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Research Article

MYCODEGRADATION OF MALATHION BY A SOIL FUNGAL ISOLATE, *ASPERGILLUS NIGER*

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ABSTRACT

Fungi are known to degrade or cause to deteriorate and processes a wide variety of materials and compounds, known as Mycodegradation. We report here, the degradation of malathion, an organophosphorous insecticide by a filamentous fungal isolate *Aspergillus niger,* isolated from soil samples from malathion contaminated cotton cultivated field soils of Guntur district. The fungal strain were cultured in the presence of malathion under aerobic and optimization of energy limiting growth conditions. The factors affecting degradation efficiency, effect of malathion concentrations on degradation rate were investigated. GC-MS studies revealed the degradative studies in different conditions. The impact of the study describes a fungal isolate *Aspergillus niger*, *as* an attractive alternative to other conventional technique may be utilized for the bioremediation of malathion contaminated residue soils.

Key Words: *Mycodegradation, Malathion, Aspergillus niger*

INTRODUCTION

Mycodegradation is a deterioration phenomenon causing degradation by fungi in a wide variety of materials and compounds (Singh H, 2006). Biological degradation studies of any pesticides is an attractive alternative to other conventional technique and is important to our understanding of the environmental fate of manmade chemicals. This process is dealing in degrading molecules to smaller compounds which may be toxic or non toxic itself or removal of pesticide molecule through simple absorption or adsorption mechanism (Adhikari, 2010). A very desirable spin-off such studies would be the development of efficient and specific biological waste treatment systems in which microorganisms especially selected for individual substrates are used (Bujacz *et al.*, 1995).

Malathion[S-(1,2-dicarbethoxyethyl)-O,O-dimethyldi-thiophosphate], also known as carbophos, maldison and mercaptothion is a nonsystemic, wide-spectrum organophosphorus insectiscide used to control the household and agricultural pests. It was recognized as the first organophosphorous insecticide with highly selective toxicity (Singh *et al.*, 2012). Malathion in soil undergo a variety of transformations that provide a complex pattern of metabolites and its fate in soil is controlled by chemical, biological and physical dynamics of this matrix. Malathion is available in two forms, a pure form as colorless liquid and a technical grade solution (brownish-yellow liquid) which smells like garlic. Malathion is short-lived and generally persist for only a few weeks to a few months (Cope *et al.*, 2004). Degradation products of malathion include dimethyl phosphate, dimethyldithiophosphate, dimethylthiophosphate, isomalathion, malaoxon and malathion mono and dicarboxylic acid due to enzymatic activity of cutinase, carboxylesterase, phosphatase (Singh, 2006) and are generally the result of impurities or exposure to extreme storage conditions (PAN, 2005). Excessive and frequent application of pesticides in high levels of its residues accumulated on agricultural crops, which possess a potential health hazard to consumers. Residues of malathion were found exceeding the MRL (5 ppb) as proposed by the Ministry of Commerce, Government of India (Goda *et al.*, 2010, Mukherjee, 2010). Soil micro flora is another potential candidate for detoxification of pesticides. Some investigators found that soil contaminated with pesticides could be possibly decontaminated by inoculation with specifically adapted microorganisms (Kamal *et al.*, 2008). Degradation by *Aspergillus flavus* and *A. sydowii* in Soil was previously reported by Hasan (1999). The

current status of species recognition and identification in *Aspergillus species* was reviewed by Geiser *et al.* (2007). Molecular Methods for Identification of *Aspergillus species* which was reported by Deak and Balajee (2010).

MATERIALS AND METHODS

Malathion is extensively used in cotton cultivated soils of Guntur district of Andhra Pradesh. The aim of the present study was to isolate and characterize, investigate their ability to degradation of malathion, that may be very well adapted to the climatic and environmental conditions from cotton cultivating soils from different sites.

Chemicals

Malathion (Technical grade) with Pesticide reference standards of organo phosphate pesticides obtained from Excel Industries Ltd, Mumbai, India (purity 98%). All other reagents are purchased from Sigma chemicals, Bangalore.

Soil

Soil samples collected from cotton cultivating fields by Random sampling method (surface layer 0-30 cms depth) in different areas of Guntur, Tenali, Mangalagiri, Nambur, chilakaluripet where extensive usage of organophosohorous pestiscide malathion is more. Physical and chemical charecterisation was determined by recommended procedures by RARS, Tirupati.

Isolation and Optimisation of fungal strain

The soil samples were collected from a depth of 10 to 15 inches in the soil where there was high moisture content. Humidity, temperature, pH and composition of soil nutrients play important roles in the efficiency of microorganisms to degrade pesticides (Goda *et al.*, 2010). The fungi need moisture for their growth. The soil samples were collected and dispensed in sterile bags which were sterilized with UV rays and X-rays to avoid external contamination. These samples were brought to laboratory and then the soil samples were screened for fungal species.

The desired fungal isolate *Aspergillus niger* were obtained by enrichment culture on the Potato Dextros Agar (PDA) medium after a test run in different physicochemical parameters such as pH, temperature (25-30°C, 35-37°C, 40-45°C) salinity (2.5%, <5%), Inorganic Sulphur (0ppm, 20ppm, 40ppm, 60ppm), Inorganic phosphorous (0ppm, 20ppm, 40ppm, 60ppm) and 2% PDA medium.

Biodegradation assay

5gms of soil was used to inoculate 100 ml of enrichment medium amended with 25 mgl^{-1} malathion and kept for incubation at room temperature (27-29^{o}C) for 6days. On next day, the enriched samples were transferred to sterile medium containing same malathion concentration i.e., 25mgl^{-1} concentration of pestiscides was increased in stepwise manner (25mgl^{-1} to 250mgl^{-1}) serial dilution technique (Aneja, 2005). Treatments were triplicated (Lourdes *et al.*, 2004).

Optimization of the medium for degradation studies: The isolate were cultured on Potato Dextrose Agar (PDA) medium (Liu *et al.*, 2001). Levels of pH 4, 4.5, 5, 5.5, 6and 6.5 were adjusted (pH was adjusted by either 0.5M NaOH or 0.5M HCl) before autoclaving. The medium was then autoclaved at 121°C at 15 lb inch-2 for 15 minutes. The sterilized medium was cooled and 250 mg of chloromycetin per 200 mL of medium was used as antibacterial (Noomrio and Dahot, 1992). Each treatment was triplicated. Three wells in each plate were made and inoculated with test strains suspension of $5x10^{5}$ conidia mL^{-1} concentration. The plates were incubated at 30 ± 2°C for five days to seven days. The extent of growth of representative strains of *A. niger* was evaluated qualitatively by visual inspection. Microscopic mounts are best made using a cello tape flag and slide culture preparation mounted in Lacto phenol cotton blue (Gillman, 1959 and Bapat *et al.*, 2003). A drop of alcohol is usually needed to detach the cellotape flag from the stick, and to act as a wetting agent.

Preparation of spore suspension, Sporulation level

Erlenmeyer flasks containing 50 ml of 1ml medium were inoculated with *A. niger* (Omar, 1998). To stock culture 10 ml of autoclaved distilled water was added and the surface was gently peeled with sterilized wire loop (Noomrio and Dahot, 1992). The inoculum added was 1 x 10^{6} spores/ml, and this was followed

by incubation during 4 days at 30ºC. Spores were harvested, and counted using a Neubauer chamber. Growth was calculated using colony forming unit (CFU/ml of culture) method (Singh B *et al.*, 2012).

Gas Chromatography (GC) studies of organophosphorus pesticides

The degree and extent of degradation of various pesticides can be very well studied using GC analysis (Andreu and Pico, 2004). The GC analysis of malathion for the standard solutions and the samples (aliquots) was performed using an Agilent 7890 gas chromatography (Agilent Technologies, Inc, Santa Clara, USA), equipped with a flame photometric detector, a capillary column (DB-1701, 30 m×0.250 mm×0.25 µm), and nitrogen as the carrier gas at a flow rate of 3 ml min^{-1}. Quantification of the pesticides was performed by comparing the peak areas of the pesticides to a calibration curve of the standards (Frenich *et al.*, 2005). Percentages of recovery were given in the respective data with technical standards. The observations in each experiment were periodically recorded. The retention indices of samples taken after different time intervals (24 hrs,72hrs,120 hrs) were matched and compared for areas (Wang *et al.*, 2010).

RESULTS

Morphological and biochemical analysis of Isolated fungal strain

Fig 1:(A) Scanning electron micrograph of hyphae of *Aspergillus niger* (B) Micrograph *A. niger* colony. (C) Lactophenol Blue staining.

Physico-chemical properties		Growth
Temperature	25-30ºC	++
	35-37ºC	+
	40-45ºC	--
pH	2.5-3.5	--
	4.5	+
	5.5	++
	6.5-8.5	+
Nacl %	2.5	+
	<5	--

Note: ++ = extensive growth
+ = moderate growth
- = no growth

Aspergillus niger was identified, based on their morphological and biochemical characteristics of *A. niger* such as conformation by cotton blue staining and Scanning Electron Microscope (SEM studies by ***Hitachi S-3500 N scanning electron microscope***). Identification of fungal strain from soil sample strain, identified by physiological and morphological tests as depicted by Lourdes *et al.*, (2004).

The present study was carried out to determine the performance of growth of screened test fungi on different media and an attempt to develop a field model for degradation of pesticides, for identifying a suitable laboratory medium, the medium that produce maximum growth will be selected for the further studies. We found that the optimal condition for the increase in biomass weight of the isolate. The optimum lies in a narrow region viability defined by the process parameters (Omar, 1998). The result of this conceptual study clearly reflect that pH value, Temparature, salinity, Phosphorous and Sulphur at multiple concentrations induced in the medium integrated with malathion at different levels had shown the ability to affect the mycelial growth rate and consequently on proliferation of *A. niger*. The factors affecting biodegradation efficiency were investigated and effect of malathion concentration on degradation rate was also determined. After inoculation of *A. niger* using the potato dextrose broth in separate conical flask and incubated at different temperatures (25, 28, 30, 35, 37, 40 and 45) for seven days, maximum growth was observed at 25°C, 28°C and 30°C and was minimal

growth was observed at temperatures of 35°C and 37°C and there was no growth at and above the temperatures of 40°C. This reveals that the optimum temperature for maximal growth of these organisms was 25°C to 30°C. To determine the role of pH (Ramasamy, 2011), the medium with different pH range has been prepared (4, 4.5, 5.5, 6.5, 7.0, 7.5, 8.5) and inoculated with *A. niger* were incubated for seven days. The growth was not observed in the flask of pH 2.5 and 3.5. The maximum growth was observed at pH 5.5 and the moderate growth was observed at pH 4.5, 6.5, 7.0, 7.5 and 8.5. These results indicate that the optimum pH for the growth of fungi was 5.5 and moderate growth in between 6.5 to 8.5. Growth medium with different salt concentrations (0, 0.5, 5, 7.5 and 10% NaCl) for salinity was selected and were inoculated with *A. niger* and *R. microsporus* separately and incubated for seven days. The growth was observed in all the inoculants of NaCl concentrations (0, 2.5% and >5) where as the growth was not observed in 5%, 7.5% and 10% of NaCl concentration containing the inoculants. It is clear evident that the maximum growth of all the fungal organisms can be achieved using 2.5% NaCl concentration.

Degradation studies of malathion enriched samples

The soil micro organisms can play an important role in the intermediate degradation and subsequent mineralization of many pesticides. Microbial degradation of a pesticide may be of a co metabolic or of incidental nature or may be linked with energy production or nutrient procurement and thus support growth of the degrading population (Sinclair *et al.*, 2006).

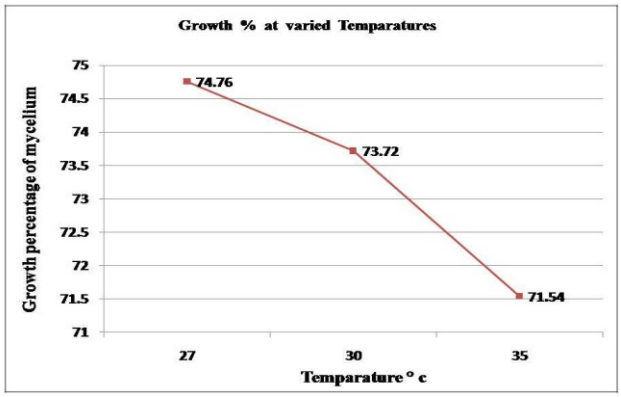

Fig 2:Percentage growth *Vs* Temparature

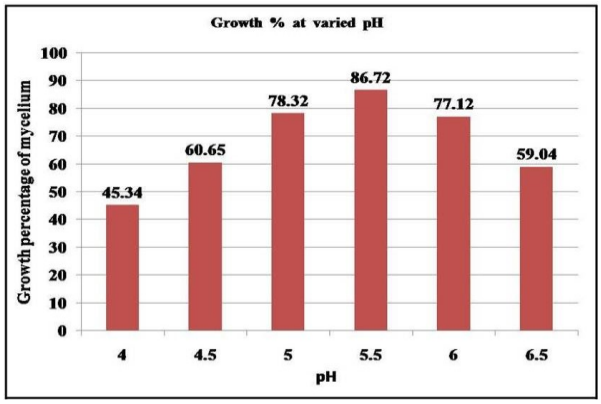

Fig 3 : Percentage growth *Vs pH*

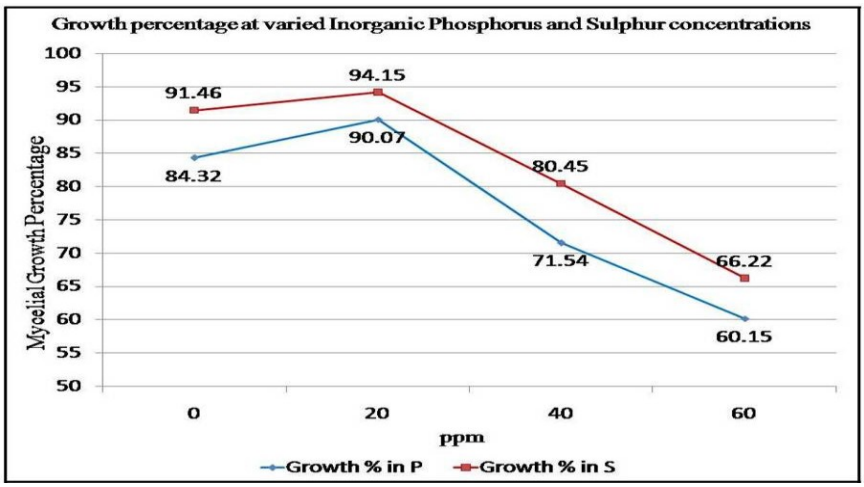

Fig 4: **Mycelial growth at Different concentrations of inorgaric Phosphorous, Sulphur**
Effect of temperature, pH, inorganic phosphorus and Sulphur for degradation studies

Optimization of dextrose concentration for degradation studies

Dextrose is an essential nutrient for the growth of fungi. It supplements the carbon source in Potato dextrose broth. All the selected fungi were grown with potato dextrose agar with 5% dextrose and amended with malathion showed maximum degradation. With the increase in percentage, the degradation was noticed with increase in percentage of dextrose from 2% to 5% and with further increase in percentage of dextrose above 5% i.e., to 6% the trend to be in a decreasing level with respect to percentage of degradation.

The predominant metabolic activities in the microbial world are meant for production of energy. Maximum degradation was taken place at room temperature of 27^{o}C. The degradation rate was about 74.76% (Fig 2). It was found that maximum degradation was achieved at a pH of 5.5 (86.72). The degradation rate was found to be increased with the increase in pH from 4.0 to 5.5 and a decreased with increased pH from 5.5 to 8.0 (Fig 3).

In the present study, it was observed that the biodegradation of Malathion was achieved to the maximum point at a phosphorus (90.07%) and sulphur (94.15%) concentration at 20ppm. The degradation rate was found decreasing with the increase in phosphorus and Sulphur concentration from 20ppm to 60ppm. Organic sulfur mineralization by the used fungal species paralleled, to some extent, organic P mineralization (Fig 4).

DISCUSSION

Pesticides which enter the soil environment are subjected to a variety of degradative and transport processes. The overall dissipation of a pesticide from soil results from a combination of loss mechanism such as microbial degradation, chemical hydrolysis, photolysis, volatility, leaching and surface runoff (Singh and Walker, 2006). The degree to which each mechanism will contribute to the overall loss of the pesticide is in turn dependent on the physicochemical properties of the pesticide (water solubility, sorptive affinity), characteristics of the soil (pH, organic content, biomass, redox status), environmental conditions (temperature, moisture). Since the conditions in soil are much more complex than those in synthetic media, the ability for pestiscide degradation in non sterile soil was investigated (Hasan, 1999).

In addition to the potential bioremedial use of microbes and enzymes for dealing with organophosphorus contamination in the environment, there has been considerable interest in the use of organophosphorus degrading enzymes prophylactically and therapeutically for organophosphorus poisonings. Future areas of research include increasing enzyme activity against poor substrates and improving enzyme catalytic activities in mixtures of chemicals (Pujari, 2011). Role of enzymes in the remediation, decontaminating

agents of polluted environments was studied by Rao (2010). Recent advancements in amperometric, potentiometric, and optical biosensors using genetically engineered microorganisms expressing organophosphate hydrolyzing enzyme intracellularly or anchored on the cell surface for the detection of organophosphate pesticides (Mulchandani and Rajesh, 2011).

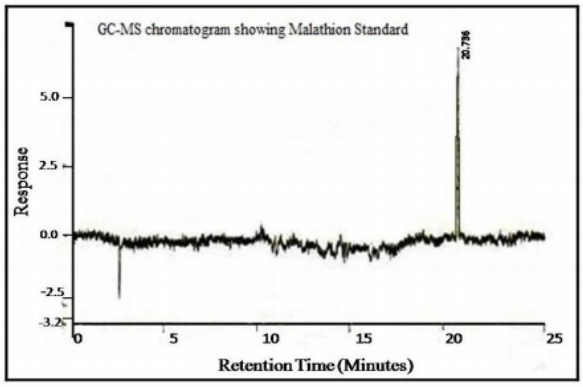

Fig 5(A). Chromatogram of Malathion standard

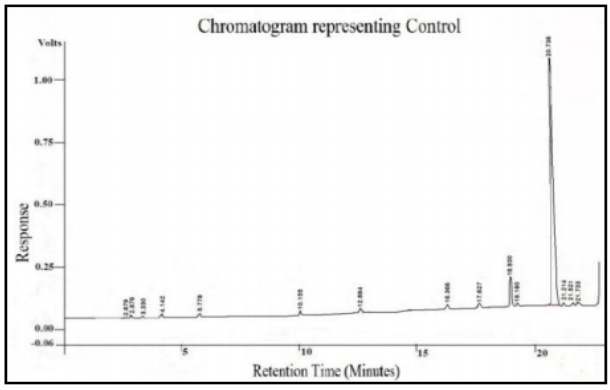

Fig 5(B). Chromatogram of Control

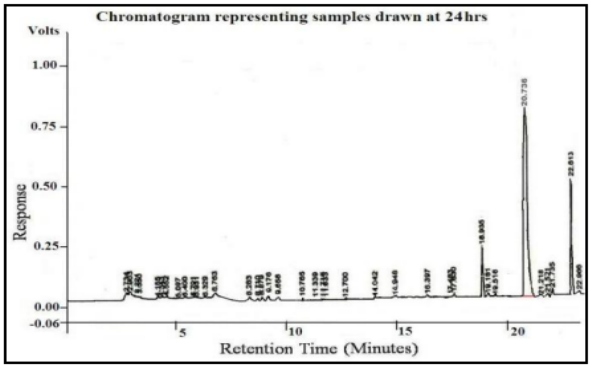

Fig (5)C. Chromatogram at 24 hour incubation.

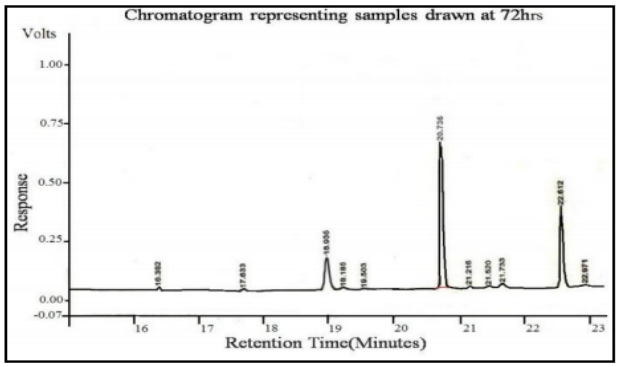

Fig (5)D. Chromatogram at 72 hour incubation.

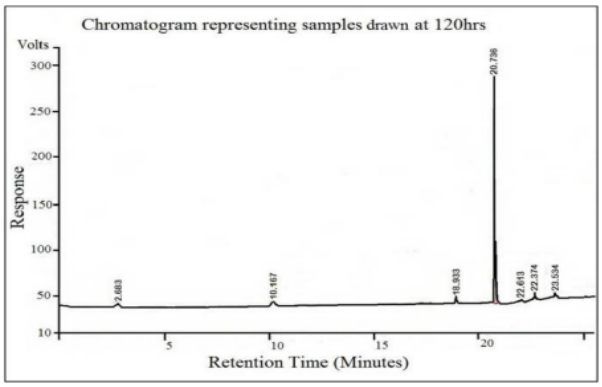

Fig (5)E. Chromatogram at 120 hour incubation.

Fig 5 (A,B,C,D,E): Comparitive studies of GC-MS chromatograms at different intervals revealed that the area of the peak of the malathion with retention time (20.736 min) decreased with increase in incubation time (24hrs to 120 hrs).

CONCLUSION

The study has elucidated that the fungal species *Aspergillus niger* are effective biodegrading species and by implementing the large scale culture of these organisms by suitable culture methods, the identified and selected fungi can be very effectively used for cleaning the contaminant soils by developing these fungal organisms as an effective biocontrolling agent for control of environmental pollution particularly contaminated soils in this area .Our fungal isolate *Aspergillus niger* could prove valuable in on-farm pesticide bioremediation systems in malathion contaminated soil. Due to their high biodegradation activity, the fungal strain isolated from this work merit further study as potential biological agents for the remediation of soil, water, or crops contaminated with the pesticide malathion.

Despite the fact that several malathion degrading fungi have been isolated, the new isolate *Aspergillus niger* from this work may be better suited to the climate and environment conditions in Southern parts of india where cotton is cultivated. This area of research needs concerted efforts as degradation products of several compounds are pollutants and may have deleterious effects on the environment and non-target organisms. Natural, inexpensive, and eco-friendly microbes endowed with pestiscide degrading potential could be an ecologically good alternative in detoxifying soil residues encourage the farmers to use natural pesticides rather than chemical pesticides.

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