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ISOLATION AND IDENTIFICATION OF POTENTIAL METHYL PARATHION DEGRADING BACTERIA FROM GWALIOR ARABLE SOIL

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ABSTRACT

Twenty one bacterial isolates were isolated from methyl parathion treated agricultural soil from in and around the Gwalior city, Madhya Pradesh to study the degradation of Methyl Parathion. Isolation was done by enrichment in Basal mineral medium with 50 mg/L methyl parathion as sole carbon and energy source. No supplement as a carbon and phosphorus source was added in medium. Methyl parathion utilizing capacities of twenty one isolates were studied with variable concentration of methyl parathion, ranging from 50 mg/L to 1600 mg/L in basal mineral medium. Three isolates EMP11c, EMP12a and EMP12b showed the highest degradation i.e. 97%, 96.4% and 99.9% of 1200 mg/L methyl parathion respectively after 48 hrs at 30°C and 7 pH. The degradation of methyl parathion was analysed by GC-MS. These bacterial isolates were identified as *Pseudomonas diminuta* (EMP11c), *Pseudomonas putida* (EMP12a) and *Pseudomonas aeruginosa* (EMP12b), on the basis of biochemical characteristics and 16S rRNA sequence analysis.

KEYWORDS: Pesticide, Methyl parathion, biodegradation, GC-MS, *Pseudomonas* sp.

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INTRODUCTION

Indiscriminate use of pesticides throughout the World has rendered the biochemical process of soil, air and water into eco-physiological imbalance[1]. The qualities of soil, ground water, inland and coastal waters and air are severely affected by pesticide contamination[2]. Removal of pesticide residuals from environment through microorganisms by biochemical and molecular modes have been well documented by many authors[3, 4].Methyl parathion (*O, O*-dimethyl-*O*-p-nitrophenol phosphorothioate, MP) is an important broad-spectrum organophosphorus insecticide, used extensively and globally for the control of a wide range of insect pests. Its residues are detected in fruits and vegetables and classified by the World Health Organization (WHO) in the class of extremely hazardous pesticides[5]. Methyl parathion owe its toxicity in biological system due to inhibition of acetylcholine esterase activity, and its LD_{50} is reported as low as 14 to 24 mg/kg of body weight while its close analogue parathion possess still higher toxicity (LD_{50} 4 to 13 mg/kg)[6]. Microbial degradation of pesticides applied to soil is the principal mechanism which prevents the accumulation of these chemical in the environments[7]. Soil microflora is potential candidate for detoxification of pesticides. Some workers found that soil microbes attack on wide range of organophosphorus insecticides[8,9,10,11,12,13,14]. Earlier, certain micro-algae, cyanobacteria and bacterial species have been isolated from soil which utilized methyl parathion as a source of energy for their growth[5,12,15,16,17,18]. The present study aimed at isolation and identification of potential methyl parathion degrading bacteria from soil from agriculture field of Gwalior and nearby places.

MATERIALS AND METHODS

(i) Collection of Soil Samples

The soil samples were collected from agricultural field of Gwalior and nearby region of Madhya Pradesh, India where methyl parathion is in use continuously and extensively for several years. Soil samples were collected from 15-20 cm depth. These samples were homogenized by sterile spatula and stored at 4°C in closed sterile plastic containers in the dark for further analysis.

(ii) Pesticide used

Commercial grade methyl parathion (MP) was used which was manufactured by Dhanuka Agritech limited, Gudgoan-Haryana (India). It contains 62.5% MP. A stock solution of 1 mg/L was prepared for enrichment of the bacteria isolated from soil.

(iii) Isolation of Methyl parathion degrading bacteria from soil by enrichment method

Ten gram soil sample from the homogenized mixture was suspended in 30 ml of sterile basal mineral medium (BMM)[19,20] containing 0.3 ml from stock MP solution, in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (200 rpm) at 30°C temperature and pH 7. Every two weeks, an additional amount of MP was supplied in the flasks at increasing concentrations by adding 0.6, 0.9, 1.2 and 1.5 ml of the stock MP solution. After 2.5 months of incubation 10 ml sample from flaks was transferred to 90 ml of sterile BMM containing 50 mg/l of MP in Erlenmeyer flasks, and was shaken at 30°C temperature (200 rpm). After two weeks, 10 ml samples from flask were transferred to fresh BMM containing 50 mg/L MP. After three consecutive transfers, mixed bacterial cultures were collected on BMM agar plates containing 50 mg/L MP and tested for MP degrading ability, and pure culture were isolated[19,21].

(iv) Purification of bacterial colonies

After enrichment process, isolates were collected on BMM agar plates containing 50 mg/L MP and streaked separately on Luria bertani (LB) agar plates containing 50 mg/L MP, and incubated at 37°C for 24-48 hrs. This procedure was repeated several times to ensure purity of isolated colonies.

(v) Inoculum preparation

Isolates were streaked separately on LB Agar plates incubated at 37°C for overnight. After incubation a single colony inoculated in 5 ml LB broth and this was incubated in shaking incubator (200 rpm) at 37°C for overnight in. After growth, 1.5 ml culture was taken in appendorf and cells were separated by centrifugation. These were washed twice with sterile normal saline (0.85%) at 4°C on 12000 rpm for 15 minutes and re-suspended in sterile BMM. Suspended cells of each isolate were added to 100 ml of BMM containing 50 mg/L (Standard MP Concentration) MP and incubated at 30°C at 200 rpm, maintaining pH at 7. This process of inoculum preparation was used for each experiment.

(vi) Time study for the growth of Methyl parathion degrading bacteria

5 ml culture from prepared inoculum (discuss above) was inoculated in fresh 95 ml BMM (V/V), containing 50 mg/L MP and incubated at 30°C and 200 rpm, at pH 7. Media without inoculum served as controls for every test run. The same procedure was applied for each isolate. Microbial growth was checked spectrophotometrically 600nm after incubation at 0, 2, 4, 6, 24, 36, 48, 72 and 120 hrs of inoculation respectively.

(vii) Determining Utilization of Methyl parathion by Isolates

In order to investigate, bacterial isolates utilized MP as sole carbon and energy source this experiment was performed. 5 ml culture from prepared inoculum of each isolates were inoculated separately in fresh 95 ml BMM (V/V), containing 50 mg/L MP. Control was also prepared separately for each sample which as devoid of MP. Flasks were incubated at 30°C and 200 rpm, at pH 7. The growth was checked by spectrophotometer at 600nm after 48 hrs of inoculation.

(viii) Screening and selection of potential Methyl parathion degrading bacteria

For screening, 5 ml culture from prepared inoculum was inoculated separately in 95 ml BMM containing 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L, 1000 mg/L, 1200 mg/L, 1400 mg/L and 1600 mg/L of MP. Same procedure was adopted for each isolate. Flasks were incubated at 30°C, pH 7 and 200 rpm. Bacterial growth was checked at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 120 hrs of inoculation by spectrophotometer at 600nm.

(ix) Quantification of Methyl parathion concentration by GC-MS

Degradation of MP was analysed by gas chromatography coupled to mass spectrophotometer (GC-MS). Isolates were taken which showed maximum growth at high MP concentration. Parellal controls (without culture) were also run and taken for comparison purposes. Tubes were centrifuged at 12000 rpm for 15 min at 4°C, supernatant was collected in tube and extracted with dichloromethane. Dichloromethane extracts were analysed by GC-MS (Agilent Technologies, USA) in full scane mode.

(x) Identification of potential Methyl parathion degrading bacteria

Identification of MP degrading bacteria was carried out by several morphological and biochemical tests such as Catalase, Oxidase, MR-VP, urease, Indole etc. The procedure of *Bergey's Manual of Systematic Bacteriology*[22] for identification was followed. For molecular identification, DNA was isolated from each isolate as described earlier[23]. The 16S rRNA was amplified through PCR using universal 16S1 (forward) and 16S2 (reverse) primers. The amplified products were purified by fermentas kit (Gene JET™ Gel, Extraction Kit # K0692) and sequencing was carried out using the same PCR primer on a 96 capillary model 373xl system using the Big Dye Terminator kit from Applied Biosystems (Applied Biosystems, Foster City, CA, USA).

All experiments were carried out in triplicates.

RESULTS

1. Isolation of Methyl parathion degrading bacteria from soil sample

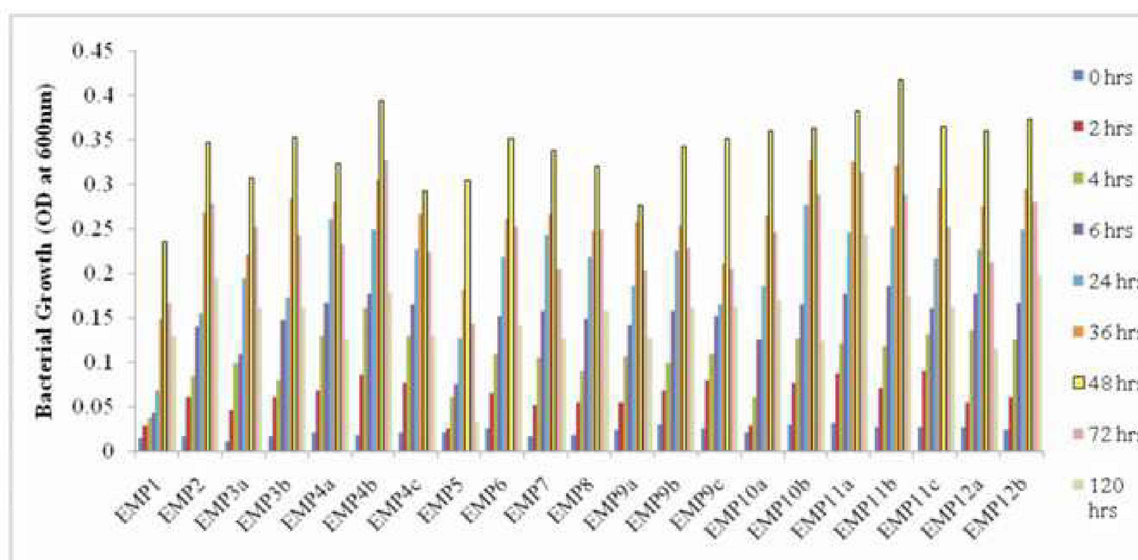
The mixed bacterial growth in BMM containing 50 mg/L MP, the faint lemon color of the

medium (the original color of commercial MP) disappeared within 24 hrs and turbidity developed in medium from 24 to 48 hours. The color of the medium was changed from faint lemon color to white. 21 isolates were isolated by enrichment method, which were capable for growing in BMM with 50 mg/L MP. These isolates were purified in LB agar plates containing 50 mg/L MP. The names of the different isolates were coded as EMP1, EMP2, EMP3a, EMP3b, EMP4a, EMP4b, EMP4c, EMP5, EMP6, EMP7, EMP8, EMP9a, EMP9b, EMP9c, EMP10a, EMP10b, EMP11a, EMP11b, EMP11c, EMP12a and EMP12b.

2. Time study for the growth of Methyl parathion degrading bacteria

When twenty one bacterial isolates inoculated in BMM containing 50 mg/L MP the maximum growth was recorded after 48 hrs of incubation. This growth was confirmed by the spectrophotometer at 600nm (Graph 1). No growth was observed in uninoculated control.

Graph 1
Time study for the growth of Methyl parathion degrading bacteria

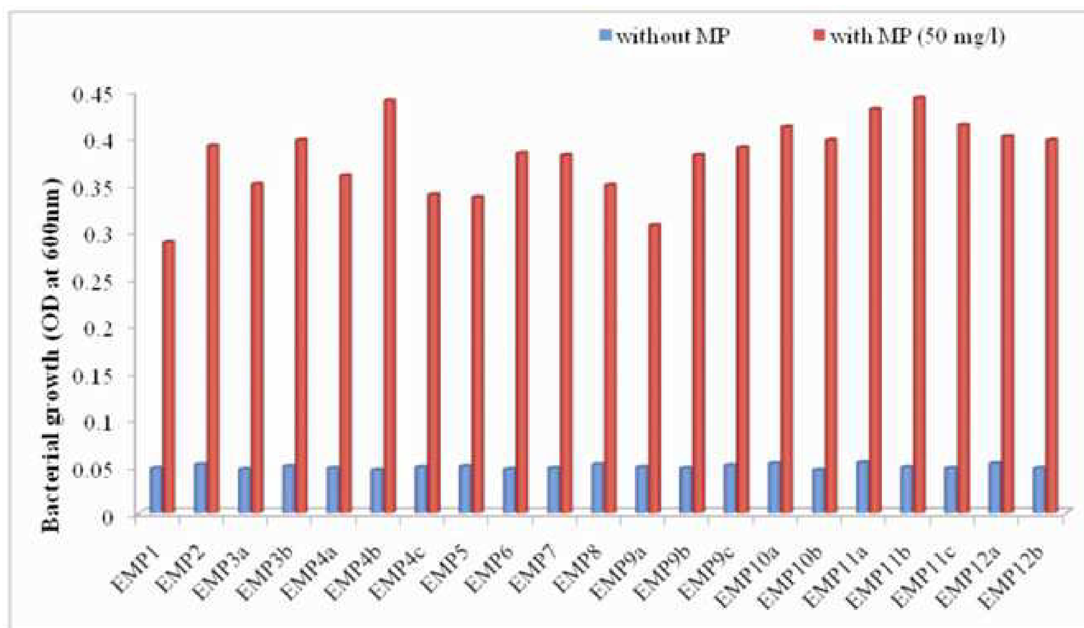


The growth of pesticides degrading bacteria in BMM containing 50 mg/L MP at different time (0, 2, 4, 6, 24, 36, 48, 72, 120 hrs) at 30ºC, pH 7, 200 rpm.

3. Isolates utilize Methyl parathion as a source of carbon and energy

Bacteria utilized MP as a carbon and energy source was confirmed by this experiment. The growth was observed after 48 hrs of incubation in BMM containing 50 mg/L MP but this growth was not recorded in the control which was devoid of MP (Graph 2).

Graph 2
Isolates utilize Methyl parathion as a source of carbon and energy

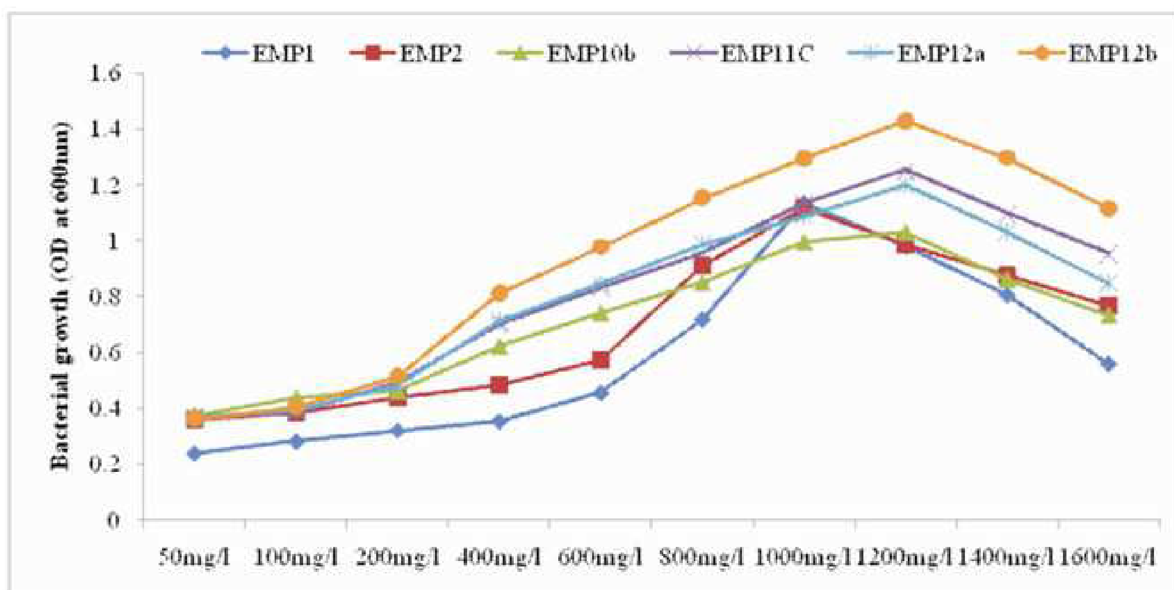


Growth of isolates in BMM in the absence and presence of (50 mg/L MP) at 30ºC, pH 7, and 200 rpm after 48 hrs.

4. Screening and selection of potential Methyl parathion degrading bacteria

From the group of 21 isolates, six bacterial isolates- EMP1, EMP2, EMP10b, EMP11c, EMP12a, and EMP12b were selected on the basis of their growth at higher concentration of MP i.e. 1000 mg/L, 1000 mg/L, 1200 mg/L, 1200 mg/L, 1200 mg/L and 1200 mg/L respectively. Maximum growth indicated the highest degradation of MP. The maximum growth was observed after 48 hrs of incubation (Graph 3).

Graph 3
Screening and selection of potential Methyl parathion degrading bacteria



Growth of potential MP degrading bacteria in BMM at different concentration of MP (50 mg/l to 1600 mg/l) at 30°C at 200 rpm and pH 7.

5. Analysis of methyl parathion degradation by GC-MS

EMP11c, EMP12a and EMP12b showed almost the complete degradation (97%, 96.4% and 99.9%) at 1200 mg/L MP after 48 hrs (Fig 1, 2, 3 and 4) but EMP1, EMP2, and EMP10b showed lower degradation (44.9%, 62.25% and 54.42%) at 1000 mg/L, 1000 mg/L, 1200 mg/L MP after the similar exposure. No degradation was observed in the control without any bacterial culture.

Analysis of methyl parathion degradation by GC-MS

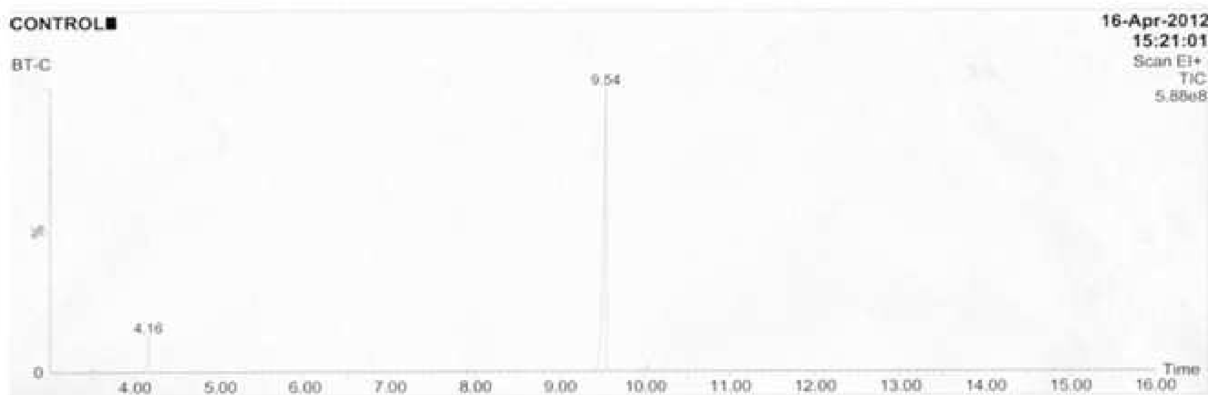


Figure 1
GC-MS spectrum of standard methyl parathion.

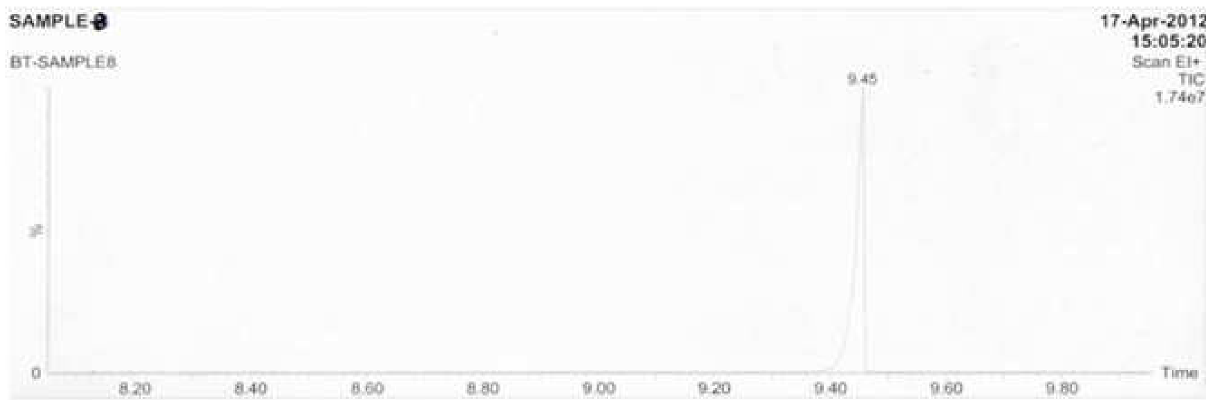


Figure 2
GC-MS chart showing degradation of methyl parathion by EMP11c (Pseudomonas diminuta) after 48 hrs.

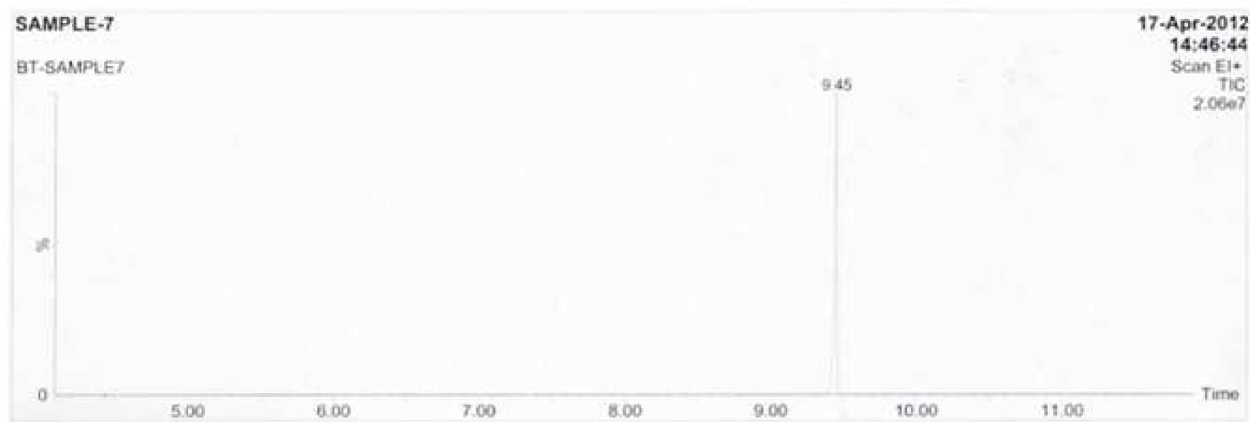


Figure 3
GC-MS chart showing degradation of methyl parathion by EMP12a (Pseudomonas putida) after 48 hrs.

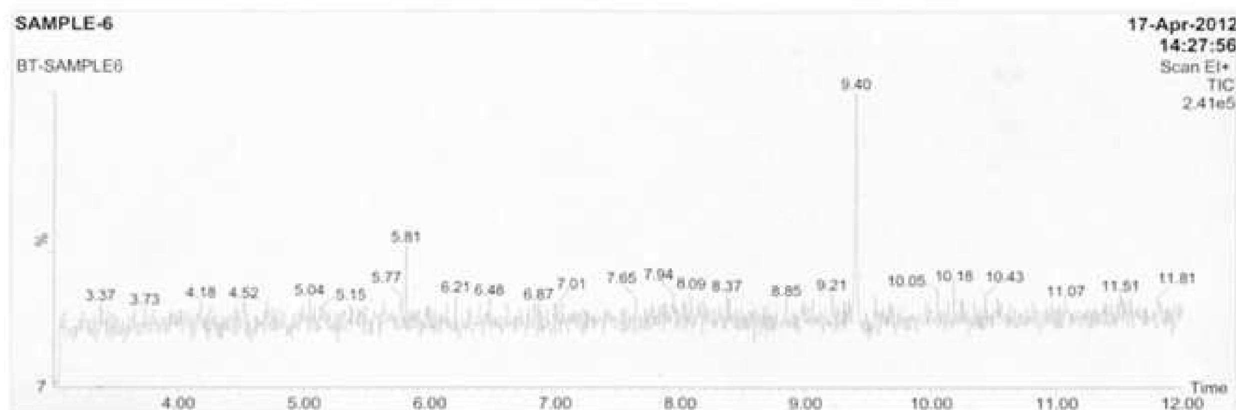


Figure 4
GC-MS chart showing degradation of methyl parathion by EMP12b (Pseudomonas aeruginosa) after 48 hrs.

6. Identification of Methyl parathion degrading bacteria

After GC-MS analysis, EMP11c, EMP12a and EMP12b were identified by morphologically, biochemical test and 16S rRNA sequence analysis. Identification was carried out by observing colony characters, growth, motility, Gram's staining and biochemical tests like Catalase, Oxidase, MR-VP, urease, Indole test etc. (Table 1). On the basis of morphological and biochemical characteristics, isolates EMP11c, EMP12a and EMP12b were identified as a member of the genus *Pseudomonas.* This was further confirmed by complete sequencing of the 16S rRNA analysis and was identified as *Pseudomonas diminuta*, *Pseudomonas putida* and *Pseudomonas aeruginosa*, respectively.

Table 1
Biochemical characterization of Methyl parathion degrading isolates, isolated from the soil sample.

Test	EMP11c	EMP12a	EMP12b
Gram's reaction	-	-	-
Cell shape	Rod shaped	Rod shaped	Rod shaped
Motility	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Methyl-red	-	-	-
Voges-Proskauer test	-	-	-
H_2S production	-	+	-
Indole test	-	-	-
Citrate test	+	+	+
Hydrolysis of starch	-	+	-
Urease test	-	-	-
Utilization of glucose as carbon source	-	+	-
Gelatin liquefaction	-	-	+

EMP11c = Pseudomonas diminuta, EMP12a = Pseudomonas putida and EMP12b = Pseudomonas aeruginosa. + = Present, - = Absent

DISCUSSIONS

In the present study, twenty one bacteria were isolated by enrichment method from pre-treated with MP agricultural soil of Gwalior and nearby places, Madhya Pradesh (India). These twenty one isolates were capable of biodegrading the MP. Several investigators have also isolated different bacterial species from organophosphorus pre-treated agricultural soil by enrichment method. Four[24] and thirteen[16] bacterial isolates were isolated from organophosphate treated soil. Two bacterial species were isolated from organophosphorus treated soil by many authors[17,19]. Mixed bacterial cultures were also obtained from MP treated soil by some researchers[5,15,25]. Twenty one bacteria were isolated in this study and they were capable for utilizing 50 mg/L MP as a carbon and energy source for their growth. Some investigators have studies the biodegradation of organophosphorus pesticides in the presence of addition energy sources such as glucose, maltose, yeast extract, $NaNO_3$, nutrient broth in medium which support the bacterial growth and enhanced biodegradation[18,26,27,33].Glucose supported the growth of *Bacillus* sp. and two other unknown bacterial species in the mixed culture in the BMM[15].These species were capable of degrading the commercial grade MP only and the biodegradation of pure MP compound remained unaffected. In the present study, three isolates showed the highest degradation of 1200 mg/L MP after 48 hrs, which were not reported in previous studies. In earlier works, the *Flavobacterium* species was isolated from soil, using parathion as a source of carbon and phosphorus[28] but this species was not able to degrade the diazinon. The result of this study did not show similarities with the findings of other investigation on microbial degradation of MP[21]. They isolated the microalgae and cyanobacterial species from pesticides contaminated soil. These isolates used the MP as a source of phosphorus and nitrogen. *Pseudomonas putida* was also capable of degrading the MP[29]. The methyl parathion was also hydrolysed by *Plesiomonas* species strain M6[12]. The MP degrading *Bacillus* species and other two unknown bacteria were isolated from MP pre-treated agriculture soil sites at Talingchan, Bangkoknoi, Bankok and Thiland[15]. The *Burkholderia cepacia* showed comparatively higher MP degradation and were able to degrade 50μg/ml MP in BMM. They obtained the different result from present study and reported that the *Burkholderia cepacia* which was degrading the commercial grade MP in the presence of glucose in BMM was unable to degrade the analytical grade in the similar conditions. A bacterial species *Ochrobactrum anthropi* was also isolated from MP treated soil[16]. But they could not isolate *Pseudomonas*

species in such soil. *Ochrobactrum anthropi* degraded MP completely in medium. A Gram negative *Serratia* sp. from MP contaminated soil was isolated[13]. This strain utilized MP as a source of carbon and energy. *Bacillus* and *Pseudomonas* species isolated from ground nut fields pre-treated with organophosphorus pesticides[30]. These species were degrading several insecticides such as chlorpyrifos, phorate, dichlorvos, methyl parathion and methomyl. The *Pseudomonas aeruginosa* and *Trichoderma viridae* were isolated from the soil containing MP and monochrotophos[17]. Both species showed higher degradation of MP than monochrotophos but *Pseudomonas aeruginosa* degraded the MP within 24 to 48 hrs and *Trichoderma viridae* from 48 to 72 hrs. Biodegradation of MP has been also reported in which the *Bacillus pumilus* Ti showed the ability to degraded the 500 ppm concentration of MP in the presence of glucose[18].

CONCLUSION

This study reports three *Pseudomonas* species, *Pseudomonas diminuta*, *Pseudomonas putida* and *Pseudomonas aeruginosa* for the first time degrading the MP in the same soil. These species showed higher degradation of MP reported till date and were capable of degrading the MP up to (1200 mg/L) within 48 hrs. Present study is useful in the detoxification of organophosphorus contaminated soil and may lead to development of a possible bioremediation in the near future for reclamation of contaminated soil. Environmental factors such as physical and chemical characteristics of the substrate, nutrients status, pH, temperature, biotic factors and inoculum density interfere the accomplishment of any bioremediation process[26,31,32]. Optimization of environmental conditions, substrate concentration and related supplements are required in the agricultural fields to enhance the biodegradation activity of these three species.

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