Bull. Environ. Contam. Toxicol. (2001) 67:98–105

DOI: 10.1007/s00128-001-0096-9

Bulletin of Environmental Contamination and Toxicology

Bioremediation of Methylparathion by Free and Immobilized Cells of *Bacillus sp*. Isolated from Soil

C. Sreenivasulu, Y. Aparna

Department of Microbiology, Vignan School of Postgraduate Studies, Palakalur Road, Guntur 522 005, India

Received: 15 November 2000/Accepted: 15 April 2001

Bioremediation, the clean up of xenobiotic environmnetal pollutants by microbial means, is an increasingly important scientific challenge and also a rapidly burgeoning business. Bioremediation may merely involve the manipulation of environmental parameters to favour biodegradation (Bartha 1986). The use of pesticides through out the world has been expanding quite rapidly since the first introduction of synthetic chemicals for use in crop protection, and growth rates of 6-8% per year are expected for the next several years. Pesticides are used in the control of pests of man, animals and plants with the result that many of these chemicals reach the soil and persist for long periods causing harm to soil microorganisms and plant growth. Organic pesticides applied to soil may be used as substrates by microorganisms and undergo degradation resulting in the formation of new compounds which may prove to be more deleterious to plants than the parent molecule.

Hazardous wastes are generated in the pesticide Industry at both consumer and producer levels and in order to ensure safe production, handling and utilization of these agricultural chemicals, environmental guidelines and regulations governing these procedures have been established. Producers of pesticides and their formulations have seemingly side range of pesticide waste disposal technology available for meeting these air and water point source discharge requirements. However once the pesticide has been distributed to the individual consumer the methods available for the disposal and/or detoxification of excess pesticides, pesticide containers, spray tanks and spray solutions are severely reduced and in most situations no detoxification technology exists.

There have been several reports on the degradation of Methylparathion (Adhya *et al.*, 1987; Sharmila *et al.*, 1988). The present study aims at bioremediation of methylparathion by free and immobilized cells of *Bacillus sp.* in the laboratory and pi-let plant experiment in the contaminated sites.

MATERIALS AND METHODS

Metacid EC 50 a commercial formulation of Methylparathion (*O-O* dimethyl *O-p*-nitrophenyl phosphorothionate) was obtained from Bayer India Ltd.,

Correspondence to: C. Sreenivasulu

Bombay. *p*-Nitrophenol (PNP) was purchased from Sigma Chemical Company, St. Louis USA. Aqueous solutions were prepared by dissolving the above chemicals in sterilized distilled water.

Soil was collected from fallow Cotton field area to a depth of 12 cm in Guntur District, Andhra Pradesh, India. Soil was passed through a 2 mm sieve and preserved at 4°C. Twenty gram portions of soil was taken into pre-sterilized test tubes(200 x 25 mm) and maintained at 60 percent water holding capacity (WHC) (Venkateswarlu *et al.*, 1977). Aqueous solutions of Methylparathion (MP) were added to the soil sample separately to provide a final concentration of 200 μM which nearly corresponds to 25 mg per kg soil or 2.5 kg per hectare (Rangaswamy *et al.*, 1994). Untreated soil samples maintained at 60% WHC served as controls. Soil enrichments were developed by four subsequent additions of 200 μM MP at 10 day intervals (Adhya *et al.*, 1987). Aqueous solutions were prepared with sterile distilled water after the fourth addition of toxicant.

Soil suspensions were prepared with the MP-enriched soil sample. Dilutions of soil suspension were prepared upto 10^{-7} dilution. To isolate bacteria capable of degrading MP, suspensions of soil enrichments from 10^{-3} dilution were placed on nutrient agar medium and kept at 37°C for 24 h. After 24 hours , single colonies were tested (Lenke *et al.*, 1992) for their ability to use MP as carbon source in mineral salts medium (MSM) supplemented with 200 μM MP. One bacterium which occurred predominantly and exhibited a greater potential in degrading MP was isolated and identified as *Bacillus sp.* on the basis of morphological, cultural and biochemical tests. Stock cultures were maintained on mineral salts medium supplemented with 200 μM MP as the sole source of carbon. Cultures were also preserved by mixing the medium containing cultures and glycerol in 3:1 ratio.

The antibiotic resistance/sensitivity of the *Bacillus* sp. towards various antibiotics was studied by culturing the organism on nutrient agar medium by placing antibiotic discs carrying concentrations of 30 μg/disc (Tetracycline, Neomycin, Kanamycin) 10 μg/disc (Streptomycin and Penicillin) and 5μg/disc (Rifampicin) (Megharaj *et al.*, 1989)

The MP degradation by *Bacillus sp* was studied by inoculating the culture suspension to 50 ml sterilized MSM supplemented with 200 μM MP (Siddaramappa *et al.*, 1973). Bacterial cell suspension was prepared by growing the culture to high cellular densities at 37°C. The cells were harvested by centrifugation at 6000 rpm for 10 min. Supernatants were discarded and the cells were suspended in sterile distilled water. Bacterial cell densities were measured by taking optical density at 600 nm. The cell density of 1.76×10^6 colony forming units (CFU)/ml at the beginning of experiment was maintained unless otherwise mentioned as inoculum in degradation studies.

Methylparathion degradation was studied repeatedly at regular intervals. Suitable aliquotes from the culture medium was taken and centrifuged for 5 minutes at

6000 rpm to settle the bacterial cells. Supernatant was taken and used for analysis of MP and PNP. Supernatant was treated with Sodium hydroxide and the yellow colour was read at 410 nm and calibrated the amount of MP degraded by the organism. Supernatant without treating with Sodium hydroxide with yellow colour PNP released was used as blank.

The rate and extent of MP degradation was studied in pure culture. Fifty milliliters of MSM containing different concentrations of MP (100, 200, 400, 1000 μM) taken into 250 ml Erlenmayer flasks were inoculated with 2 ml of cell suspension. Controls were maintained without bacterial inoculation to detect abiotic degradation or volatilization of MP. Triplicate flasks, maintained for each treatment including uninoculated controls, were maintained at 37°C. At regular time intervals, triplicate samples were taken for each treatment, and the amount of MP remained and the released PNP was estimated (Siddaramappa *et al.*, 1973). Residues in the medium were extracted thrice with 25 ml of chloroform: diethylether (1:1) and solvent fraction was pooled. The residues were evaporated to dryness at room temperature and dissolved in 2 ml of methanol. The residues were spotted on silica gel plates and developed for a distance of 20 cm by employing hexane-chloroform-methanol (7:2:1) as a developing agent. After drying the authentic compound of MP and PNP were located by spraying chromatophore with 0.5% palladium chloride in 25% HCl followed by 2.5 N NaOH. The silica gel areas of the sample corresponding to Methylparathion were scrapped carefully and transferred to a test tube. One millilitre of 2.5 N NaOH was added to each tube, and MP was converted to PNP by alkaline hydrolysis in a water bath for 1 hour. After cooling, the volume was made upto 25 ml. Silica gel was removed by centrifugation and the supernatant was read at 420 nm. The amount of MP in the samples was determined as per the calculations of Siddaramappa *et al.* (1973). PNP in the silica gel areas of the samples opposite to the authentic compound was directly eluted in 0.1 N NaOH. After centrifugation of the silica gel suspension, PNP in the supernatant was determined colorimetrically measuring absorbance at 420 nm.

To study the effect of temperature on degradation of MP, 50 ml portions of MSM with MP were inoculated with 2 ml of culture suspension and incubated at 15, 37 and 45°C in Bacteriological Incubators. At regular intervals, aliquotes from triplicate samples for each flasks were withdrawn and released PNP was estimated. Effect of pH on degradation was also studied by inoculating the culture to 50 ml MSM with MP adjusted to different pH levels (6.5, 7.0, 7.5, 8.0) and incubating at 37°C. At regular intervals of time the amount of MP remained and the amount of PNP released were quantified.

Degradation of MP by immobilized cells of *Bacillus sp.* was carried out by the slightly modified method of Megharaj *et al.*, (1992). Cells of *Bacillus sp.* grown in MSM containing 200 μM MP and 0.1% yeast extract, were aseptically harvested by centrifugation at 6000 rpm for 20 minutes and suspended in 50 ml sterilized distilled water. To another 50 ml sterilized distilled water, 6% (w/v) sodium alginate was added and gently stirred for 1 hour, boiled and cooled. Bacterial suspension was then added to sodium alginate solution and

stirred for 30 min to ensure proper mixing. All these steps were done under aseptic conditions in laminor flow hood. The mixture of sodium alginate (3% w/v) and bacterial cells was run down, dropwise, through a burette into 0.4 M $CaCl_2$ solution for 1 h for stabilization. The beads were washed twice with sterilized distilled water before use. Single alginate bead dissolved by immersing in 1 ml of 0.2 M sodium phosphate buffer, pH 6.9 for 30 min for obtaining the bacterial count (Chibata and Tosa, 1977). After dissolving the beads, dilutions were made further and triplicates of each 0.1 ml suspension from suitable dilution was plated on nutrient agar and incubated at 37°C. Discrete colonies developed after 48 h incubation at 37°C, were counted to determine the cell density entrapped in a bead. About 220 beads with a stocking density of 4 x 10^6 cells $bead^{-1}$ of *Bacillus sp.* were transferred to 50 ml MSM supplemented with 200 µM MP containing in 250 ml Erlenmayer flasks. Flasks were incubated at 37°C. The amount of MP present and the amount of PNP accumulated were monitored at regular intervals.

A surface sterilized tank with one liter capacity, with an outlet to collect the sample, was filled with water sample collected from MP contaminated site and inoculated with immobilized culture to test the ability to degrade the pesticide under field conditions *in vitro*. Aeration was maintained during the incubation period. At regular intervals the amount of MP present in the sample was monitored as mentioned above by collecting the sample through outlet.

The data were subjected to analysis of variance, and the means were compared by Duncun's Multiple Range (DMR) test at 5% level.

RESULTS AND DISCUSSION

The indigenous microbial communities in soil were enriched with Methylparathion in laboratory at 37°C to use MP as carbon source. MP degrading bacterium was isolated by the method of serial dilutions and plating from soil enriched with 200 µM MP. Based on morphological, cultural and biochemical characteristics (Gram +ve, sporulative, motile, no fluorescence, no growth on McConkey agar, strict aerobe, acid produced from glucose, citrate utilisation +ve, gelatin liquefaction +ve, Starch hydrolysis -ve, Urea hydrolysis -ve, Casein hydrolysis +ve, Catalase +ve, Oxidase +ve, H_2S production -ve, Nitrate reduction -ve), the bacterium isolated from soil was identified as species of *Bacillus*. The sensitivity/resistance of *Bacillus* sp. towards antibiotics revealed that the organism is sensitive to Streptomycin and Tetracycline and resistant to Penicillin.

The degradation rate in terms of MP disappearance and PNP accumulation was directly related to the densities of bacterial population in the inoculum used (data not shown). The time required for complete degradation of 200 µM MP by *Bacillus* sp. is 36 h and stoichiometric amounts of PNP were released. The accumulated PNP is not further metabolized by the organism. In contrast to this Chaudhry *et al.*, (1988) isolated *Pseudomonas* sp., which hydrolyzed the pesticide to PNP but required glucose or another carbon source for growth. Another bacterium isolated from mixed culture, a *Flavobacterium* sp., used PNP for

growth and degrade it to nitrite. A *Flavobacterium* sp., isolated from paddy water by enrichment culture technique decompose diazinon in MSM as sole source of carbon. The bacterium converted 540 µg parathion to 280 µg PNP (Sethunathan and Yoshida 1973). A mixed culture was adapted to grow on parathion to determine the feasibility of using microorganisms to detoxify concentrated parathion in agriculture wastes. The culture was able to degrade 50 mg of parathion per liter per hour (Munnecke and Hsieh 1974). Microalgae and Cyanobacteria have also been implicated in the metabolism of MP (Megharaj *et al.*, 1994)

An attempt was made to determine the toxic concentrations of MP employing the step-up levels of 400, 800, 1000 µM. The use of these concentrations is based on the fact that biodegradation of test compound will be totally inhibited by a concentration always above the level employed for enrichment (Zeyer and Kearny 1984). The toxic level of the compound was determined by inoculating the culture in MSM with different concentrations of MP and then monitoring the released PNP from MP. The lag period in the degradation of MP was reduced when the culture medium was supplemented with a step-down concentration of 100 µM MP (Table 1).

Table 1. Degradation of different concentrations of MP by *Bacillus* sp. isolated from soil

Incubation (h)	MP concentrations (µM)				
	100	200	400	800	1000
6	98	198	398	799	987
12	82	180	392	798	990
18	48	146	396	794	993
24	0	102	379	798	995
30	0	52	342	792	994
36	0	0	282	765	996
48	0	0	124	656	996
60	0	0	32	512	998
66	0	0	0	425	996
90	0	0	0	126	998
102	0	0	0	0	998

Thus 100 µM MP was degraded within 24 h of incubation whereas 200 µM MP degraded after 36 h of incubation. There was an increase of 30 h in complete degradation when MP was supplemented at 400 µM MP. However the initiation of MP degradation was delayed until 36 h in culture medium treated with 800 µM MP. Complete degradation was observed at 102 h after incubation. There was no onset of degradation at 1000 µM concentration of MP, and appeared to be toxic to *Bacillus sp.* The above results show that the lag period occurred during

degradation of MP was directly proportional to increasing MP concentration. Munnecke and Hsieh (1974) observed the PNP-induced lag period in growth of *Pseudomonas* sp. on PNP. The degradation of parathion, MP and Fenitrothion were rapid under flooded conditions than under non-flooded condition in four out of five soils.Degradation of these insecticides accelerated after repeated applications to flooded alluvial soil (Adhya *et al.*, 1987). River population pre-exposed to MP adapted to degrade the pesticide more rapidly; salt marsh population did not adapt to degrade MP (Spain *et al.,* 1980).

Immediately after the disappearance of MP, at varying concentrations the culture medium was tested for metabolites. Except PNP no other metabolite was detected. At the time of MP disappearance, the stoichiometric amounts of PNP was released. The isolated *Bacillus sp.* hydrolysed MP to PNP and the released metabolite PNP could not be further degraded by this organism. A *Flavobacterium* sp. ATCC 27551 hydrolyzed MP and parathion while *Pseudomonas* sp. ATCC 29353 hydrolyzed only parathion (Adhya *et al.*, 1981). Glucose inhibited the hydrolysis of parathion by *Pseudomonas.*

Optimum pH for degradation of MP by *Bacillus sp.* was 7.5. MP hydrolysing bacterium *Pseudomonas* grew optimally at pH 7.0 to 7.5 in LB or Minimal medium in the presence of glucose as a carbon source (Chaudhry *et al.,* 1988). Crude enzyme preparations has a broad pH profile and maximum activity was observed at pH 7.5 to 9.5. Optimum temperature required for MP degradation by *Bacillus* sp. was established by incubating the culture flasks at 15, 37 and 45°C. Virtually there was no degradation of MP at 15°C. The complete disappearance of MP possibly by the end of 36 h when the bacterium was grown at 37 °C. Chaudhry *et al* (1988) reported the optimum temperature for the degradation of MP by *Pseudomonas* was 28°C, but the organism also grow at 37°C. Crude enzyme activity was increased with an increased with an increase in the temperature, with maximum activity occuring at 35 to 40°C (Chaudhry *et al.*, 1988).

To study the degradation of MP by immobilized cells of *Bacillus sp.,* calcium alginate was used to entrap the cells. This bacterium degraded 200 µM MP within 30 h of incubation with a stocking density of 4×10^6 cells $bead^{-1}$ and released the stoichiometric amounts of PNP (Table 2). A comparision with free cells and immobilized cells indicate that the rate of MP degradation by immobilized cells is high. Thus 200 µM MP degraded completely by immobilized cells after 30 h incubation as opposed to 36 h incubation period required for complete loss of 200 µM MP by the same number of resting cells. The present investigation indicates that greater potential of *Bacillus sp.* in MP degradation which could be largely exploited for effective bioremediation of the habitats contaminated with high concentrations of MP.

In an attempt to study the use of immobilized cells for the effective bioremediation of contaminated sites, one liter sample was collected from the contaminated site and placed in a tank with outlet facility to collect the

sample. Control was maintained without inoculation of immobilized cells to check for the biotic/abiotic conversion of the toxicant. The initial concentration of MP in the sample was 256μM. Proportionate number of beads were inoculated to the tank. The complete hydrolysis of MP was observed only after 40 h. Degradation of MP was not observed in the control tank. There was

Table 2. Degradation of MP (200μM) by Immobilized cells of *Bacillus* sp. isolated from soil

Incubation (h)	MP(μM)	PNP(μM)
6	198	0
12	165	20
18	102	98
24	52	148
30	0	198
36	0	198
42	0	198

no leaking of the cells from the beads. The beads were stable and the activity was as it was at the initial stage even after one year of beads preparation. This results clearly show that the immobilized cells in the beads can be effectively used for the bioremediation of MP contaminated sites. A bioreactor can be constructed with the immobilized cells and the sample can be continuously pumped through the system and the toxicant can be removed. Thus from the stand point of Environmental Contamination of Pesticides, the present observation of using immobilized cells for the remediation of MP may be exploited further in Environmental Biotechnology for the effective detoxification of the MP polluted areas.

Acknowledgments: We thank Dr. Lavu Rataiah, Chairman & Managing Director and Sri Paturi Koteswar Rao, Executive Director, Vignan Group of Educational Institutions, Guntur, Hyderabad, Vijayawada and Visakhapatnam, India for providing necessary facilities to do this research work. We are also thankful to Prof. N. N. Prasad, Head Department of Microbiology for his encouragement in doing this work.

REFERENCES

Adhya TK, Waid PA, Sethunathan N (1987) Persistance and biodegradation of selected organophosphorus insecticides in flooded versus nonflooded soils. Biol Fertil Soils 5: 36-40

Bartha R (1986) Biotechnology of petroleum pollutant biodegradation. Micro

Ecol 12: 155-172
Chaudhry RG, Ali AN, Wheeler FW (1988) Isolation of methylparathion degrading *Pseudomonas* sp. that possess DNA homology to the *opd* gene from a *Flavobacterium* spp. Appl Environ Microbiol 54: 288- 293
Chibata I, Tosa T (1977) Transformation of organic compounds by immobilized microbial cells. Adv Appl Microbiol 12: 1-27
Lenke H, Pieper DH, Bruhn C, Knackmuss HJ (1992) Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2 Appl Environ Microbiol 58: 2928-2932
Megharaj M, Pearson HW, Venkateswarlu K (1992) Removal of nitrogen and phosphorus by Immobilized cells of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil. Enzyme Microb Technol 14: 656-658
Megharaj M, Madhavi DR, Sreenivasulu C, Umamaheswari A, Venkateswarlu K (1994) Biodegradation of methylparathion by soil isolates of *Microalgae* and *Cyanobacteria.* Bull Environ Contam Toxicol 53: 292-297
Munnecke DM, Hsieh DPH (1974) Microbial decontamination of parathion and *p* -nitrophenol in aqueous media. Appl Microbiol 28: 212-217
Rangaswamy V, Reddy BR, Venkateswarlu K (1994) Activities of dehydrogenases and protease in soil as influenced by monocrotophos, quinalphos, cypermethrin and fenvalerate. Agric Ecosys Environ 47: 313-326
Sethunathan N, Yoshida T (1972) Conversion of parathion to *para* nitrophenol by diazinon-degrading *Flavobacterium* sp. Proc Inst Environ Sci New York Meet 18:255-257
Sharmila M, Ramanand K, Adhya TK, Sethunathan N (1988) Temperature and persistence of methylparathion in flooded soil. Soil Biol Biochem 20: 399-401
Siddaramappa R, Rajaram KP, Sethunathan N (1973) Degradation of parathion by bacteria isolated from flooded soil Appl Microbiol 26: 846-849
Spain JC, Pritchard PH, Bourquin AW (1980) Effect of adaptation rates in sediment, water cores from estuarine and freshwater environments Appl Environ Microbiol 40: 726-734
Venkateswarlu K, Gowda TKS, Sethunathan N (1977) Persistence and biodegradation of carbofuron in flooded rice soils J. Agric Food Chem 25: 533-536
Zeyer J, Kearney PC (1984) Degradation of *o*-nitrophenol and *m*-nitrophenol by *Pseudomonas* putida. J Agric Food Chem 32: 238-242