

Isolation and characterization of monocrotophos degrading activity of soil fungal isolate *Aspergillus Niger* MCP1 (ITCC7782.10)

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ABSTRACT

Monocrotophos (MCP) is both a systemic and control pesticide useful for the protection of various cash crops. The use of pesticide degrading microbial system for the removal of pesticide residue and other products from contaminated systems requires an understanding of the growth requirements of degrading organisms. Monocrotophos (MCP) degrading fungal strain was isolated from soil by enrichment culture. The fungal strain was identified by colony morphology, staining and 18S rDNA analysis as *Aspergillus niger* (MCP1) (ITCC 7782.10) GenBank accession no JQ660373. Monocrotophos degrading ability of the MCP 1 was characterized as assessment of two parameters i.e growth kinetics of fungal strains in the presence of monocrotophos by dry mass estimation method and degradation kinetics of monocrotophos by spectrophotometric method of estimation at 254nm. Growth kinetics revealed that monocrotophos had induced the growth of MCP 1 to approximately 2 folds. Culture conditions for the growth of MCP 1 were optimized by growing it under different physical and chemical parameters. Optimum conditions for growth were found to be pH-8, Temperature 25-30°C and monocrotophos concentration 150 mg L⁻¹. *Aspergillus niger* is capable of degrading 90% monocrotophos under optimized conditions in 10 days. Degradation kinetics of monocrotophos followed straight line equation and half life was thus calculated. Constant of degradation K_{deg} was 0.009212 and the half life of monocrotophos was 75.87 hrs. In our study it is the first report showing the potential of *Aspergillus niger* as a potential degrader of monocrotophos and can be used as a candidate for removal of monocrotophos from agricultural sites.

Keywords: Monocrotophos, Biodegradation, *Aspergillus niger* (ITCC 7782.10, NCBI GenBank Accession No. JQ 660373).

1. Introduction

Monocrotophos [dimethyl-(E)-1-methyl-2-(methylcarbamoyl) vinyl phosphate] (MCP) is an organophosphorus insecticide with nonspecific systemic and acaricide action, used to control common mites, ticks, and spiders, pests of cash crops such as cotton, sugarcane, groundnut, tobacco, maize, rice, soybeans, and vegetables by contact and stomach action (Anonymous, 2001, Vig *et al.*, 2001; Bhadbhade *et al.*, 2002a). It is water-soluble thus weakly sorbed by soil particles and quickly penetrates into plant tissue (Tomlin, 1995).

Microbial activity has been proved so far as the most influential and significant cause of organophosphorus pesticide removal. *Pseudomonas aeruginosa*, *Clavibacter michiganense* *Arthrobacter atrocyaneus*, *Agrobacterium radiobacter* *Bacillus megaterium* and *Pseudomonas mendocina* (Ramanathan and Lalithakumari, 1999, Bhadbhade *et al.*, 2002b,

Horne *et al.*, 2002, Singh and Singh, 2003) have been reported to degrade MCP in solutions and soils. Although, fungal degradation of pesticides is not well known but many fungi, including the white-rot fungi and *Aspergillus sp.* have been tested for their ability to degrade organophosphate pesticides (Omar, 1998, Hasan, 1999, Yu *et al.*, 2006). Therefore, the present study is designed to isolate and screen the most efficient monocrotophos degrading fungi and optimize the favorable growth conditions for it.

2. Materials and methods

2.1 Chemicals

Monocrotophos of analytical grade (sigma, 99.5%) was used in this study. A stock solution of 1mg ml⁻¹ was prepared in 100% ethanol. Working solutions were prepared by appropriate dilution of stock. All the other chemicals used in this study were of analytical grade and were obtained from Sigma, Himedia, Rankem, India.

2.2 Media

Modified Czapekdox medium (M1) used for the enrichment of monocrotophos degrading fungal strains contained sucrose, 30 g; NaNO₃, 2 g; KCl, 0.5 g; MgSO₄.7H₂O, 0.5 g; glucose, 10 g; FeCl₃, 10 mg; BaCl₂, 0.2 g; CaCl₂, 0.05 g, per litre distilled water and monocrotophos was supplemented as an additional source of phosphorus

2.3 Soil sampling

Soil samples were collected from various agricultural farms within 10 km range of Banasthali campus, Rajasthan. The collected samples were mixed, air dried and sieved through 2mm mesh prior to use. Samples were stored at 4°C for further use.

2.4 Isolation of microorganisms

Isolation of fungal organisms was done by enrichment Culture Technique (Megharaj *et al.*, 1987). For this the 1 gm soil sample was serially diluted in 10 ml of 0.85% saline followed by dilution to appropriate concentration (10⁻⁴). 100µl of this soil sample was spreaded individually to plates containing M1 medium. To inhibit the growth of bacteria, effective antibiotics at their optimum concentration (30 mg l⁻¹) were mixed in the growth medium. 0.1 ml of each suspension was placed on synthetic solid medium. The plates were then incubated at 28±2°C for 5 days and growth of fungi was observed. Fungal colonies were isolated by streaking the isolated fungal strains on M1 medium and identified on the basis of colony morphology and staining properties.

2.5 Screening of monocrotophos degrading fungal strains

Further, for screening of monocrotophos tolerant fungi, these fungal isolates were grown in M1 medium (modified Czapekdox), containing monocrotophos as a sole source of phosphorus. Medium was inoculated with 100µl of soil suspension (10⁻⁴) in 0.85% saline containing gradient of monocrotophos concentrations (50, 100, 150, 200 250 and 300 mg l⁻¹) and incubated for 5 days at 28±2 °C. Pure culture of the isolated strain was maintained on the agar slopes of the same medium. Growth was observed both by number of colonies and dry mass (mg ml⁻¹). The most tolerant culture MCP1 was deposited in Indian Type Culture Collection, Indian Agricultural Research Institute (IARI), New Delhi, India.

2.6 Molecular characterization of MCP 1

For the molecular characterization, genomic DNA of MCP1 was isolated by CTAB method (Doyle and Doyle, 1987) and the interspecific transcribed spacer (ITS) was amplified by polymerase chain reaction (biometra, Germany, model personel) using the forward primer: ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3' and reverse primer ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'. The amplified gene fragment was purified by agarose gel electrophoresis and cloned into pGEM-T vector (Promega scientific, Santa Barbara, California). Both the strands of cloned 18S rDNA fragment were sequenced using sequencing facility provided by Bangalore Genei, India. The sequence were compared to those in GenBank using Blast Alignment Tool and deposited to the GenBank, EMBL and DDBJ library.

2.7 Optimization of culture conditions for the growth of MCP 1

Culture conditions for the growth of isolate MCP1 was optimized by growing the pure culture isolate in M1medium under different growth parameters for different duration of time (5, 10, and 15). Spore suspension of pure culture isolate was made about 1×10^8 spores ml^{-1} in 0.85% saline and 100 μl of this spore suspension was inoculated into 100 ml of medium and growth was observed in the form of dry mass (mg ml^{-1}).

2.7.1 Temperature

Temperature was optimized by growing the isolated strain MCP1 at various temperatures such as 10, 25, 30 and 37°C under optimized pH-8 and 150 mg L^{-1} monocrotophos concentration. Medium without monocrotophos was also incubated at different temperature as control.

2.7.2 pH

For the optimization of ionic strength, the isolated strain MCP1 was allowed to grow in different ionic concentrations with pH- 5, 6, 7, 8, 9 and 10 in M1 medium containing 150 mg L^{-1} concentration of monocrotophos. Similarly medium of different ionic concentration without monocrotophos served as control.

2.7.3 Concentration of Monocrotophos

Monocrotophos concentration was optimized by growing the isolate MCP1 at 50, 100, 150, 200 250 and 300 mgL^{-1} concentrations of monocrotophos. Control without monocrotophos was run in parallel.

2.8 Growth kinetics of MCP1 under optimized culture conditions

In order to determine the growth pattern, 1-ml spore suspensions (1×10^8 spores ml^{-1} in 0.85% saline) of isolated strain MCP1 was inoculated into a series of flasks each containing 125ml of M1 medium with and without monocrotophos at optimum pH 8 and incubated at optimum temperature 28°C in an orbital shaking incubator at 90 rpm for optimum incubation duration 10 days. The mycelial mass from each flask was separated by filtration using Whatman filter paper no. 1 and washed with deionized water. The dry weight of fungal biomass was determined by drying it at a constant temperature of $80 \pm 2^\circ\text{C}$ for 2 hrs. in preweighed filter paper.

2.9 Degradation kinetics of MCP1

For the study of degradation kinetics of MCP1 pure culture of MCP1 was suspended in 1 ml 0.85% saline to make a cell suspension of 1×10^8 cells per ml and 100 μ l of this suspension was inoculated in 125 ml of M1 medium containing 150 mg L⁻¹ concentration of monocrotophos and incubated at 28 ± 2 °C for 15 days in an orbital shaking incubator at 90 rpm under aerated culture conditions. Thereafter, monocrotophos was extracted at time interval 5, 10 and 15 days twice with equal amount of ethyl acetate (1:1). The solvent was evaporated and the residue was re-dissolved in 3 ml of ethyl acetate. Amount of monocrotophos was estimated at 254nm. The residual amount of monocrotophos was calculated by molar absorbtion coefficient. Degradation kinetics of monocrotophos follows first order kinetics. Degradation constant K_{deg} was calculated by drawing straight line curve between log of concentration of monocrotophos at certain incubation duration Vs incubation duration and the slope of the curve was multiplied by 2.303. Thus, the degradation coefficient was calculated by using straight line equation

$$y = -mx + c$$

$$K_{deg} = -2.303 \times -m$$

Half life of monocrotophos in M1 medium by MCP1 was calculated by the following formula-

$$t_{1/2} = 0.699 / K_{deg}$$

The value was expressed in hours.

2.10 Statistical analysis

The statistical analysis was done by using Statistical Package for the Sciences System (SPSS). The variables were subjected to Student t-test.

3. Results and discussion

3.1 Isolation and screening of fungal isolates

In comparison to the control the number of fungi was decreased from 12 to 8 isolates in the medium contained 50 mg L⁻¹ concentration of monocrotophos. Further, increase in the concentration of monocrotophos led to survival of only 5 isolates at 150 mg L⁻¹. At the highest concentration of monocrotophos 300 mg L⁻¹ only one isolate survived. This isolate was considered as the most tolerant fungal isolate and designated as MCP1 (table-1). Therefore, MCP1 was considered and selected as potential monocrotophos degrader and used for further studies.



Figure 1: Conidial head of *Aspergillus niger* as visible by phase contrast microscope 40x.

Earlier studies had also shown that the application of different concentration of organophosphates led to decrease in the number of population of fungal strains (Khallil and

Omar, 1993). Identification and characterization of MCP1 was carried out on the basis of colony morphology and staining by lactophenol cotton blue. The colonies were started to develop in pale yellow but quickly turned into jet black spherical profused conidia, the back was buffed. All the characteristics of fungal colony were matched with that of *Aspergillus niger* (Figure 2). The fungus, *Aspergillus niger* (MCP1) was designated as 7782.10 by the Indian type culture collection, IARI, New Delhi, India.

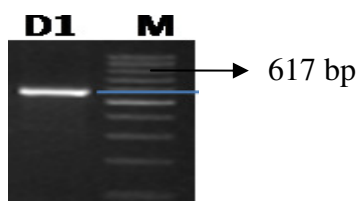
Table 1: Number of colonies observed after 7 days of incubation in M1 medium containing various concentrations of monocrotophos.

Concentration of Monocrotophos	Number of colonies
0	12
50	8
100	7
150	5
200	4
250	3
300	1

3.2 Molecular characterization of MCP 1

PCR product of the amplified DNA appeared as intense bands on agarose gels (Figure 2). The approximate size of the amplified PCR product was 617 bp. The sequence and comparative phylogenetic analysis of PCR amplified 18S rDNA gene fragment confirmed MCP1 as closest homologue of *Aspergillus niger* NCBI Genbank Accession No. JQ 660373.

(a)



(b)

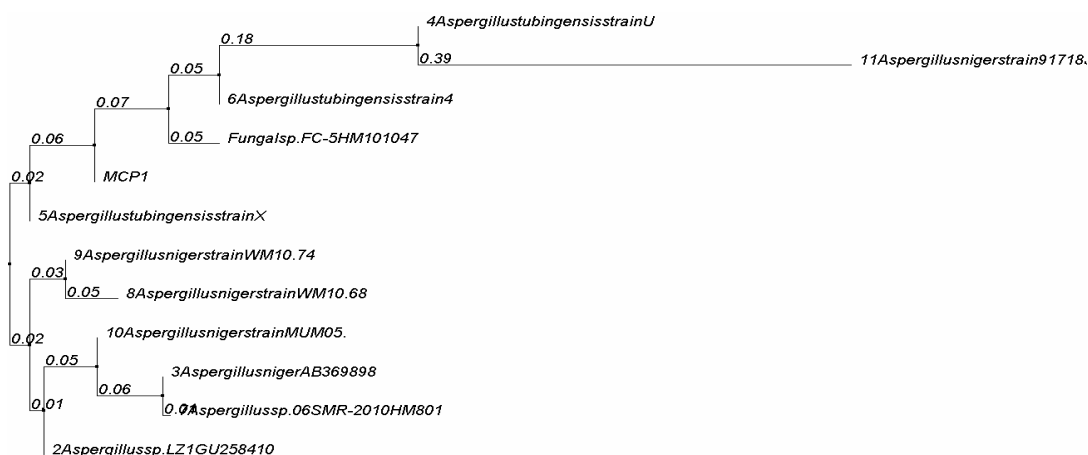


Figure 2: (a) PCR amplification of 18S rDNA fragment of MCP1 Lane M- marker, Lane D1- Amplified 617bp 18S rDNA Gene Fragment from Genomic DNA of MCP1.

(b) Neighbour joining Phylogenetic Tree constructed between ten closest homologues of MCP 1 by Clustal W program showing distance among different strains.

3.3 Optimization of culture conditions for the growth of fungal isolates

It is a well known fact that the change in physical and chemical factors directly affects the growth of microorganisms. Hence, in this study the isolated strain was allowed to grow at different culture conditions and its growth was observed in liquid medium as dry mass (mg ml^{-1}).

3.3.1 Temperature

3.3.1 Temperature

When isolate was subjected to temperature variance for a period of 15 days, minimum growth 0.25 mg ml^{-1} at temperature 10°C was observed on day 5. Increase in growth was continued till day 10 with increase in temperature to $25\text{-}30^\circ\text{C}$ to 2.14 mg ml^{-1} and then declined gradually till day 15 along with increasing temperature to 37°C (Figure 3). Hence, it could be concluded that the optimum growth of MCP1 was achieved on day 10 at temperature $25\text{-}30^\circ\text{C}$.

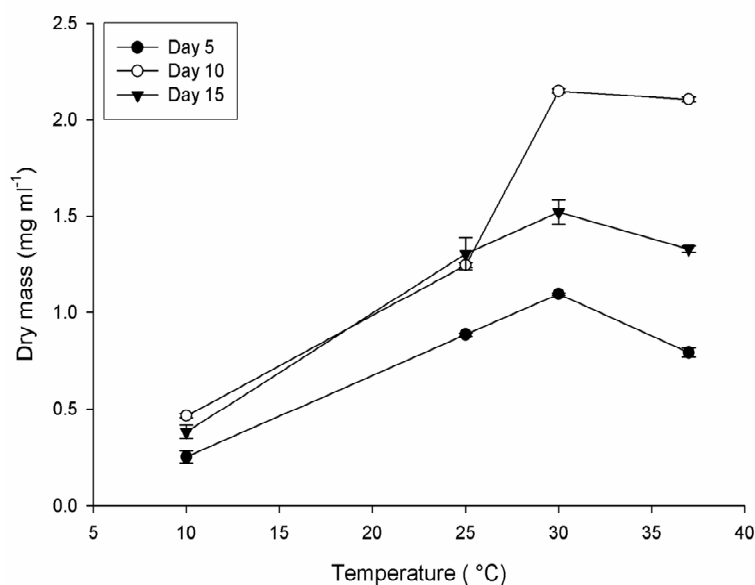


Figure 3: Effect of temperature on the growth of *Aspergillus niger* in M1 medium supplemented with 150 mg L^{-1} monocrotophos at various incubation duration. Error Bars indicate standard deviation. Variables were subjected to t-Test and were found significant at $P \leq 0.05$ level.

3.3.2 pH

Increased pH of growth medium resulted in visible increase in the growth of MCP1 (Figure 4). At pH-5 the growth was minimum 0.565 mg ml^{-1} after 5 days of incubation. Fungal growth was increased 3 folds with change in pH from 5-8. Growth was continued till 10th day and maximum growth observed was 2.9 mg ml^{-1} at pH-8. Further, rise of pH to 10 resulted in inhibition of growth to 2.32 mg ml^{-1} after 10 days of incubation. It has also been reported by Wheeler *et al* (1991) that favorable growth of fungal strains is achieved at alkaline pH 8-9 and after this the ionic concentration begins to interfere with the growth of fungi and results in decreased growth.

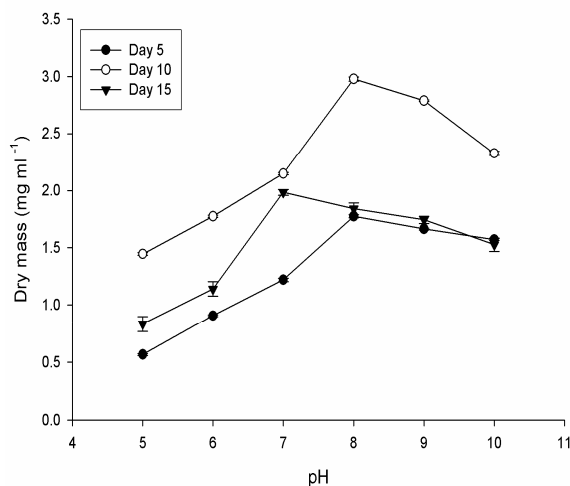


Figure 4:- Effect of pH on the growth of *Aspergillus niger* in M1 medium supplemented with 150 mgL⁻¹ of monocrotophos at different incubation duration. Error Bars indicate standard deviation. Variables were subjected to t-Test and were found significant at P≤0.05 level.

3.3.3 Monocrotophos concentration

150mg L⁻¹ concentration of MCP was found to be the optimum concentration of applied pesticide. At 50 mg L⁻¹ concentration of MCP, the growth of MCP1 was minimum 0.33 mg ml⁻¹ which was increased 2.5 folds i.e 0.88 mg ml⁻¹ at concentration 150 mg L⁻¹ but further increases in MCP led to decrease in the growth of fungi after 5 days of incubation (Figure 5). Further, the growth of MCP1 increased to 1.34 mg ml⁻¹ at 150 mg L⁻¹ after 10 days of incubation and started to decline afterwards. This could be due to nutrient limitation or due to product accumulation N-methylacetoacetamide (Gundi *et al.*, 2006).

Therefore, from the above study it was concluded that MCP1 grow optimally at 25-30°C temperature, pH- 8, and 150mg/L concentration of monocrotophos in a period of 10 days incubation.

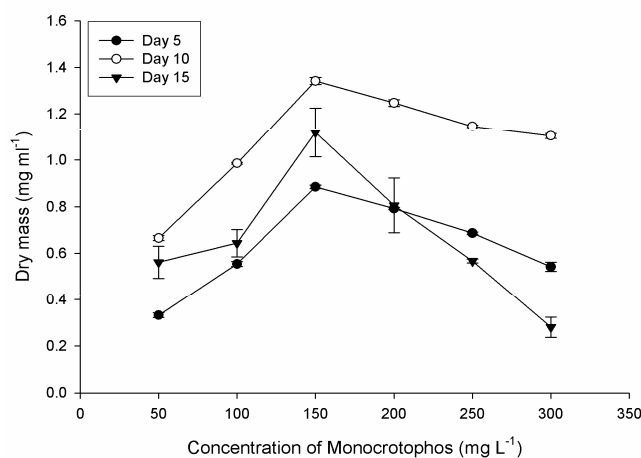


Figure 5:- Effect of different concentrations of monocrotophos on the growth of *Aspergillus niger* in M1 medium (pH-8) at different intervals of incubation. Error Bars indicate standard deviation. Variables were subjected to t-Test and were found significant at P≤0.05 level.

3.4 Growth kinetics of MCP1 under optimized culture conditions

In order to ascertain the viability of fungal isolate MCP1 (7782.10) in monocrotophos containing medium, the growth kinetics was studied. Application of 150 mg L^{-1} concentration of monocrotophos at pH-8, temperature 28°C to the isolated fungal strain resulted in visible increase in the dry mass of fungal mycelia. Data depicted in Figure 6 show that under optimized culture conditions and in the presence of monocrotophos the growth of MCP1 was 0.454 mg ml^{-1} whereas, it was 0.338 mg ml^{-1} in monocrotophos free medium. There was an increase of 37% in the growth, when it was compared with the growth of MCP1 in the absence of monocrotophos. Hence, it was concluded that monocrotophos induced the growth of MCP1 by serving as a source of phosphorus and energy.

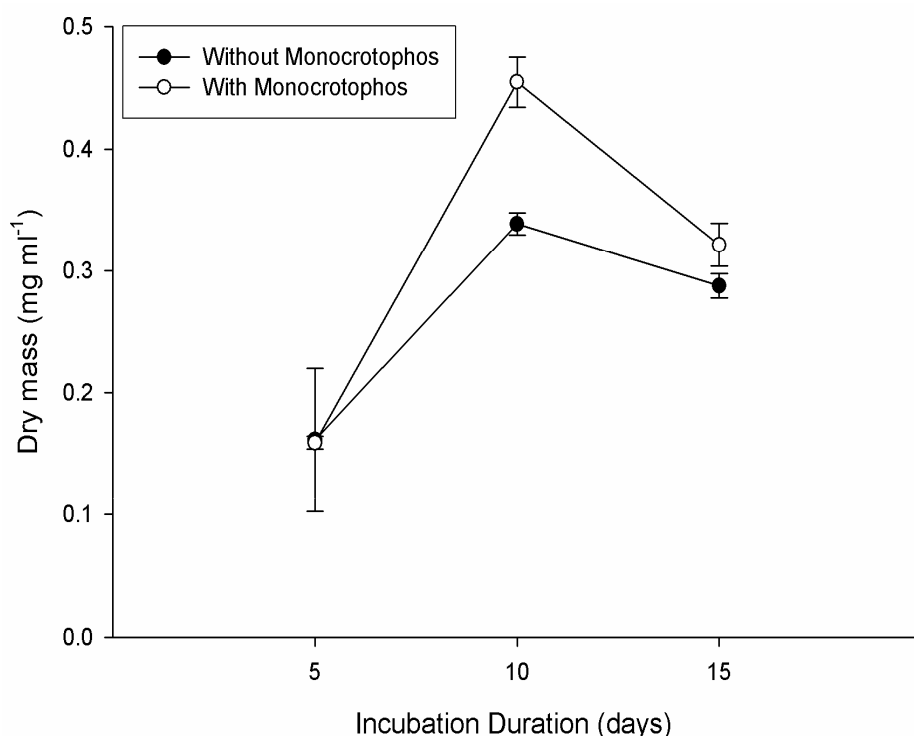


Figure 6: Growth kinetics of *Aspergillus niger* in M1 medium with and without monocrotophos at various intervals of incubation. Error Bars indicate standard deviation. Variables were subjected to t-Test and were found significant at $P \leq 0.05$ level.

3.5 Degradation kinetics of MCP1

In order to find out the half life of monocrotophos in the presence of MCP1, the degradation kinetics was studied. The results of monocrotophos degradation kinetics well correlated with its growth kinetics. It was found that more the growth of MCP1, higher the rate of degradation of monocrotophos. This could be due to utilization of monocrotophos by MCP1 as sole source of phosphorus and additional energy source during its growth period. After 10 days of incubation the recovery of monocrotophos was 9.7% i.e 14.45 mg L^{-1} from its initial applied concentration 150 mg L^{-1} and the percentage of degradation of monocrotophos was found to be approximately 90%. The degradation constant K_{deg} calculated from straight line curve was 0.009212 and half -life was 75.87 hrs (Figure 7). Whereas, in control sample the kinetic constant K_{deg} was 0 and thus the half life was infinite. Hence, it was concluded that MCP1 is an efficient degrader of monocrotophos.

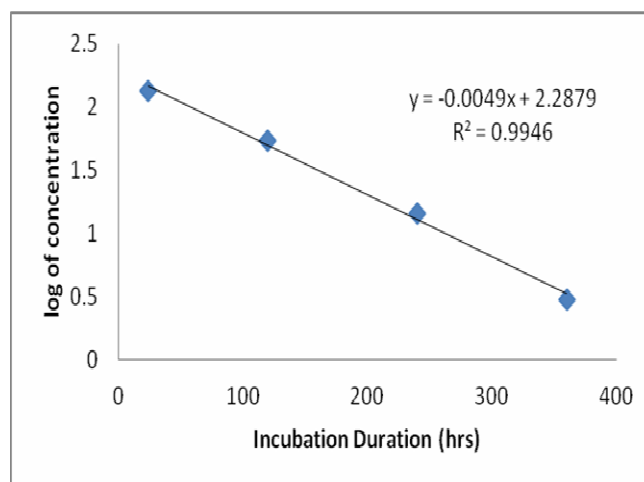


Figure 7: Degradation kinetics of monocrotophos by *Aspergillus niger* in M1 medium under optimized culture conditions. Straight line equation shows that degradation of monocrotophos follows first order kinetics. Variables were subjected to t-Test and were found significant at $P \leq 0.05$ level.

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4. References

1. Anonymous, (2001), Year Book in Pesticide Information. January–March Issue, pp. 6–7. New Delhi: Food and Agricultural Organization (FAO).
2. Bhadbhade. B.J, Dhakephalkar. P.K, Sarnaik. S.S and Kanekar. P.P., (2002a), Plasmid-associated biodegradation of an organophosphorus pesticide, monocrotophos, by *Pseudomonas mendocina*. *Biotechnology Letters*, 24, pp 647–650.
3. Bhadbhade. B.J, Sarnaik. S.S, Kanekar. P.P., (2002b), Bioremediation of an industrial effluent containing monocrotophos, *Current Microbiology*, 45, pp 345–349.
4. Doyle. J.J and Doyle. J.L., (1987), A rapid DNA isolation procedure for small quantities of fresh leaf tissue, *Phytochemical Bulletin*, 19, pp 11-15.
5. Hasan. H.A., (1999), Fungal utilization of organophosphate pesticides and their degradation by *Aspergillus flavus* and *A. sydowii* in soil, *Folia Microbiologica*, 44, pp 77–84.
6. Horne. I., Sutherland T.D., Harcourt R. L., Russell R.J. and Oakeshott J.G., (2002), Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate, *Applied Environmental Microbiology*, 68, pp 3371–3376.
7. Kathryn .A. Wheeler., Beverly. F .Hurdman., Pitt J.I., (1991), Influence of pH on the growth of some toxigenic species of *Aspergillus*, *Penicillium and Fusarium*, *International journal of food microbiology*, 12(2-3), pp 141-149.

8. Megharaj. M., Venkateswarlu. K, Rao A.S., (1987), Metabolism of monocrotophos and quinalphos by algae isolated from soil, *Bulletin of Environmental Contamination and Toxicology*, 39, pp 251-256.
9. Ramanathan M.P. and Lalithakumari. D., (1999), Complete mineralization of methylparathion by *Pseudomonas sp.* A3, *Applied Biochemistry and Biotechnology*, 80, pp 1-12.
10. Khallil. A.M.A and Omar. S.A., (1993), Influence of the insecticide Dimethoate on some metabolic activities of five zoosporic fungi, *Journal of Basic Microbiology*, 33(6), pp 405-411.
11. Omar S.A., Moharram A.M., Abd-Alla M.H., (1993), Effects of an organophosphorus insecticide on the growth and cellulolytic activity of fungi, *International biodeterioration and biodegradation*, 31(4), pp 305-310.
12. Singh. S., Singh. D.K., (2003), Utilization of monocrotophos as phosphorus source by *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* sub sp. *iniosidiosum* SBL 11, *Canadian Journal of Microbiology*, 49, pp 101–109.
13. Tomlin. C., (Ed.) (1995), *The Pesticide Manual*, 10th ed. British Crop Protection Council and Royal Society of Chemistry.
14. Vig. K., Singh. D.K., Agarwal. H.C., Dhawan. A.K., Dureja. P., (2001), Insecticide residues in cotton crop soil. *Journal of Environmental Science and Health B*, 36, pp 421–434.
15. Vijay. A.K.B. Gundi., B.R. Reddy., (2006), Degradation of monocrotophos in soils, *Chemosphere*, 62, pp 396–403.
16. Yun. Long. Yu., Hua. Fang., Xiao. Wang., Xiao. Mao. Wu., Min. Shan. and Jing. Quan. Yu., (2006), Characterization of a fungal strain capable of degrading chlorpyrifos and its use in detoxification of the insecticide on vegetables, *Biodegradation*, 17, pp 487–494.