

Molecular Characterization of Isolated Methyl Parathion Degrading Bacteria and Gene

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Abstract— The burning problem in present era is pesticide residue in fruits and foods. present study focus on degradation of pesticide in contest to that total 45 methyl parathion(MP) degrading bacteria were isolated from sludge of MP producing industrial waste. According to screening 21 highly efficient pesticide degraders were selected at 500ppm concentration of standard methyl parathion as a sole carbon source. All isolates were characterized by RAPD and based on the RAPD result all 21 bacterial isolates were grouped in to 2 main clusters with 58% similarity. Characterization of MP degrading gene was carried out by using specific primer. Out of 21 isolates, 14 isolates were containing mpd gene and 4 isolates were containing opd gene while remaining 3 isolates which did not show amplification with opd/ mpd primer so it may contain other unreported gene responsible for methyl parathion degradation. Isolates were identified based on 16S rRNA sequence and one highly efficient bacterium selected for genome characterization.

Key words: Methyl Parathion, RAPD, opd/ mpd gene

I. INTRODUCTION

Organophosphorus compounds (OP) are widely used as pesticides to control agricultural and household pests. Overall, OP compounds account for 38% of total pesticides used globally [1].

The excessive use of natural resources and large scale synthesis of OP compounds have generated a number of environmental problems such as contamination of air, water and terrestrial ecosystems, harmful effects on different biota, and disruption of biogeochemical cycling [2].

Microbial degradation is a common method for the removal of pesticide because of its low cost and low collateral destruction of indigenous animal and plant organisms. The first microbial degradation was reported in 1973 by *Flavobacterium* spp. [3]. Other than the bacteria bioremediation can be relies on fungi, and plants to alter contaminants in liquid cultures and soil systems as these organisms carry out as a normal life functions.

Pseudomonas putida able to degrade 110.24mg/l isolated and characterized form waste of pesticide producing industry [4].

In this present study identify microbes capable to metabolizing methyl parathion and to provide new aspects to study on the genetic diversity of microbial populations with their catabolic function. This provides an environmentally friendly, cost effective and rapid method of in situ detoxification.

Molecular characterization of bacteria done by using the RAPD-PCR and for identification of bacteria by amplified 16S rRNA sequencing. these were used to conduct genetic fingerprinting and obtain specific molecular markers for the studied isolates [5].

Analysis of the 16S rRNA gene sequence indicated that the isolates were related to members of the genera *Pseudomonas*, *Enterobacter* etc. [6].

II. MATERIALS AND METHODS

A. Soil Enrichment Technique For Isolation Of Methyl Parathion Degrading Bacteria:

Soil samples were taken by a core sampler from the top 10 cm depth of industrial sludge area and from the field. For enrichment 50gm of each sample was placed in glass dishes (5 cm deep × 9.5 cm diameter). Then soil samples were treated with an aqueous suspension of methyl parathion to give a final concentration of 500µl/100ml (commercial grade) in soil and the contents were mixed gently and incubated at room temperature (28°C) for eight weeks.

B. Isolation and Screening Of Methyl Parathion Degrading Bacteria:

After soil enrichment, a pinch of enriched soil was taken in mineral salts medium (MSM, pH 7.0) [7] supplemented with different concentrations of 0.15, 0.25, 0.35ml/l (commercial grade) of insecticide and incubated on a shaker. After a week, it was serially diluted and pour plated on to solid media for the isolation of methyl parathion degrading bacteria [8]. the bacterial isolates were screened by growing on higher concentration of methyl parathion-standard (50, 100, 150, 200ppm and up to 500ppm). Finally, the isolates capable of growing at 500ppm methyl parathion concentration were selected and used for further studies.

C. Random Amplified Polymorphic DNA (RAPD):

RAPD-PCR was carried out according to the procedure [5,9]. with some suitable modifications. RAPD-PCR experiments were done using 14 OPERON oligonucleotide primers of OPA, OPC and OPO series as Table 1.

PCR reaction was carried out in a total volume of 20µl. Each reaction mixture contained 1.0µl genomic DNA(25ng/µl), 2.0µl decamer oligonucleotide primer (25pmoles/µl), 0.3µl Taq polymerase (3U/µl), 2.0µl of 10X buffer, 0.06µl dNTPs mix (2.5mM each), 0.6µl MgCl₂(5mM) and 14.04µl Millipore Sterile distilled water. PCR amplification was performed for 45 cycles after an initial denaturation step for 5 min at 94 °C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 36 °C for 1.5 min, extension at 72 °C for 2 min. An extension step was performed for 7 min at 72 °C in the final cycle. The amplification products were resolved by electrophoresis in a 1.2% agarose gel.

The data was analyzed using NTSYSpc version 2.02 [10]. The data matrix was read by NTSYS-pc version 2.2 (Numerical Taxonomy and Multivariate Analysis System for Personal Computers, Exeter Software).

D. Characterization of Methyl Parathion degrading gene in Efficient Bacteria:

The Specific Primer for *opd/mpd* gene of 900-1000bp which encodes enzymes which are involved in methyl parathion degradation was amplified from all the isolates. Primers and PCR condition used for *opd/mpd* gene amplification described by [7,11]. As per Table 2.

PCR reaction was carried out in a total volume of 20 μ l. Each reaction mixture contained 1.0 μ l genomic DNA(25ng/ μ l), 1.0 μ l forward primer (25pmoles/ μ l), 1.0 μ l reverse primer (25pmoles/ μ l), 0.3 μ l Taq polymerase (3U/ μ l), 2.0 μ l of 10X buffer, 0.06 μ l dNTPs mix (2.5mM each), 0.6 μ l Mgcl₂(5mM) and 14.04 μ l Millipore Sterile distilled water. The primers were added individual to each tube.

PCR amplification was performed for 30 cycles after an initial denaturation step for 2 min at 95 °C. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 62 °C for 45 sec., extension at 72 °C for 2 min. An extension step was performed for 7 min at 72 °C in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel.

E. 16S r RNA Sequencing:

Identification of efficient pesticide degrading bacteria by 16S rRNA sequencing using 3130 xl gene sequencer and as per the recommended protocols.

III. RESULT

A. Soil Enrichment Technique For Isolation Methyl Parathion Degrading Bacteria

Five different soil samples (Ahmedabad, Ankaleshwer, Vadodara, Kalol and Field soil) were collected from industrial waste of pesticide and field soil of different areas of Gujarat were enriched with commercial grade pesticide for 8 weeks. By performing enrichment technique bacterial isolates were able to utilizing methyl parathion as the sole carbon source.

Isolation and screening of methyl parathion degrading bacteria

As many as 45 methyl parathion degrading bacterial isolates were obtained from 5 different soil samples. All the bacterial isolates showed growth on MSM agar containing methyl parathion up to 0.35ml/l methyl parathion (commercial grade). out of 45 isolates 21 isolates selected for further studies based on capability to MP degradation.

B. Random Amplified Polymorphic DNA:

Total 14 RAPD primers were amplified which gave a total of 124 bands/alleles with an average of 8.85 bands per primer. Out of 122 polymorphic bands, 114 shared band within two or more isolates, while 9 bands were unique-polymorphic. The percent polymorphism obtained for RAPD primers varied from 88.9% (OPC-05 and OPO-02) to 100% (OPA-01, OPA-3, OPA-04, OPA-05 (Fig.1), OPA-06, OPA-07, OPA-9, OPA-10 (Fig.2), OPA-11, OPA-19, OPC-5, OPC-11 (Fig.3) OPA-18 and OPO-02 (Fig.4) with an average value of 98.42% per primer. as shown in table-3. Identification of genotype specific marker from RAPD primers

In the present study, among the screened primers OPA-03, OPA-06, OPA-07, OPA-9, OPA-10, OPA-11 and OPC-11 amplified some of the unique genotype specific bands. Primer OPA-03 produced 1 unique band of M. wt. 169bp for MPD-10. A 345bp band in MPD-11 was produced by primer OPA-06. In case of primer OPA-07, 3204bp band was produced in MPD-14. Primer OPA-09 produced 2 specific bands of M. wt. of 753bp in MPD-11 and 1315bp in MPD-16. OPA-10 produced specific band of M. wt. of 376bp in MPD-10. OPA-11 produced specific bands of M. wt. of 519bp in MPD-13 and 636bp in MPD-15. In case of primer OPC-11 2224bp band was produced in MPD-3.

C. Cluster analysis of RAPD:

The RAPD data were subjected to statistical analysis for Jaccard's similarity coefficient and cluster analysis by UPGMA using NTSYSpc-2.02i software. The dendrogram was constructed using UPGMA based on Jaccard's similarity coefficient for RAPD data of 21 methyl parathion degrading bacterial isolates. The genotypes were grouped into two main clusters viz. cluster-A and cluster-B shared 58 % similarity. Fig-6 shows the dendrogram based on RAPD data.

Characterization of methyl parathion degrading gene in efficient bacteria

In present investigation, 21 methyl parathion degrading isolates were subjected to analysis using 5 different specific *opd/ mpd* primers. Out of 5 primers 4 primers were specific to *opd* gene as reported by Singh *et al.*, 2005 and 1 primer was for *mpd* gene as reported by Yang *et al.* 2006. Among 4 *opd* gene specific primer only 1 primer OPD-3 was amplified. Fig-5 shows *opd/mpd* gene profile.

IV. DISCUSSION

In screening of Methyl Parathion degradation 21 (twenty one) highly efficient isolates were selected according their growth on 500ppm concentration of standard methyl parathion pesticide. During the RAPD analysis the highest numbers of twelve bands were produced by primer OPA-07 whereas the lowest (seven) bands were produced by OPA-03, OPA-5 and OPA-10. The largest fragment of 3899bp was amplified by OPA-04 primer and the smallest fragment of 169bp was obtained with OPA-03 primer. The PIC values for RAPD marker ranged from 0.770 (OPA-10) to 0.989 (OPA-09) with an average value of 0.853 per primer and RAPD primer index (RPI) differed from 5.390 (OPA-10) to 10.53 (OPA-07) with an average value of 7.58 per primer. Thus, RPI showed that OPA-07 gave best result among the primers used. based on the dendrogram The cluster-A consisted 20 isolates, while cluster B consisted 1 isolate. Cluster A consisted 2 sub-cluster A1 which consisted 11 isolates MPD- 1, MPD-2, MPD-10, MPD-17, MPD-5, MPD-6, MPD-7, MPD-3, MPD-4, MPD-8 and MPD-9 while, sub-cluster A2 consisted 9 isolates MPD-11, MPD-21, MPD-12, MPD-16, MPD-18, MPD-13 and MPD-15 while cluster B consisted only 1 isolate MPD-14. In sub-cluster A1 2 isolates (MPD-1 and MPD-2) was 94% similar, while MPD-14 was 54% similar. Thus similarity range was found between 54% - 94 %.

The complete nucleotide sequence of the amplified opd gene was around 936bp. Out of 21 isolates four isolates (MPD-5, MPD-6, MPD-11 and MPD-15) showed amplification in OPD-3 primer. However, mpd gene was amplified in 14 isolates (MPD-1, MPD-2, MPD-3, MPD-4, MPD-8, MPD-9, MPD-10, MPD-12, MPD-13, MPD-16, MPD-17, MPD-18, MPD-20 and MPD-21) by using MPD primer at around 903bp which indicates that these organisms contain mpd gene which is responsible for methyl parathion degradation. While other organisms (MPD-7, MPD-14 and MPD-19) not shown amplification which indicates that genes differ from those identified previously for methyl parathion degradation.

V. CONCLUSION

Based on the work on the methyl parathion degradation it was concluding that the bacteria were capable to degrade pesticide and bacteria have genes responsible for the degradation of pesticide.

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