as biodegradation of pesticide. Even effective treatment process for *in-situ* or *ex-situ* bioremediation could be developed through immobilized culture stock of these bacterial isolates.

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