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Plasmid Borne *opd* Gene in *Myroides odoratimimus* SKS05- GRD for Degradation of Dimethoate

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Myroides odoratimimus **SKS05-GRD (Nucleotide sequence accession no. JQ178355) was able to utilize Dimethoate as phosphorus source and it can tolerate Dimethoate up to 6.25mg/ml. The ability of the organism to produce Phosphatase enzyme was screened by agar plate method and Phosphatase activity. The organism consists of an indigenous plasmid of size more than 21kb and effective plasmid curing was observed at higher concentration of Ethidium Bromide (300µg/ml and 500µg/ml). Phosphatase enzyme activity and Organophosphate (*opd*) gene was located on the indigenous plasmid in *M. odoratimimus* SKS05-GRD. HPLC analysis of the degradation assayed at intervals of 10days revealed a decrease in concentration of Dimethoate in the soil from 0.0312mg at the 10th day to 0.0239mg at the end of 40 days.**

Key words: Dimethoate, *Myroides odoratimimus,* Organophosphate, Phosphatase, *opd* gene.

Pollution is one of the biggest threats the world faces today as huge number of pollutants in the form of chemical compounds continued to be dumped in to the environment. An organophosphate pesticide (OP) is one of the most extensively used groups of pesticides that possess a phosphoric acid derivative as its chemical structure. Dimethoate ($C_5H_{12}NO_3PS_2$), which belongs to the organophosphate group of compounds, is a wide-ranging insecticide used to eradicate mites and insects. It inhibits the activity of acetyl cholinesterase, an enzyme that is required for the proper functioning of the central nervous systems; therefore rendering it extremely toxic and easily absorbed through the skin (Khusnul *et al*., 2008).

Although organophosphates are biodegradable in nature, frequent use of pesticides retains some traces in the environment which turns out to be pollutant. Recent studies have reported that microbial metabolism has proved to be very versatile and diverse (Hayatsu *et al*., 2000). Generally, organophosphate compounds do not harmfully affect bacteria, because bacteria do not possess acetylcholinesterase enzyme, and several microorganisms utilize organophosphates as an energy source (Singh and Walker, 2006).

An *opd* gene which is responsible for hydrolyzing organophosphate pesticide was first reported in Pseudomonas and in Flavobacterium species (Serdar *et al*., 1982). Somara and Siddavattam (1995) reported *opd* genes in Pseudomonas are plasmid borne. The present study focused on the study of Minimal Inhibitory Concentration (MIC) of Dimethoate against *M. odoratimimus* and its ability to utilize dimethoate as phosphorous source. The study also investigated the presence of *opd* (organophosphate degrading) gene responsible for

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the hydrolyzing dimethoate and efficiency of dimethoate degradation in soil by the organism.

MATERIALS AND METHODS

Bacterial strain and Enrichment medium

M. odoratimimus SKS05-GRD (Nucleotide sequence accession no. JQ178355) was first grown in Davis Mingioli's medium (DM medium) containing KH_2PO_4 - 3g, K_2HPO_4 – 7g, $MgSO_4.7H_2O$ – 0.01g, $(NH_4)_2SO_4$ – 1g, pH- 6.9 per liter. DM medium was enriched by Peptone – 1g/l and 0.5mM dimethoate and incubated at 30ºC with agitation. Dimethoate resistant culture was subcultured for two generations on DM agar plates containing dimethoate.

Minimal Inhibitory Concentration

MIC of dimethoate against *M. odoratimimus* SKS05-GRD was determined by tube dilution method as described by Andrews (2001).

Utilization of Dimethoate

Modified DM medium was used to determine the ability of *M. odoratimimus* SKS05-GRD to utilize dimethoate as phosphorous source. KH_2PO_4 and K_2HPO_4 were replaced by dimethoate in DM medium with a final concentration of 0.5mM/l. *M. odoratimimus* SKS05-GRD was inoculated in to 100ml of modified DM medium and incubated at 30ºC with agitation for 5 days. A control was maintained without inoculum. Growth was monitored measuring the turbidity at 600nm. Phosphate released into the culture supernatant was estimated by the method of Olsen *et al*., (1954).

Screening for the Phosphatase enzyme

M. odoratimimus SKS05-GRD was initially screened for phosphatase enzyme with the modified MGP method. Tryptose Phosphate agar was added with 50µg of Methyl Green (MG) per ml and 500µg of a phosphate substrate per ml. Both dye and substrate from stock solutions filtered through 0.22-µm-pore-size filters were added to Tryptose Phosphate agar which had been melted and kept at 44°C. Phenyl phosphate disodium salt and potassium hydrogen phosphate were used as the phosphate substrate. Isolated colonies to be tested were spot inoculated on the agar surface. Colonies showing any degree of green color after incubation at 37°C for 24 h were recorded as phosphatase positive

Enzymatic activity

Phosphatase activity of *M. odoratimimus* SKS05-GRD was examined by measuring the amount of phosphate released. In this assay, a volume of 5ml of three day old culture was centrifuged at 5000rpm for 10 min at 4 ºC. Phosphatase activity in pesticide hydrolysis was determined using dimethoate as substrate. The reaction mixture consisting of 2.5 ml of the substrate (1mM of dimethoate in 0.1 mol/l of sodium acetate-acetic acid buffer in varying pH 3, 3.5, 4, 4.5, 5, 5.5, and 6) and 0.5 ml of enzyme preparation. After 1 h at 25 ºC the released phosphates were determined as described by Olsen *et al*., (1954). One unit of phosphatase activity (1 nkat) is the amount of the enzyme which produces 1nmol of phosphorus per second. Specific activity is given in nkat per mg of protein.

Plasmid curing

M. odoratimimus SKS05-GRD maintained on the dimethoate containing DM medium was subjected to plasmid curing by Ansari and Khatoon, (1997). Ethidium bromide at various concentrations (30µg/ml - 700µg/ml) were added to 5ml of DM broth and inoculated with culture. A control broth lacking ethidium bromide was maintained. The tubes were incubated at 37°C overnight and plasmid isolation was performed to confirm the plasmid curing. Plasmid cured culture was subjected to phosphatase assay by agar plate method.

DNA isolation

Genomic DNA was isolated from the plasmid cured *M. odoratimimus* SKS05-GRD by the method described by Takahashi and Nagano (1984) with modifications.

PCR amplification of opd genes

PCR amplification of *opd* gene was performed using primers [*1f* (AAA GGC TGT GAG AGG ATT), *2f* (GAC GTC AGT TTA TTG GCG A), *3f* (TGG TTC GAC CCG CCA CTT TC), *1r* (TAG CTC GAA AAC CCG AAC AG), *2r* (CAA GAG AGC CCK TGT TTG C), *3r* (CTT CTA GAC CAA TCG CAC TG)] as described by Singh *et al*., (2003).

DNA and plasmid extracted from the organism were diluted, and PCR amplification was attempted by using all possible paired combinations of forward and reverse primers. The PCR running conditions were setup with an initial

denaturation of 94°C for 5min followed by a denaturation for 45s. The annealing temperature was varied from 53°C to 58°C for 45s for the different primer combinations. This was followed by an extension at 72°C for 45s for 35 cycles and a final extension for 5mins. The products of the PCR reaction were analyzed by agarose gel electrophoresis (2% gel).

Degradation in soil

Clay soil from the Tamil Nadu Agricultural University (TNAU, Coimbatore) was used for this study. The soil had physiochemical properties of clay loam with nitrogen (Kg/ ha) -213, phosphorous (Kg /ha) - 23.0 and potassium (Kg /ha) - 353 and alkaline pH. The soil was air-dried, passed through a 4 mm sieve, and remoistened with sterile distilled water up to 20 % to permit good aeration. Amounts of 100 g of non-sterile soil were packed in polythene bags. The following variants were set up in triplicate: soil + pesticides (control), soil + Dimethoate + inoculum (test). Dimethoate was added at a concentration of 300ppm to each soil package. 2% of overnight grown *M. odoratimimus* culture was added to the test samples. The bags were incubated at room temperature for 50 days.

Extraction of Dimethoate from soil

To determine the degradation of dimethoate in soil, dimethoate was extracted from the soil samples at every 10 day interval. 10g of soil sample was taken and extracted in a soxhlet extractor. The solvents used for the extraction process was hexane:acetone (90:10). The extraction process was carried out for a period of 6 hours. The extract was concentrated in rotary vacuum evaporator until completely dry and reconstituted with 5 ml acetone.

HPLC analysis of Dimethoate

The samples were submitted for HPLC analysis at the Department of Pharmaceutical Analysis, College of Pharmacy, SRIPMS, Coimbatore. The analysis was done at 37ºC using a Shimadzu liquid chromatograph LC 10ATVP with Shimadzu diode array detector. The analytical column was Hibar column (5µm particle size) and the UV detector wavelength was set at 229nm for dimethoate. In order to separate the pesticides, a mobile phase constituting a mixture of acetonitrile/ water/acetic acid 39:59:2 (v/v/v) was used. The chromatographic system was controlled by software package ClassM10A.

RESULTS AND DISCUSSION

Minimal Inhibitory Concentration

The potential of *M. odoratimimus* SKS05-GRD to utilize and degrade dimethoate was assessed in this study. Exploiting the ability of microorganisms to tolerate and utilize high concentrations of pesticides shows possible ways to eliminate hazardous pesticide residues (Guha *et al*., 1999; Deb Mandel *et al*., 2005). *M. odoratimimus* SKS05-GRD showed a slow growth on DM medium supplemented with 0.5mM dimethoate. The organism was subcultured for two generations to familiarize the organism to the presence of dimethoate. The organism tolerated Dimethoate up to 6.25mg/ml which was determined by tube dilution method beyond which the

Table 1. Phosphatase activity of the *M. odoratimimus* SKS05-GRD hydrolyzing Dimethoate at 30ºC

pH of the Sodium Acetate-Acetic Acid Buffer	Phosphorous produced*(µmol/h)	Enzyme activity* (nkat)	specific activity (nkat/mg)
3	2068	1149.2	821
3.5	1485	825	589
4	839	466.2	333
4.5	1485	825	589
5	646	358.7	256
5.5	*1162*	646	461
6	904	502	358

*Enzyme activity, Phosphorus (in nkat) released by 1ml extract

organism started showing inhibitory effects. This is the first report for *Myroides odoratimimus* SKS05-GRD to tolerate and utilize dimethoate as phosphorous source. Similarly, wild type *P. putida* is able to utilize dimethoate as phosphorous source than the orthophosphates (Aboalfazl, 2007). In another study by Sethunathan and Yoshida (1973) isolated a species of *Flavobacterium* that could use parathion as source of phosphorous but not diazinon as carbon source. Walker and Kaesling (2002) reported the ability of *Pseudomonas putida* to utilize parathion as carbon and energy source.

Dimethoate utilization

Growth on dimethoate supplied as sole phosphorous source was examined in modified DM medium. The *M. odoratimimus* SKS05-GRD was found to utilize 0.5mM dimethoate as phosphorous source and the growth was observed after 24h followed by exponential growth compared to the organism grown in media with no dimethoate. Utilization of dimethoate was measured by released phosphorous in the medium. We found an increase in the concentration of released phosphorous in the medium which confirms the utilization of dimethoate as energy source by the organism.

The metabolism of organophosphate pesticide is catalyzed by the presence of enzymes in bacteria (Ziv and Celia, 2007). Bacterial enzymes play an important role in detoxifying pesticides (Yanez- Ocampo *et al*., 2009). This reaction is achieved by esterases or phosphotriesterases in different genera of bacteria and fungi (Singh and Walker, 2006). Interestingly, the metabolism of dimethoate in the present study is governed by the phosphatase enzyme. Phosphatase enzyme hydrolyses dimethoate in to phosphorous which in turn is utilized as an energy source. Similarly, Hasan (1999) reported that phosphatase enzyme produced by *Aspergillus syndowii* plays an important role in hydrolyzing organophosphate pesticides. In the same way, the utilization of chlorpyrifos as phosphorous source may be mediated by phosphodiesterase and phosphomonoesterase activity in the Enterobacter B-14 strain (Singh *et al*., 2004). Screening for the phosphatase enzyme in *M. odoratimimus* SKS05-GRD showed green colored colonies on the plate containing phenyl phosphate disodium salt as substrate. The organism that does not utilize potassium hydrogen phosphate as phosphate substrate showed no green colored colonies. Since the phenyl phosphate disodium salt is used for the determination of acid phosphatase, this study confirms the organism producing acid phosphatase. The ability of phosphatases in the metabolization of dimethoate assessed is shown in Table 1.

Table 2. Degradation of Dimethoate in soil as analyzed by measuring the peak area of HPLC chromatogram

Days of incubation	Area of the Peak	Height of the peak	Concentration of Dimethoate residues (mg)
Control	24203070	524119	0.0342
10	21982999	541952	0.0312
20	22492184	567870	0.031
30	21258903	590694	0.030
40	16820070	433298	0.0239

Plasmid curing

Bacterial plasmid plays an important role in the pesticide degradation. Another significant observation in this study was the molecular level evidence for the dimethoate degradation. We found the phosphatase enzyme activity was lost up on the plasmid loss in the organism. *opd* gene that codes for the organophosphorous hydrolase enzyme in *M. odoratimimus* SKS05-GRD is located on the plasmid. The plasmid content of wild type and cured strains were examined. The wild type *M. odoratimimus* SKS05-GRD contained a single band of more than 21kb in size. Among the concentrations of ethidium bromide (EtBr) used for curing 100µg/ml and 200µg/ml showed no effect of curing. The cured culture was found to lose the plasmid bands at the concentrations of 300µg/ml and 500µg/ml. The plasmid cured *M. odoratimimus* SKS05-GRD was then assessed for the enzymatic activity by agar plate method. The organism showed no green colored colonies in the agar plate after plasmid curing. This result reveals that phosphatase activity in *M. odoratimimus* SKS05-GRD is plasmid mediated. This is also the first report for the plasmid mediated phosphatase enzyme and *opd* gene in *M. odoratimimus* SKS05-GRD. Similar plasmid borne *opd* gene have been identified in *Pseudomonas, Flavobacterium balustinum* and *Bacillus licheniformis* (Serdar *et*

al., 1982; Somara and Siddavattam 1995; Deb Mandal *et al*., 2005) and *opdA* gene was reported in *Agrobacterium radiobacter's* which is located on the chromosome (Horne *et al*., 2002).

PCR amplification of opd gene and HPLC analysis of Dimethoate

Dimethoate degradation in soil by *M. odoratimimus* SKS05-GRD was found to be slow at static condition at room temperature. The slow rate of degradation of dimethoate may be due to the lack of aeration and agitation. PCR amplification of both genomic DNA and plasmid DNA with 9 different combinations of *opd* primers showed amplification in 3 of the primer combinations in plasmid DNA (Fig 1 & Fig 2).

A study had reported an effective degradation of organophosphate pesticides in clay soil by *Aspergillus flavus* and *Aspergillus syndowii* at static condition (Hasan 1999). The static condition did not support the effective degradation by the organism in our study. This may be attributed to the condition provided as degradation in soil is much more complex compared to degradation in synthetic medium. Organism might take time to adapt to the complex environment and to metabolize dimethoate. Table 2 shows the degradation of dimethoate in soil by *Myroides odoratimimus* SKS05-GRD detected by HPLC. The degradation of dimethoate was analyzed by comparing the degradation in the test sample (Soil+ dimethoate+ inoculum) against the control (Soil + dimethoate) at regular intervals. Result indicates a gradual reduction in the dimethoate concentration from 0.0342mg/ml to 0.0239mg/ml in 40 days.

CONCLUSION

Our findings have reported for first time that *M. odoratimimus* SKS05-GRD can tolerate and utilize dimethoate as phosphorous source with the phosphatase enzyme activity and *opd* gene encoding for degradation in the organism is plasmid mediated.

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