Isolation, Characterization and Identification of Dimethoate Degrading Bacteria from Soil Series of Tamil Nadu

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ABSTRACT

Bacteria play a very important role in degrading various compounds which pollute the environment. Some of the bacteria belonging to the genus Bacillus, Pseudomonas, Rhodococcus, and many other bacterial species have the capacity to degrade large number of xenobiotic compounds, petroleum products, pesticides, industrial effluents, etc. Dimethoate is an organophosphate compound which is used extensively in agriculture. In this study, dimethoate degrading organisms has been isolated and identified by biochemical and 16s rRNA gene amplification. In Total 44 isolates of dimethoate degrading bacteria were isolated from dimethoate contaminated soils. Among the 44 isolates 33 isolates were Bacillus, 7 isolates were Enterobacter, 3 isolates were Pseudomonas and 1 isolate was Aeromonas. Bacillus pumilus predominantly degraded dimethoate which has a higher growth rate of OD range 0.36 at 6 hours. The phylogenetic tree was constructed in order to identify the closely related species within the bacterial population.

Key words: Pesticide, Biodegradation, Bacillus, Dimethoate, Enrichment.

INTRODUCTION

India is one of the largest consumers of pesticide in South Asia. Pesticides are chemicals compounds which include insecticides, fungicides, herbicides, nematicides and few more. They are applied to the environment as a control measure against the pests and the parasites to have a secure agricultural and industrial produce. Persistence of pesticides in the soil can vary from few hours to many years as in case of Organochlorine (OC) pesticides. Even though OCs had been either banned or restricted in many countries, there are reports of their residue in soil [18, 25, 27, 2]. The organophosphorus pesticides (OP) are all esters of phosphoric acid and are also called organophosphates, which includes aliphatic, phenyl and heterocyclic derivatives. Organophosphate compounds (OPCs) when exposed to sunlight, air and soil degrade rapidly by...
hydrolysis, though minute quantity of residue may be detected in food and drinking water. But the most important drawback of OPcs is that they pose the problem of acute toxicity on sudden exposure to larger amounts. Dimethoate (O, O-Dimethyl-S-(N-methylcarbamoylmethyl) phosphorodithioate) is an organophosphorous insecticide that is used worldwide in agriculture and urban areas due to its high efficacy and rapid environmental degradation. It was registered in 1962 and has been used to control a wide range of insects including mites, flies, aphids, and plant hoppers [16]. Dimethoate can be applied to many crops such as fruits, vegetables, grains and ornamentals, in addition to non-agricultural applications for landscape maintenance and structural pest control. However, in 2000, all non-agriculture uses of dimethoate including residential uses were cancelled. Roughly 816,466 kg’s of active ingredient is applied annually on agricultural sites with the highest applications being on alfalfa, wheat, cotton, and corn [24]. In California, its use has decreased approximately 90% on alfalfa, oranges, and grapes between the years 1990 and 2011 [6]. Dimethoate is one of the most important organo phosphorous insecticides and its poisoning is usually associated with the neuromuscular transmission block in both animals and humans [7]. Immunological effects due to Dimethoate have also been reported [12]. These pesticides represent a group of pesticides that is widely used and has been shown to have toxic effects in man [1, 7]. Toxicity of organo phosphorous pesticide in any organs results in effects on many organs [1]. Microorganisms can utilize pesticide as a source of energy. In fact, soil bacteria constitute the principle biological agents that are responsible for accelerated biodegradation [4]. Especially saprophytic microorganisms perform the major degradation of pesticides in the soil and many microorganisms degrade organic pesticides, because of their comprehensive enzyme systems, which have the capacity to hydrolyze, reduce and oxidize these compounds [22, 13]. Microbial degradation of pesticides involving their biochemical and molecular mechanisms has been well documented earlier [5, 20]. Use of microorganisms to remove the pollutants from contaminated sites is an effective, minimally hazardous, versatile and environment-friendly strategy. The main objective of the present study described the isolation and molecular characterization of organo phosphorous pesticide (Dimethoate) degrading bacterial strain which was isolated from the dimethoate contaminated soil using an enrichment culture technique, and to optimize the growth of the dimethoate degrading bacterial isolate has been investigated under aerobic conditions.

MATERIALS AND METHODS

Collection of Sample
The soil samples used in this study were collected from different regions of Tamil Nadu, (Table.1). These samples were collected using auger up to a depth of 15cm. The collected samples were air dried, ground, passed through 2 mm sieve to get fine soil particles and stored with in sealed plastic bags at room temperature. These stored samples were used for microbial study.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Geographical area</th>
<th>Standing crop</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Achuvayal – Ramnadhapuram, Tamil Nadu</td>
<td>Paddy and Chilies</td>
</tr>
<tr>
<td>2.</td>
<td>Pannivayal – Pattukottai, Tamil Nadu</td>
<td>Paddy</td>
</tr>
<tr>
<td>3.</td>
<td>Irugur – Coimbatore, Tamil Nadu</td>
<td>Banana and Brinjal</td>
</tr>
</tbody>
</table>
Pesticide selected for the study
The pesticide selected for the present study is Dimethoate which is basically an organo phosphate (OP) pesticide, chemically termed as O, O - dimethyl - S - methyl carboxyl - methyl phosphorodithioate (Chemical Abstracts Service (CAS) number 60-51-5) widely used in Tamil Nadu (Personal Survey). The pesticide was purchased from the local pesticide supplier.

Amendment of Pesticides in soil
100g of soil samples were amended with 1% of Dimethoate pesticide and incubated at room temperature with appropriate soil moisture content. The samples were withdrawn at regular intervals of 0, 2, 7, 15, 30, 45, 60, 90, and 120 days.

Isolation and purification of dimethoate degrading bacterial strain
1g of pesticide contaminated soil sample was suspended in 9 ml sterile distilled water in a sterile test tube to get 10 % soil suspension. From that, 1 ml was transferred to 9 ml of sterile distilled water in another tube to make $10^{-2}$ dilution. Following above procedure, the soil suspension was serially diluted up to $10^{-5}$ dilution. Then from each dilution 100µl of soil suspension was poured on N-Agar plates (Hi-media) and incubated at 37°C for 48 hours to get isolated colonies of dimethoate bacteria. The well isolated colonies were grown on sterile nutrient agar slants as pure cultures and maintained at 4°C streaked nutrient agar slants at as stock culture. This stock culture was used for the further experimental procedures.

Biochemical Characterization
Biochemical analyses of all isolates were carried out according to Bergey’s Manual of Determinative Bacteriology and classified primarily through morphology, physiological and biochemical observations [19].

Isolation of genomic DNA
The isolates were used to inoculate in Nutrient broth (Hi-media) and incubated overnight at 37°C and 200 rpm. The resulting bacterial suspension was pelleted at 10,000 rpm for 5 min and the genomic DNA was extracted using the method outlined by using Phenol: Chloroform: Isoamyl alcohol [23].

PCR amplification and sequencing of 16S rRNA encoding genes
In order to amplify ~ 0.4kb gene from the isolated genomic DNA, 16S rRNA gene primers are [FP: 5’- CCTACGGGCGGCAGCAG- 3’ and RP: 5’- GGATTAGATACCCGTGGTAGTC- 3’] used by Klindworth [3], were used. A 20µl reaction was set with 10µl of 2X Master mix (1 U Taq DNA polymerase, 1.5mM MgCl$_2$), 1µl (10pmol) each of Forward and reverse primers, 2µl of DNA and 6µl of NFW. PCR was performed in Eppendorf Master Cycler (Eppendorf,
Germany). PCR conditions were set as follows: initial denaturation at 95°C for 5 minutes, followed by 25 cycles of denaturation at 95°C for 40 seconds, annealing at 55°C for 2 minutes and primer extension at 72°C for 1 minute ending with final elongation step at 72°C for 7 minutes. PCR product obtained was gel purified and sent for sequencing with 16S rRNA primer. DNA alignments were made and the sequences were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to identify the isolate.

**Phylogenetic analysis**
The 16S rRNA gene sequences of all isolates were compared to references of 16S rRNA gene sequences of other bacterial isolates retrieved from NCBI database. Phylogenetic analysis was performed for gram positive and gram negative bacteria separately. Mega6.0 software was used to construct phylogenetic relationship with our isolated strain by neighbor-joining method.

**Growth curve**
Nutrient broth was prepared in 250 ml Erlenmeyer flasks and autoclaved at 121°C, 15 psi for 20 minutes. After cooling each flask was inoculated with purified bacterial isolates. Control tubes were also maintained. The bacterial tubes were incubated at 37°C for 24 hrs. OD was measured at 600nm with duration of 0, 10, 30, 1, 2, 3, 6, 12 and 24 hrs.

**RESULT**

**Isolation of Bacterial cultures**
In Total 44 bacterial isolates were obtained from the collected soil samples. They were morphologically identified and gram stained. Out of 44 isolates 33 isolates were identified as Gram positive rod shaped bacteria and 11 Gram negative rod shaped bacteria and all were pure cultured.

**Biochemical Characterization**
The biochemical analysis showed that all the 33 isolates of Gram positive bacteria were identified as *Bacillus*. From the 11 isolates of gram negative bacteria, 7 isolates were identified as *Enterobacter*, 3 isolates were *Pseudomonas* and 1 was *Aeromonas* (Table. 2 & 3).

**Table 2.0: Physical and morphological characterization of the bacterial isolates**

<table>
<thead>
<tr>
<th>Size</th>
<th>Margin</th>
<th>Elevation</th>
<th>Surface</th>
<th>Opacity</th>
<th>Pigmentation</th>
<th>Form</th>
<th>Bacterial Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>Entire</td>
<td>Convex</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Light yellow</td>
<td>Circular</td>
<td>Aeromonas</td>
</tr>
<tr>
<td>Large</td>
<td>Undulate</td>
<td>Convex</td>
<td>Smooth</td>
<td>Opaque</td>
<td>Light yellow</td>
<td>Circular</td>
<td>Bacillus</td>
</tr>
<tr>
<td>Small</td>
<td>Entire</td>
<td>Flat</td>
<td>Smooth</td>
<td>Opaque</td>
<td>Bright yellow</td>
<td>Irregular</td>
<td>Enterobacter</td>
</tr>
<tr>
<td>Small</td>
<td>Entire</td>
<td>Raised</td>
<td>Smooth</td>
<td>Opaque</td>
<td>Brown</td>
<td>Circular</td>
<td>Pseudomonas</td>
</tr>
</tbody>
</table>
### Table 3.0: Biochemical characterization and identification of the bacterial isolates

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Gram stain, Shape</th>
<th>MacConkey agar</th>
<th>Motility</th>
<th>Litmus milk-agar</th>
<th>Dextrose Fermentation</th>
<th>Lactose</th>
<th>Sucrose</th>
<th>H₂S production</th>
<th>Indole production</th>
<th>Nitrate reduction</th>
<th>MR reaction</th>
<th>VP reaction</th>
<th>Citrate use</th>
<th>Gelatin liquefaction</th>
<th>Starch hydrolysis</th>
<th>Catalase test</th>
<th>Oxidase test</th>
<th>Urease test</th>
<th>Endospore formation</th>
<th>Lysine decarboxylase</th>
<th>Identification</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>-ve Rods</td>
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<td>3</td>
<td>-ve Rods</td>
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<td>4</td>
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**PCR amplification**

The PCR products were run on 1.5% Agarose gel and documented in gel doc system (BIO-RAD Gel doc XR, USA) (Fig: 1, 2 & 3). A bright band near 500 bp was observed.

**Fig 1:** PCR amplification 16s rRNA Gene

**Fig 2 & 3:** PCR amplification 16s rRNA Gene
Phylogenetic analysis
The amplified PCR products were purified using gel extraction kit (QIAGEN, Inc) and sequenced. All the sequences were edited manually and trimmed to remove ambiguous region. Only sequences which read ~ 464 bp were analyzed and submitted to NCBI (GenBank accession Nos KU720338 – KU720357, KU645317 – KU645323, KU672333 – KU672335, KU761821 – KU761830, KU870425 – KU870427, KU933683). The 16s rRNA sequences were BLAST searched against GenBank data base in the NCBI. The sequences shows in which 14 isolates were Bacillus pumilus, 7 isolates were Bacillus licheniformis, 4 isolates were Bacillus cereus, 3 isolates were Bacillus amyloliquefaciens, 2 isolates were Bacillus megaterium, 1 isolate each of Bacillus xiamenensis, Bacillus altitudinis and Bacillus manpovensis, 2 isolates were Pseudomonas otitidis and 1 isolate was Pseudomonas stutzeri, 7 isolates of Enterobacter cloacae and 1 Aeromonas veronii was identified. Then the sequences were aligned by multiple sequence alignment with other bacterial isolates and phylogenetic tree was constructed using Mega 6.0 software (Supplementary 1 & 2). The phylogenetic analysis revealed that our isolates were all closely associated with each other. From the gram positive bacteria phylogenetic analysis all the isolates of Tamil Nadu are closed related as the strains of Bacillus are in the same lineage. From the gram negative bacteria phylogenetic analysis all the Enterobacter from Tamil Nadu fall in same lineage and closely related to Enterobacteraceae.

Growth Curve
All the bacterial isolates had their log phase peaking at 6 hours. Aeromonas and Enterobacter had their highest peak OD range of 0.25-0.29. Pseudomonas stutzeri and Pseudomonas otitidis had peak OD values of 0.25 and 0.31 respectively. Whereas isolates of Bacillus had the OD value ranges from 0.27 to 0.36 except B. manpovensis, which has the highest peak range of 0.24 (Fig. 4 & 5). These results imply that the growth rate of Bacillus species in the dimethoate contaminated soil was higher when compared to other bacterial species.
DISCUSSION

The present study was carried out for the isolation and identification of dimethoate degrading bacterial species from the soil. It was found that majorly *Bacillus* were good pesticide degrading organisms. Predominantly *Bacillus pumilus* was isolated from the soil. Reports shows that the *Bacillus pumilus* naturally exhibits resistance to much environmental stress such as non availability of nutrients, irradiation, Toxic compounds, etc [26]. The 16s rRNA gene shows 99% similarity of the 14 isolates of isolated *B. pumilus*. Since many isolates are *Bacillus*, 3 isolates of *B. amyloliquefaciens*, 7 isolates *B. licheniformis* and 4 isolates of *B. cereus* has been isolated in this study. It was reported that *B. licheniformis* and *B. cereus* has the capacity to grow in heavy polluted soil and has heavy metal tolerant capacity [10]. These bacterial species were extensively used in many industries. It was reported *Bacillus licheniformis* has the ability to decolorize textile dyes [17]. Studies degradation of dimethoate by *B. licheniformis* was studied and reported that degradation upto 3.5 mg/ml in nutrient broth [15]. Thus *B. licheniformis* had been identified with the property to degrade different varieties of compounds. Recent reports also showed that *B. pumilus* degrade Malathion and Dimethoate with 45% and 37% degradation [21]. *B. amyloliquefaciens* and *B. licheniformis* has the capacity to degrade the organophosphate compounds, Malathion and Quinalphos [10]. A recent study endophytic quinclorac-degrading bacterium strain Q3 from the root of tobacco grown in quinclorac contaminated soil has been isolated and it was conformed as *Bacillus megaterium*. The effects of temperature, pH, inoculation size, and initial quinclorac concentration on growth and degrading efficiency has been calculated and found that the bacterium could degrade 93% of quinclorac from the initial concentration of 20 mg/L in 7 days [27]. Thabit and Naggar were reported that effective bacterial strains that degrade malathion are S5 (*Bacillus licheniformis*), S4 (*Bacillus pseudomycoides*) and S1 (*Pseudomonas aeruginosa*), and their residue half life values (RL½) were 12.49, 12.72 and 16.68 days respectively, when compared to that of control strain (27.50 days) [14]. From the above reports it is clear that the genus *Bacillus* is capable of degrading various compounds including OPCs. Thus it can be concluded that the isolates obtained from this study posses the quality to degrade dimethoate. Usually the genus *Pseudomonas* has the ability to degrade several compounds. Three isolates of *Pseudomonas* were obtained from this study. It was reported that *Pseudomonas otitidis* has the capacity to degrade triphenyl methane dyes [8]. *Pseudomonas stutzeri* has degraded naphthalene and survived in petroleum contaminated soils [11]. Genome data of *P. stutzeri* YC-YH1 to possess methyl parathon hydrolase (mpd, EF515812) and a gene of organophosphorus hydrolase (ophC2, EU651813), which are regarded to degrade most of
OPPs [28]. A strain of *P. stutzeri* capable of degrading dimethoate (OPC) had been identified. In this present study *Enterobacter* has been also isolated from the pesticide contaminated soil. Previous report of degrading organophosphate compound chlorpyrifos by *Enterobacter* strain was produced by [9]. In his study it was found that six - chlorpyrifos degrading bacteria were isolated. From this study, various strains of bacteria that were capable of degrading dimethoate contaminated soil were identified. Of all the isolates *Bacillus* was found to be most potent against dimethoate. Out of various *Bacillus* species, *B. pumilus* was found to be more predominant in the dimethoate contaminated soil samples. The isolates which followed *B. pumilus* in terms of predominance in dimethoate degradation were *B. licheniformis, Enterobacter cloacae, B. cereus, B. amyloliquefaciens, B. megaterium, Pseudomonas otitidis* and *Pseudomonas stutzeri*

CONCLUSION
The present study was carried out to isolate and identify dimethoate degrading bacterial species from the soil. *Bacillus* was found to be an excellent pesticide degrading organism.

ACKNOWLEDGEMENT
The support extended by Sri Krishna Arts and Science College and Tamil Nadu State Council for Science and Technology (TNSCST) for the conduction of this study is highly acknowledged.

REFERENCE


Supplementary 1: Phylogenetic analysis of dimethyl ether-degrading Gram Positive bacterial isolates
Supplementary 2: Phylogenetic analysis of dimethoate degrading Gram negative bacterial isolates