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Microbial Degradation of Chlorpyrifos by *Myroides odoratimimus* SKS05-GRD

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There has been a rapid increase in the percentage of pesticides accumulated in soil leading to deleterious effects to our ecosystem. Chlorpyrifos is one of the commonly found pesticides that have been treated by chemical procedures as a means of remediation. This present study focuses on bioremediation of chlorpyrifos by using *Myroides odoratimimus* SKS05-GRD (JQ178355). The bacterium was allowed to treat pesticide residues obtained from treated soils to adjudge the potential of the bacterium for remediation studies followed by molecular analysis of its bioremediation potency. The bacterium was found to remediate increasing concentrations of chlorpyrifos and molecular analysis revealed the genetic basis for degradation to be mpd functional gene of *Myroides odoratimimus* SKS05-GRD. This study concludes the use of bacteria that possess the degradation capability at the molecular level increases the efficiency of bioremediation although the present study is to be extended for cloning and expression analysis.

Key words: Chlorpyrifos, *Myroides odoratimimus*, pesticide, degradation, gene.

Environmental pollution is one of the biggest threats the world faces today. A huge number of pollutants in the form chemical compounds are dumped in to the environment either directly or indirectly. Pesticides easily find their way into soils, where they may be toxic to arthropods, earthworms, fungi, bacteria, and protozoa. Small organisms are vital to ecosystems because they dominate both the structure and function of ecosystems. Pesticides being toxic in nature do not differentiate between targeted and non-targeted species and threaten the health and well-being of human and wildlife in every region of the world (Koirala *et al*., 2000).

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Consumption of pesticide is prone to increase atleast two to three times more in future. However, there has been a substantial qualitative and quantitative change in pesticide use in the last few years worldwide and in India as well. Different pesticides of this group like methyl parathion and malathion slowly gained their markets replacing the organochlorines and carbamates (Roberts *et al*., 2012). Among the pesticides, monocrotophos, quinalphos and chlorpyrifos top the list of organophosphorus pesticides in the Indian market.

Chlorpyrifos (diethyl (3, 5, 6 – trichloro – 2 - pyridyl phosphorothioate) is a broad-spectrum pesticide with trade names Dursban, Lorsban and Spannit, displaying insecticidal activity against a wide range of insect and arthropod pests. In view of pesticide toxicity and the damages caused to the environment and health, essential technologies

have been developed that guarantee their elimination in a safe, efficient and economic way. A treatment that promises to be efficient, economic and safe is the biological treatment, because several reactions catalyzed by enzymes of specific microorganisms take place. This kind of treatment has been approached from a biotechnological point of view in order to be able to have a methodology that is safer and more economic than the conventional treatments, as well as avoiding additional damages to the environment.

Microorganisms play an important role in degrading synthetic chemicals in soil. The importance of the microorganisms is emphasized on their great diversity and metabolic plasticity, which allows them to use diverse ecological niches (Choudhry *et al*., 2005). This trait has allowed many different bacterial and fungal genera to evolve activities capable of degradation and offers an important source of alternatives for conventional treatment. A large group of bacterial genera such as *Flavobacterium, Pseudomonas* and *Bacillus* has been reported to degrade organophosphates. The metabolism of chlorpyrifos by microorganisms in soil has been reported (Rouchaud *et al.,* 1991) with 3, 5, 6-trichloro-2-pyridinol (TCP) as the primary breakdown product. Use of pesticide degrading microbial systems for degradation, thus, receives attention because of its cost effectiveness and eco-friendly nature.

The ability of microorganisms to degrade pollutants and growth of cells are strongly influenced by nutritional and environmental parameters such as carbon sources, nitrogen sources, inorganic salts, temperature, and pH. However, as far as we know, there is limited knowledge about nutritional and environmental requirements for pesticide degradation by *M. odoratimimus*. Therefore, this study is aimed to design an appropriate process for maximizing the removal of chlorpyrifos by *M. odoratimimus* SKS05-GRD.

MATERIALS AND METHODS

Culture maintenance

Myroides odoratimimus SKS05-GRD (JQ178355) was obtained from chicken samples sourced from Coimbatore. The organism was cultured and maintained on Food Flavobacterium medium (Beef extract - 1g, Peptone - 1g, Calcium chloride - 0.5 g, NH4SO4 - 0.5 g, Olive oil - 1%, Glucose - 0.5 g, Distilled water - 100 ml, pH - 7.5).

Minimal Inhibitory Concentration

Minimal Inhibitory Concentration of chlorpyrifos against *M. odoratimimus* SKS05-GRD was determined by tube dilution method. 100mg/ml of chlorpyrifos as stock solutions was prepared and overnight culture of *M. odoratimimus* SKS05-GRD was used as the inoculum. Twelve sterile test tubes were taken, nine of which were marked 1,2,3,4,5,6,7,8,9, and the rest three were assigned as T_M(medium), T_{MC}(medium + compound) and T_{MI}(medium + inoculum).

1 ml of sterile nutrient broth medium was poured to each of the 12 test tubes by a process of serial dilution. 10µl of inoculum was added to each of 9 test tubes and mixed well. To the control test tube T_{MC}, 1 ml of the chlorpyrifos was added, mixed well and 1 ml of this mixed content was discarded to check the clarity of the medium in presence of chlorpyrifos. 10µl of the inoculum was added to the control test tube T_{MI}, to observe the growth of the organism in the medium used. The control test tube T_M, containing medium only was used to confirm the sterility of the medium. All the test tubes were then incubated at 30 ºC for 18 hours.

Enrichment medium

M. odoratimimus SKS05-GRD was first enriched in Mineral Salts Medium broth to familiarize the organism to the pesticide environment. Mineral Salts Medium (pH 7.0) containing (g/L) K_2HPO_4 - 1.5; KH_2PO_4- 0.5; $(NH_4)_2SO_4$- 0.5; NaCl- 0.5; $MgSO_4$- 0.2; $CaCl_2$- 0.05; $FeSO_4$- 0.02 supplemented with peptone - 1g and chlorphyriphos – 50mg/L. The culture was subcultured for 3 generations and maintained on the same Mineral salt Agar medium supplemented with peptone and chlorphyriphos.

Growth rate of *Myroides odoratimimus* SKS05-GRD

Overnight culture of *M. odoratimimus* SKS05-GRD was inoculated into Mineral salts medium supplemented with 50mg/L of chlorpyrifos and mineral salts medium without Chlorpyrifos and incubated at 30°C in a rotary shaker for different time intervals. Medium without inoculums was maintained as control. Growth was determined turbidometrically at different time intervals by measuring the optical density at 600nm.

Biodegradation studies

Shake flask studies were carried to work out the chlorpyrifos degrading capacity of the *M. odoratimimus* SKS05-GRD. Seed culture of *M.odoratimimus* SKS05-GRD was grown in mineral salts broth containing chlorpyrifos (50 mg/L). Following 24 h of incubation, 1% inoculum of the culture was inoculated in Mineral salts medium (150 ml) containing 50mg/L chlorpyrifos and incubated at 37°C and 150 rpm on a rotary shaker. Mineral salts medium flask without inoculum was kept as control.

Spectrophotometric determination of chlorpyrifos residues

In this assay, at defined intervals, 1 ml of the sample was transferred to 1 ml centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was separated out and 100µl of supernatant was taken in a 25ml graduated tube. 1ml of 5M sodium hydroxide solution was added and the solution was incubated at 40° C for 30 mins for complete hydrolysis. 1ml of diazotized-PABA was then added and shaken thoroughly and kept at 4°C for 10 mins followed by 1 hour at room temperature for full colour development and wine red colour was obtained. The solution was then diluted to 25 ml and absorbance was measured at 520nm against distilled water as blank. The concentration of chlorpyrifos was estimated by referring to the standard curve. The standard curve was prepared by using gradient concentrations of chlorpyrifos standard ranging from 1.2µg to 18µg and measured at 520 nm

Extraction of sample (pesticide residue) for analysis

Samples were recovered from culture flasks (inoculated and uninoculated) at respective time intervals (0, 3, 5 and 15 days) and centrifuged at 10,000rpm for 10 min to obtain cell free medium. Chlorpyrifos residues was extracted twice with equal volume of dichloromethane and the collected solvents were allowed to dry the final volume of extracted residues was made in hexane. The residues were spotted on TLC plate. The plate, using the solvent system Hexane: Ethyl acetate (7:3), was observed under UV light. The standard Chlorpyrifos was also spotted, chromatogrammed and their R_f values was calculated. Thin layer chromatography was performed to identify the pesticide Chlorpyrifos and the major breakdown product, TCP (3,5,6-trichloro-2-pyridinol).

HPLC conditions and analysis

Extracted samples were analyzed on a Shimadzu liquid chromatographic system (LC-8A) equipped with multi-wavelength UV diode detector, at ambient temperature and a rheodyne injector and high pressure gradient quaternary pumps. A reverse phase C-18 column (250×4mm), 5µl (Waters Phenomix) was used for separation. LC solution (Shimadzu) was applied for the data collecting and processing. Mobile phase of a mixture of acetonitrile: water (60:40) was delivered at a flow rate of 1.0ml/min with UV detection. The column temperature was maintained at room temperature. Injected 20µl of standard preparation using a rheodyne syringe and recorded the chromatogram. Chlorpyrifos was detected at 290 and 320nm wavelengths. The retention time for Chlorpyrifos was 13.4min (Abu-Qare and Abou-Donia, 2001).

DNA extraction

DNA was extracted from *M. odoratimimus* SKS05-GRD using general procedure. Cells were grown overnight in broth medium. Cells was pelleted by centrifugation, and resuspended in 5 ml 50mM Tris (pH 8.0), 50mM EDTA. Cell suspension was frozen at -20°C. 0.5 ml 250mM Tris (pH 8.0), 5µl of lysozyme (10mg/ml) was added to frozen suspension and thawed at room temperature and placed on ice for 45 min. 1 ml 0.5% SDS, 50mM Tris (pH 7.5), 0.4M EDTA, 5µl of Proteinase K (10mg/ml) was added and placed in 50°C water bath for 60 min. Extraction was done using 6 ml Tris-equilibrated Phenol: Chloroform: Isoamyl Alcohol (25:24:1) and centrifuged at 10,000X g for 15 min. The top layer was added to new tube by avoiding the interface. 0.1 volumes of 3M Sodium Acetate was added and mixed gently and then 2 volumes of 95% Ethanol was added and mixed by inverting. The tube was centrifuged and transferred to 5 ml 50mM Tris (pH 7.5), 1mM EDTA, and 200 mg/ml RNase. Samples from all tubes were electrophoresed in a 0.8% agarose gel in TAE buffer and DNA bands were observed under UV transilluminator. The banding patterns obtained was then photographed onto gel documentation unit and the bands were analyzed.

PCR amplification of mpd gene

The *mpd* functional gene of *M. odoratimimus* SKS05-GRD was amplified by PCR with a pair of primers (Cui *et al*., 2001).The primer

F-5'-GAATTCATATGCCCCTGAAGAAC-3', and R-5'-GAATTCTCGAGCTTGGGGTTGACGACCG-3' were used. PCR was performed under 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and elongation at 72°C for 1 min. The PCR amplified products were analyzed through 2% Agarose gel electrophoresis. The band patterns obtained was then photographed onto gel documentation unit and the bands were analyzed.

RESULTS AND DISCUSSION

Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration is defined as the lowest concentration of Chlorpyrifos pesticide required to inhibit the growth of bacteria. In this study, attempts were made to exploit the role of *M. odoratimimus* SKS05-GRD in utilization and degradation of Chlorpyrifos in liquid medium. Primarily, MIC value of Chlorpyrifos against the organism was determined. Growth of the organism was inhibited by Chlorpyrifos in a concentration-dependent manner. The result indicates that the organism is able to tolerate Chlorpyrifos up to 2.5µg/ml×10^4. Value of MIC is expressed in universal unit µg/ml. Nawab *et al.*, (2003) reported that certain pesticides have inhibitory effects on bacterial growth.

Growth rate of Myroides odoratimimus SKS05-GRD

To adopt *M.odoratimimus* SKS05-GRD in the pesticide environment, the organism was enriched in Mineral salts medium supplemented with 10g/L of Peptone and 50mg/L of Chlorpyrifos. Initially, growth of organism was found after 48 hours. The culture was then sub cultured on the same medium for two generations which showed growth after 24 hours. The relationship of concentration of Chlorpyrifos to *M. odoratimimus* growth rate was determined by turbidometerically at different time intervals by measuring the optical density at 600nm. Growth pattern of the organism was in Mineral salts medium supplemented with 50mg/L of Chlorpyrifos is compared with the organism grown in medium without Chlorpyrifos.

Growth pattern of *M. odoratimimus* in mineral salts medium containing up to 50mg/L Chlorpyrifos and utilization as carbon and energy source showed an exponential growth after a late lag phase. This indicates that the adaption time of *M.odoratimimus* increases when it is introduced in to a new environment such as Chlorpyrifos containing medium. The duration of lag phase depended on substrate concentration and on adaption of the inoculum. The lag phase before microbial growth is well known from the growth curve of pure cultures. This phase is usually explained by the time necessary for cells to synthesize the enzymes essential for growth when they are introduced into a new environment

Figure 1 illustrates that the lag growth rate of *M. odoratimimus* was seen to be reduced by the presence of Chlorpyrifos in medium compared with lag growth in medium without Chlorpyrifos. Optical density Values of growth rate of the organism in medium with Chlorpyrifos obtained from turbidity measurements ranged from 0.181 (24 hours) to 1.600 (72 hours) whereas the growth rate of the organism in medium without Chlorpyrifos obtained was 1.507 (24hours) to 1.549

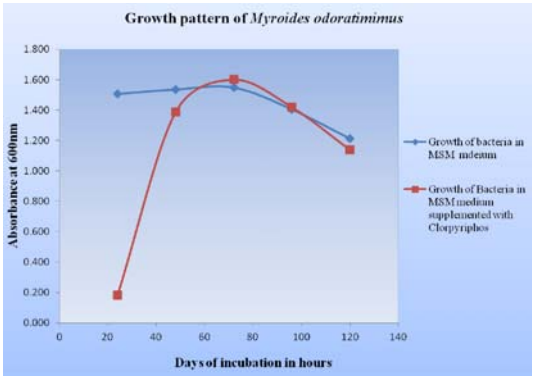

Fig.1. Growth Pattern of *Myroides odoratimimus* SKS05-GRD

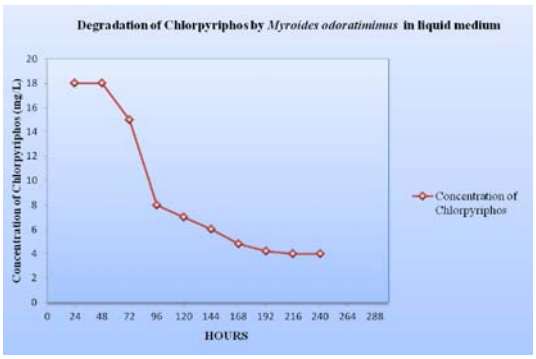

Fig. 2. Degradation of Chlorpyrifos by *Myroides odoratimimus* SKS05-GRD

(72 hours). Furthermore, the highest growth rate occurred at 72 hours in both the medium. Above this level, growth of *M. odoratimimus* was slowly inhibited in both medium with Chlorpyrifos and medium without Chlorpyrifos.

Biodegradation of chlorpyrifos by *M. odoratimimus* SKS05-GRD

Shake flask studies were carried to work out the Chlorpyrifos degrading capacity of *M. odoratimimus* SKS GRD05. Figure 2 show the gradual decrease in the concentration of Chlorpyrifos up to 4mg/L in 240 hours. Concentration of Chlorpyrifos remained same (18mg/L) till 48 hours because of slow lag growth of *M. odoratimimus*. A rapid loss in Chlorpyrifos concentration (55%) with almost constant degradation was observed after 48hours. About 78% of Chlorpyrifos (50 mg/L) in Liquid medium was degraded by *M. odoratimimus* within 10 days.

Thin layer chromatography

Ability of *M. odoratimimus* to degrade Chlorpyrifos was assessed in liquid medium for 10 days. Residual concentration of Chlorpyrifos was determined by spectrophotometric estimation up to 10days. About 77% of Chlorpyrifos degradation at concentrations up to 4mg/L in flasks, with shaking, has been achieved. Moreover the organism utilizes Chlorpyrifos as sole carbon and energy source which resulted in effective degradation. Degradation of Chlorpyrifos by *M.odoratimimus* was not reported so far. Thin layer chromatography was performed to identify the insecticide Chlorpyrifos and the major breakdown product, TCP (3,5,6-trichloro-2-pyridinol). The solvent system used was Hexane: Ethyl acetate (7:3). The Rf value of the standard pesticide was 0.72 and of the intermediate TCP was 0.28. Upto two days of extraction, a spot on TLC

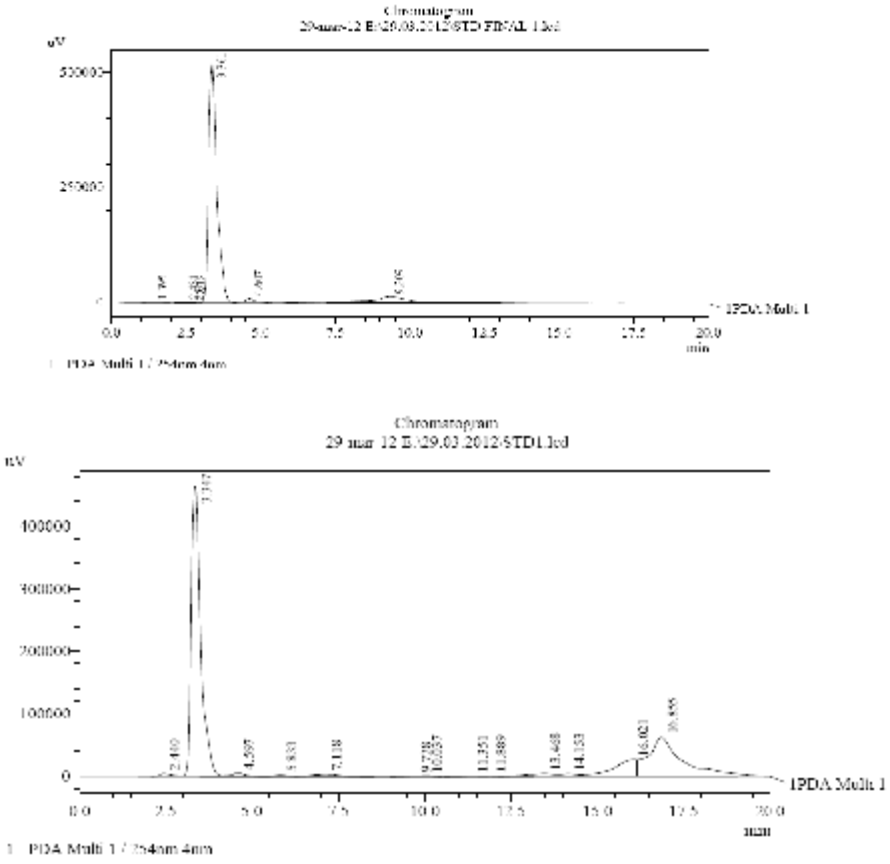

Fig. 3. HPLC chromatogram of control and test samples of Chlorpyrifos residues extracted from liquid medium

corresponding to the standard pesticide Chlorpyrifos was observed. On 5th day extraction a spot corresponding to Rf value 0.6 corresponding to an unknown intermediate metabolite was observed. On the 10th and 15th day of extraction, another spot corresponding to Rf value 0.53 corresponding to an unknown intermediate metabolite was observed. From 30th day extract three spots corresponding to the Rf value 0.72, 0.53 and 0.07 was observed.

HPLC analysis

HPLC analysis was performed for the fine confirmation of the Chlorpyrifos degradation by *M. odoratimimus* SKS05-GRD (Figure 3). From the first day solvent extraction of chlorpyrifos sample a small peak corresponding to the Chlorpyrifos retention time 13.4 along with peaks corresponding to the acetone solvent was observed in HPLC chromatogram. From the 30th day solvent extraction peak corresponding to the retention time 13.4 disappeared and few other peaks corresponding to the acetone solvent was observed in the chromatogram. The result indicates that at the end of 30th day no Chlorpyrifos residue was found in the liquid medium which is degraded by *M. odoratimimus*.

PCR amplification of mpd gene

Genomic DNA and Plasmid DNA were extracted from *M.odoratimimus* SKS GRD05. PCR amplification of *mpd* gene was carried out in Genomic DNA revealed amplification of the *mpd* gene, giving a product size of 650bp.

CONCLUSION

M. odoratimimus SKS05-GRD possess a large plasmid of size 21kb and also confers plasmid mediated antibiotic resistance such as kanamycin, gentamycin and amikacin. The same plasmid mediated resistance was therefore investigation in this study for bioremediation in the presence of methyl parathion degrading (*mpd*) gene. The detention of this gene in *M.odoratimimus* has been carried out for the first time that yielded a product of 650bp. Although future studies are to be carried out regarding the cloning and expression profiles of this gene, this study confirms the ability of the bacterium to degrade methyl parathion at molecular level.

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