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Original Research Paper

Study of Methyl Parathion Degradation by *Arthrobacter globiformis* Ad26 Isolated from Soil

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ABSTRACT

Methyl parathion (MP), a widely used organophosphorus (OP) pesticide, degrader was isolated from MP contaminated soil sample. It was identified as *Arthrobacter globiformis* AD26 on the basis of biochemical studies and 16s rRNA analysis. MP at a concentration of 1250 µg/mL was totally biodegraded by *Arthrobacter globiformis* AD26 as the sole carbon source at retention time (RT) for 72 hours on shaker. Cells grown in medium containing MP as sole carbon source, showed higher amount of protein, than the cells grown in presence of glucose as sole carbon source which was estimated by Biuret method. These results were confirmed by SDS-PAGE which showed induction of 97 kDa protein. *P*-nitrophenol (PNP), a hydrolysis product of MP, was also completely degraded by *Arthrobacter globiformis* AD26. This strain contained plasmid of approximately 47 kb size. Plasmid-curing experiments produced plasmid-free strain. This strain was unable to utilize MP demonstrating that the degradative genes for MP were located on the 47-kb plasmid.

INTRODUCTION

In India, 15-20% of all agricultural produce is destroyed by pests (Bhalerao & Puranik 2007). This emphasizes the paramount importance of pesticides in India to prevent agricultural loss and enhance production. Methyl parathion (O, O-dimethyl-O-p-nitrophenyl phosphorothioate (MP) has been increasingly used as a pesticide in agriculture and public health since the 1970s in place of an analogous chemical, parathion (O, O-diethyl-O-p-nitrophenyl phosphorothioate) which has been banned in many countries because of its higher mammalian toxicity. Methyl parathion is an important broad spectrum organophosphorus insecticide and acaricide used to control insect pests of agricultural crops, primarily on cotton and vegetables (Adhya et al. 1981). This compound is classified as a highly toxic insecticide in U.S. Environmental Protection Agency list of toxicity class I compounds.

After being ingested by insects, the parathion becomes oxidized by oxidases to give paraoxon. It kills insects by contact, stomach and respiratory action. The neuro-toxicological property of MP was to carry out irreversible phosphorylation of esterases in the central nervous system of insects and mammals which leads to suppression of the activity of acetyl-cholinesterase, and as a result, prevent acetylcholinesterase from breaking down acetylcholine at the synaptic junction (Ohshiro et al. 1999, Skripsky & Loosli 1994). It leads to an overstimulation of the nervous system, causing nausea, confusion, and at high exposures respiratory paralysis and death (Conteras et al. 1999, Rayd 1999). It was also found to cause chromosomal damage associated with bladder cancer (Webster et al. 2002). Residual MP can be found in soil and water. It has been found to be highly toxic to birds, aquatic invertebrates, bees and humans (Xiaofen & Urs 2006).

Furthermore, MP can be potentially hazardous as a result of accidental spills and discharges from pesticide containers and wastes. OPs in the aqueous environment degrade spontaneously through different pathways like hydrolysis, photolytic oxidation, microbial transformations and other biological processes. The microbial application for MP detoxification would represent a significant improvement in waste disposal technology which is efficient, convenient, economical and causes little collateral destruction of indigenous flora and fauna (Timmis & Pieper 1999). MP can be biodegraded by plants, algae, fungi and bacteria, the key players in these processes being enzymes, such as hydrolase and oxidase (Kim et al. 2002, Amitai et al. 1998).

In this study, culture capable of biodegrading MP was enriched, isolated from soil and indentified as *Arthrobacter globiformis* AD26, and the plasmid origin of MP-degradation activity was investigated.

MATERIALS AND METHODS

Chemicals and media: Commercial grade methyl parathion (MP), 2.5% w/w dust formulation, used in enrichment and MP degradation studies was purchased from Northern Minerals limited, New Delhi, India.

Basal mineral medium (BMM) contained the following

ingredients (g/L): K_2HPO_4, 4.8; KH_2PO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.04; and $FeSO_4.7H_2O$, 0.005 (Chaudhry et al. 1988, Ou et al. 1989) and pH was adjusted to 7.2. The minimal medium was either supplemented with 0.25%, i.e., 62.5µg/mL of methyl parathion or 1mg/mL of glucose as sole carbon source just before inoculation. Basal minimal medium plates were prepared by adding 15g of agar to 1 liter of the medium.

The enrichment of MP-degrading microorganisms: Soil samples were collected from methyl parathion-treated farmlands. Enrichment was carried out by suspending a 10-g soil sample in 100mL of basal minimal medium containing 62.5µg/mL (since 2.5 g of MP dust formulations was added in 1 kg of soil or 1 liter of water for application in fields, 0.25% of MP dust formulation was used for enrichment) of methyl parathion in a 250-mL flask. The flasks were incubated on a rotary shaker (200 rpm) at room temperature (26° ± 2°C) for one week. After 1 week incubation flask was left on a bench top for a few minutes to allow the soil particles to settle and a10 mL sample of each flask was transferred to 90 mL of sterile BMM containing 62.5 µg of MP/mL in 250 mL Erlenmeyer flasks, without glucose, and were shaken at room temperature. Once every two weeks, 10 mL samples of each flask were transferred to fresh media similar to those described above. After three consecutive transfers, mixed bacterial cultures from enriched broth were collected and isolated on MP-BMM solid medium without glucose.

Standard graph of PNP: A spectrometric assay was employed to determine PNP produced during MP metabolism. Stock of 20µg/mL PNP was prepared in BMM. Range of 2-20 µg/mL of PNP was used and absorbance was read at 410nm, and standard graph was plotted.

Assay of residual MP: Alternately, the samples were hydrolyzed with 2.5 N NaOH, and the resulting product, p-nitrophenol, was estimated spectrophotometrically using standard graph of PNP. Standard MP solution in the range of 12.5µg/mL to 125µg/mL with an interval of 12.5µg/mL was prepared using BMM. One mL of 2.5N NaOH was added to 1ml of the standard MP solutions and tubes were incubated for 15 minutes. After this, the tubes were centrifuged at 3000rpm for 15 minutes. Absorbance of supernatant was recorded at 410nm. The amount of PNP produced from MP for respective MP standard solutions as a result of NaOH treatment were determined from PNP standard graph. The PNP readings for supernatant of the medium without NaOH treatment were also recorded. These readings were subtracted from the PNP readings obtained by NaOH treatment, to calculate the actual concentration of PNP corresponding to MP.

Viable count of the isolate: Five mL of culture (O.D.-0.5 at 530nm) was inoculated in 95mL of BMM medium supplemented with 62.5µg/mL of MP as a sole source of carbon and biotin, thiamine, pantothenic acid and Vit B12 at a concentration of 0.2 µg/mL of each were added. Non-inoculated media served as controls. Both 1 mL and 5 mL samples were collected from each flask and bacterial populations were counted using viable count on nutrient agar plates at various time intervals (0, 24, 48 and 72hrs). The residual concentration of MP was also analyzed spectrometrically at the same time intervals.

Acclimatization of the isolate to increasing concentration of MP: BMM slant grown culture (acclimatized to 62.5 µg/mL of MP) was washed with saline and 1mL of it was added in 50mL of BMM supplemented with 125 µg/mL of MP as a sole source of carbon and all the growth factors and incubated on shaker. Again growth from this flask was inoculated serially in flasks containing 250 µg/mL of MP in BMM. Similar experiments were repeated serially with 375, 500, 625, 750, 875, 1000, 1125 and 1250 µg/mL of MP in BMM. Degradation was checked by release of PNP and estimation of it. Similarly, medium control was maintained for respective concentrations of MP. Acclimatization was also checked by observing growth on solid medium containing respective concentration of MP.

Identification of the isolate: Methyl parathion-hydrolyzing bacterium was identified using morphological, cultural and biochemical characteristics based on the classification scheme outlined in Bergey's Manual of Determinative Bacteriology, 8th edition. Subsequent characterization was done by carrying out 16S rRNA sequence analysis.

Extraction and isolation of plasmid DNA from the isolate: Plasmid DNA was isolated by alkaline lysis method and observed by DNA electrophoresis described by Sambrook et al. (1989).

Plasmid curing: The isolate was streaked on Luria Bertanii agar plate and incubated at RT for 24 hrs. Growth from this plate was again streaked on LB agar plate and incubated at RT for 24 hrs. Similar passages were carried out 30 times and then growth from last passage of LB agar plate was streaked on a plate of BMM agar medium with MP as sole carbon source. Plasmid was extracted from the culture grown on LB agar and observed by DNA gel electrophoresis.

Comparison of protein profile of MP degrader, grown in presence of methyl parathion and glucose as sole source of carbon in BMM was made by Biuret and SDS-PAGE:

***Cultivation of cell mass*:** Cultures grown in one flask having BMM containing 62.5µg/mL of MP and in another flask BMM with 1mg/mL of glucose as sole source of carbon were washed with sterile phosphate buffered saline and O.D. of both the cultures was adjusted to 0.5 at 530 nm.

***Biuret method*:** For Biuret method (Fine 1935) standard Bovine serum albumin was prepared in range of 2-10 mg/mL. Five mL of above grown cultures was taken and cells were recovered by centrifugation at 12,000 rpm for 2 minutes. The pellet was suspended in 1mL of 3N NaOH and boiled for 5 minutes and supernatant was used as sample for protein estimation.

***Separation and analysis of proteins by SDS-PAGE*:** Five mL of above grown cultures were taken. The cells were recovered by centrifugation at 12,000 g for 2minutes. The supernatant was discarded; the cell pellet was resuspended by vortexing in 50µL of water. As soon as the pellet was dispersed, 50 µL of 2X SDS gels loading buffer was added and vortexed for 20 sec. The sample was placed in boiling water bath for 15 minutes and centrifuged at 12,000 rpm for 10 minutes at RT. The supernatant was taken in fresh tube. The proteins were separated with the help of 12% SDS-PAGE at 100 V. The gel was stained with Coomassie Brilliant Blue R250 for 20 to 30 minutes (Sambrook et al.1989). The molecular weight of unknown proteins was determined by comparing with the standard molecular weight marker.

RESULTS AND DISCUSSION

The enrichment of MP-degrading microorganisms: An increase in the intensity of yellow colour of the medium, during enrichment was observed during the first 12 hrs of incubation, which indicated p-nitrophenol (PNP) production by MP catabolism. While the medium turned colourless after 24 hrs indicating utilization of PNP. A complete degradation of MP was observed after 24 hrs as detected by the quantitative alkaline hydrolysis test which directly detects their actual MP concentration. This suggests that the enriched sample is capable of degrading both MP and its intermediate PNP. While several MP and PNP degrading bacteria have been isolated from OP pesticides polluted soils, bacteria that simultaneously degrade both compounds are scarce (Pakala et al. 2007).

Determination of PNP concentration during MP degradation: The culture was grown in minimal medium with MP as the only carbon source; the culture grew by utilizing methyl parathion. Yellow color appeared which was due to conversion of MP to PNP but the yellow color of the medium (caused by p-nitrophenol) also decreased in 24 hrs (Fig. 1) suggesting the culture also can utilize PNP.

Isolation of culture: From enriched soil sample, methyl parathion-degrading culture was isolated on solid BMM medium containing MP. This culture was capable of utilizing not only methyl parathion but also p-nitrophenol as a sole source of carbon. Methyl parathion-hydrolyzing bacterium was identified as *Arthrobacter globiformis*. The bacterium was grown and maintained in minimal medium, containing 62.5µg/mL of MP which was provided as the sole carbon source. Subsequent characterization of using 16S rRNA sequence analysis and biochemical tests indicated that it belonged within the *Arthrobacter globiformis* strain AD26 group. They were characterized by pleomorphism and Gram variability (staining positive or negative). When the culture was young, cells were slender rods that stain Gram-negative. Jointed rods were observed after 1-2 days. By about 30 hours, the cells had become very short, gram-positive rods and coccoids. This organism produced yellow pigment on nutrient agar medium after 48hr of incubation. *Arthrobacter* species form part of the Gram-positive coryneform bacteria, weakly motile and are considered one of the major groups of aerobic soil bacteria. Due to their ubiquitous presence in soil and nutritionally versatility they have ability to metabolize a variety of substances such as nasty chemicals including nicotine, nucleic acids, carbamate herbicides and chlorinated biphenyls (Tam et al. 1987). It did not lose the ability to degrade methyl parathion after several transfers in minimal medium containing methyl parathion.

Corrleation between MP degradation and viable count of *Arthrobacter globiformis*: The ability of *Arthrobacter globiformis* to utilize MP as a sole source of carbon and energy was evident by residual MP concentrations quantitated spectrophotometrically and increase in viable count readings of the organism after 24, 48 and 72 hrs (Table 1). As residual MP decreased from the flask, the viable count of the isolate was also increased which confirmed that organism can utilize MP as sole source of carbon.

Acclimatization of *Arthrobacter globiformis* to increasing concentration of MP: *Arthrobacter globiformis* effectively utilized 62.5 µg/mL of MP as a sole source of carbon and energy and acclimatized to 1250 µg/mL of MP. It can utilize 1250 µg/mL of MP in 72 hrs as it was evident from the presence of growth in the same and release of PNP.

Extraction of plasmid and curing: Plasmid was isolated from *Arthrobacter globiformis* and it was found to be of 48kb size (Fig. 2). In *Flavobacterium* sp. strain ATCC 27551, a 43-kb plasmid was associated with the production of parathion hydrolase (Mulbry et al. 1986). The MP degradation gene(s) in *Arthrobacter globiformis* was found to be plasmid borne as confirmed by loss of plasmid by extensive subculturing on nutritious media and a subsequent loss of ability of the organism to grow on MP supplemented medium. Stability of plasmid requires a selective pressure to be maintained. The organophosphate degradation (opd) gene is localized either on plasmids or on the chromosomes. But in most of the studies, opd gene was found to be plasmid based and to be present on large molecular size plasmids.

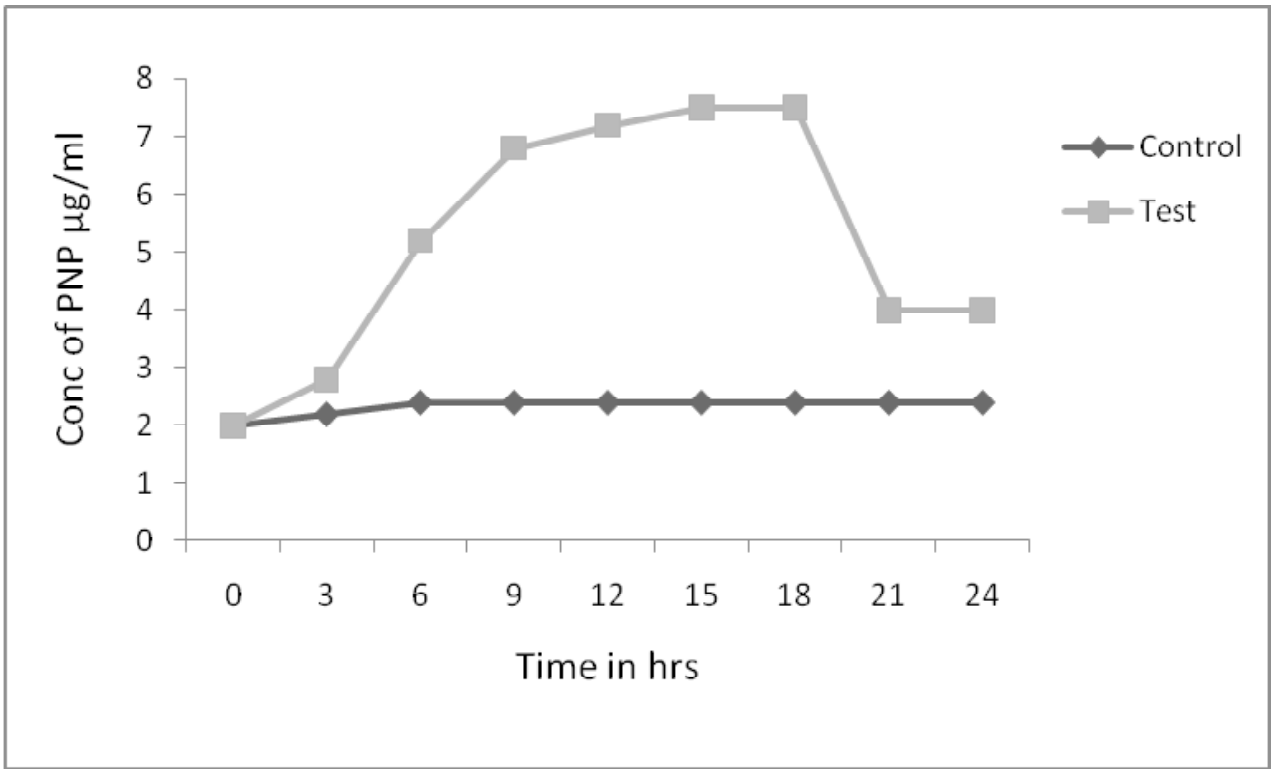

Fig. 1: Determination of PNP concentration during MP degradation.

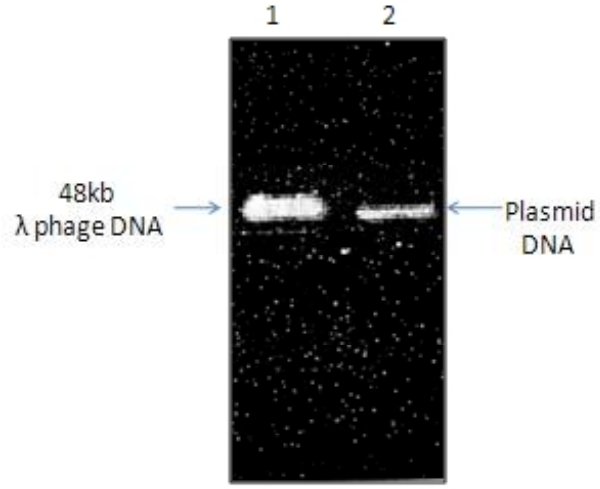

Fig. 2: DNA electrophoresis Lane 1- λ phage DNA; Lane 2- Plasmid DNA from *Arthrobacter globiformis.*

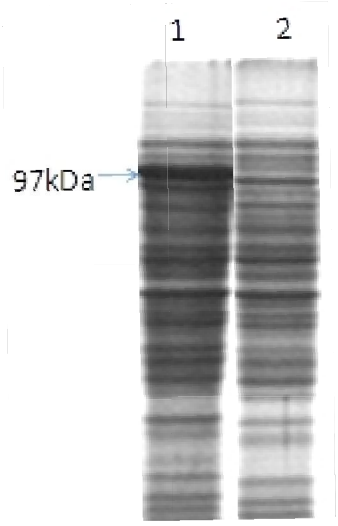

Fig. 3: SDS-PAGE (Commassie staining).
Lane1- Protein sample of *Arthrobacter globiformis* grown in BMM containing MP as sole carbon source; Lane2- Protein sample *of Arthrobacter globiformis* grown in BMM containing glucose as sole carbon source. Arrow indictes 97kDa induced protein band.

Table 1: Correlation between MP degardation and viable count of the isolate.

Incubation Hours	Control flask MP conc. µg/mL	Test flask Residual MP conc. µg/mL	Cfu/mL
0	56.25	56.25	3×10^{4}
24	56.5	15.5	15.6×10^{8}
48	54.5	5	2.5×10^{9}
72	54	0	5×10^{10}

The fenitrothion degradative capability of *Burkholderia* sp. strain NF100 was found to be associated with the two large molecular size plasmids, designated pnf1 (105kb) and pnf2 (33 kb) (Masahito et al. 2000). While two opd plasmids, ppdl2 (43kb) from *Flavobacterium* sp. and pcms1 (51kb) from *Pseudomonas diminuta*, have been isolated (Harper et al.1988). Since many degradative traits for the metabolism of xenobiotic compounds have been shown to be carried on plasmids the ability of the host organisms to degrade target xenobiotic compounds may depend upon the stability of the degradative plasmids or their genes.

Comparison of protein profile of MP degrader grown in presence of methyl parathion and glucose as sole source of carbon in BMM by Biuret method and SDS-PAGE:

Protein content of cells which were grown in presence MP as sole source of carbon in BMM was compared with protein content of cells grown in glucose as sole carbon source in BMM. It was observed that protein content of the cells grown in MP was 47% while that of the glucose was 35% obtained by Biuret method. The protein content of the cells grown in presence of MP as a sole source of carbon was higher as compared to the control, i.e, cells grown in presence of glucose. This may be due to the proteins induced in cells when grown in presence of MP. Commassie blue stained gel showed a 97kDa induced band in the protein pattern of cells grown in presence of MP as a sole source of carbon which was absent for the control as confirmed by SDS- PAGE (Fig. 3). All the organophosphorus hydrolase (OPH) proteins reported to date are in range from 30-60 kDa. OPH of about 31kDa was first discovered in soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* sp. Another class of ops degrading enzyme are identified in *Alteromonas* spp. are much larger, approximately 60 kDa. Thus, the induced band of protein for cells grown in presence of MP as sole source of carbon may be due to single protein, i.e, a novel OPH or may be due to several other proteins which play a role in MP degradation.

OPH protein is known to have a broad substrate specificity and is able to hydrolyze a number of OP pesticide; such as MP, parathion, diazinon and chemical warfare reagents like sarin and soman (Cho et al. 2004). Thus, MP degrading *Arthrobacter globiformis* could be further studied for its OP compound degradation spectrum. This would allow bioremediation of MP but also of other OP pesticides wastes and chemical warfare agents.

Thus, the findings of this study highlight the potential of *Arthrobacter globiformis* strain AD26 to be used in the cleanup of contaminated MP waste and for accurate monitoring of OP pesticides in the environment.

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