Journal of Environmental and Occupational Science
www.jenvos.com
DOI: 10.5455/jeos.20141214124733

Original Research

ScopeMed

Degradation of monocrotophos in soil, microbial versus enzymatic method

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Received: November 04, 2014
Accepted: December 14, 2014
Published: January 14, 2015

ABSTRACT

Aim: The present study has been designed to compare two commonly used methods viz. microbial and enzymatic for the degradation of monocrotophos (MCP) in sandy loam soil of Rajasthan. **Materials and Methods:** Methodology involves the use of molecularly characterized fungal strain Aspergillus niger JQ660373. For the microbial method spore suspension (1 × 108 spores/ml) was used as inoculum. Enzymatic method employ the use isolated, purified extracellular fungal hydrolases as inoculum. %age of MCP degradation was assessed in the form of residual MCP concentration and hence degradation kinetics was calculated. The results were confirmed by using analytical tools, high pressure thin layer chromatography (HPTLC) and Fourier transform infrared spectroscopy (FTIR). **Results:** The results of this study demonstrated that extracellular fungal hydrolase composed of two different subunits of 33 and 67 Kd. Both subunits synergistically degrade MCP. Residual MCP concentration was found to be 64.94 ± 0.42 and 16.95 ± 0.55 μg/ml after 15 days of incubation for microbial and enzymatic method, respectively. Degradation of MCP followed first order kinetics with rate constants of 0.002 and 0.136/day and hence the calculated half-life was found to be 12.64 and 5.14 days, respectively. HPTLC chromatograms clearly indicate the significant decrease in the standard MCP peak (rf 0.19-0.21) with increasing incubation duration. Molecular insight of MCP degradation was studied by FTIR. Degradation proceeded with hydrolytic cleavage of MCP resulting in the formation of inorganic phosphates (—PO4). **Conclusion:** The study concludes that enzymatic method of degradation was more efficient than the microbial method.

KEY WORDS: Degradation, Fourier transform infrared spectroscopy, high pressure thin layer chromatography, organophosphate, pesticide, soil

INTRODUCTION

Soil microorganisms participate in a myriad of naturally occurring processes [1-6]. Extensive use of agrochemical in soil led to adverse effects on different soil properties as well as on the microorganisms [7-13]. Considerable evidence indicates that changes in the composition of a microbial community can be used to predict and dictate alteration in soil quality [14,15].

Monocrotophos (MCP) (Dimethyl (E)-1-methyl-2 methyl carbamoyl vinyl phosphate) is a broad spectrum organophosphate (OP) insecticide widely used for agricultural and household purposes, which works systemically and on contact [16].

Owing to the extensive use of MCP in agriculture, there is a high risk of human exposure to this chemical. MCP exhibits high oral and moderate dermal toxicity. The toxicologically relevant mode of action is the inhibition of AChE. The toxicity of metabolites of MCP is comparable with the parent compound [17,18].

Microbial metabolism is probably the most important pesticide degradative process in soils [19-22].

Metabolic reactions, such as N-demethylation, O-demethylation, hydroxylation of N-methyl groups and cleavage of the phosphate-crotanamide linkage, occur during the metabolism of MCP by microbial cultures and in soils, with the formation of O-desmethyl MCP, monomethyl phosphate, dimethyl phosphate, N-methylacetoacetamide and N-methylbutyramide [23-29].

Extracellular protein also plays a major role in the degradation of pesticides [30-34].

Enzymatic mode of degradation is preferable due to their ability to perform same function as many harsher chemicals, but at a neutral pH, a moderate temperature, and their biodegradable nature [35-37]. Their specificity is another attractive feature for using them for the purpose of degradation i.e. enzymes having less specificity can degrade many different pesticides in the environment and high specificity enzymes can degrade that particular enzyme from the mixture [37-43].

MCP degradation is very well-studied in liquid culture medium by means of different bacteria [27,28,44-46], algae [47], fungi [48-51] and extracellular fungal enzymes [52]. However, the biodegradation of MCP in soil remained a mystery. Although Gundi and Reddy, [29] had reported the degradation of MCP in two naturally occurring soil samples. Jia *et al.*, [53] had also reported the degradation of MCP in the fluovo aquic soil by using *Paracoccus* sp. In view of its indiscriminative, intensive

and continuous use in Indian agriculture, kinetics of MCP degradation in soils is a need of the hour. Therefore, this study has been designed to compare the degradation process of MCP by *Aspergillus niger* JQ660373 using two different methods *viz.* microbial and enzymatic.

MATERIALS AND METHODS

Chemicals

MCP of analytical grade (99.5% purity) was procured from Sigma and its stock solution of 1 mg ml^{-1} was prepared in ethanol. All the other chemicals employed in the present study were of analytical grade and purchased from Himedia and Rankem, India.

Soil samples were sterilized at 200°C for 24 h in a hot air oven to inhibit the growth of microorganism. Thereafter, the samples were cooled down to room temperature for further use.

Physico-chemical Analysis of Soil

Physico-chemical properties of soil were analyzed using standard methods (Jain *et al.*, 2014). Type of particles in soil, organic carbon percentage, exchangeable cations and pH were as following-

Particle size: Clay- 8.9%, silt- 5.3%, sand- 85.8%, Texture class- Loamy sand

Organic carbon: 0.33%, exchangeable cations: Ca-7.5 m.e/100 g soil, Mg-2.00 m.e/100 g soil, Na-0.65 m.e/100 g soil, K-0.039 m.e/100 g soil, Soil reaction (pH) - 7.88.

Experimental Setup

Experiment was set in triplicates. 50 g of soil sample was weighed by using physical balance. Each of the samples was put in 250 ml erylmener flask.

- Microbial method: A. *niger* JQ660373 was suspended in 1 ml, 0.85% saline to make a cell suspension of 1×10^8 cells per ml and this suspension was inoculated in 50 g of sterilized soil containing 150 $\mu g\ g^{-1}$ MCP Jain *et al.* [50].
- Enzymatic method: A. *niger* JQ660373 at a concentration of 1×10^8 cells per ml was suspended in modified czapekdox medium (CZM; Composition-sucrose, 30 g; $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; glucose,10 g; $FeCl_3$,10 mg; $BaCl_2$, 0.2 g; $CaCl_2$, 0.05 g per liter and was supplemented with MCP (150 μg/ml) as a sole source of phosphorus) and incubated for 10 days in an orbital shaking incubator at 28 ± 2°C. After the completion of incubation time the flasks were withdrawn and the filrate was checked for the presence of OP hydrolases (OPH) enzyme by the assay procedure described by Jain and Garg, [52]. Extracellular enzyme was purified by AmSO4 precipitation, dialysis and G- 100 chromatography. Partially purified enzyme (G-100) was further purified by ion exchange chromatography. The concentration of the purified enzyme was optimized by estimating the enzyme activity at different protein concentrations i.e., 50, 100, 150, 200, 250 and 300 μg ml^{-1}. 1 ml (optimum concentration) of the purified enzyme fraction was inoculated in soil.

Samples were incubated at temperature 30°C under static culture conditions for 30 days. The water holding capacity of soil was maintained at 60%. The flasks were incubated in dark to rule out the possibility of photo degradation. Effective antibiotics, streptomycin and penicillin (30 mg/kg), were added in the sterile soil to avoid any bacterial contamination.

Control sample containing sterile soil + MCP (150 $\mu g/g$) was prepared simultaneously. At regular time interval of 5 days each flask was taken out and residual MCP was extracted and calculated.

Degradation Study

Residual MCP was extracted with equal amount of ethyl acetate (1 ml ethyl acetate for 1 g soil). The solvent fraction was pooled. The solvent was allowed to evaporate and residues were dissolved in 2 ml ethyl acetate. Clean up of residual MCP was done by using florosil column and cyclo hexane: Ethyl acetate (1:1 v/v) as solvent system. Again the purified fractions were collected and solvent was evaporated to dryness and residue was re-dissolved in minimum amount of ethyl acetate. Purified samples of MCP were stored at −20°C and used for further quantification by spectrophotometer. The results were confirmed by high pressure thin layer chromatography (HPTLC) and Fourier transform infrared spectroscopy (FTIR).

Residual MCP was quantified spectrophotometrically at 254 nm. The concentration of the remaining MCP was then calculated using molar absorption coefficient. From the residual MCP concentration % of MCP degradation was calculated using the formula

$$\%\ \text{of MCP Degradation} = \frac{\text{Concentration of MCP in control - Concentration of MCP in control}}{\text{Concentration of MCP in control}} \times 100$$

Degradation kinetics of MCP was studied as per the method used by Jain *et al.*, [50].

Analytical Methods

HPTLC (CAMAG Linomat 5, Switzerland) was used for analysis of samples. The aqueous samples from MCP degradation flasks were extracted with ethyl acetate. The samples (20-25 ml) were inoculated on silica gel (60 F 254) TLC plates (E. Merck, India) using an applicator system. Twin trough glass chamber (20 × 20 cm) containing dichloromethane: Methanol (9:1) as the mobile phase was used for development of the chromatogram. Spots were detected using a CAMAG TLC scanner-3 at the wavelength of 254 nm using a deuterium lamp.

Infrared spectra of the parent compound (MCP) and sample after fungal degradation were recorded at room temperature (25°C) in the frequency range of 4000-400 cm^{-1} with a FTIR spectrophotometer (8400 Shimadzu, Japan, with Hyper IR-1.7 software for Windows) with a helium neon laser lamp as a source of infrared radiation. Aqueous samples (20 days of incubation) from MCP degradation flasks were extracted with ethyl acetate and solvent was evaporated using a rotary vacuum evaporator (Rotavapor R.214, Bu chi, Switzerland). The contents were re-dissolved in acetone. A drop of this sample in acetone was placed in between two sodium chloride discs, after cleaning with ethyl acetate. The background spectrum for acetone was corrected from the sample spectrum.

Statistical Analysis

The statistical analysis was performed using Statistical Package for the Sciences System. The variables were subjected to Student's *t*-test and one-way ANOVA.

RESULTS

Purification Profile of Extracellular hydrolase from *A. niger* JQ660373

A. *niger* was grown in CZM medium containing 0.5 g/L KH_2PO_4 for the production of extracellular enzyme secreted by the isolates. After 10 days of incubation period the medium was checked for the presence of extracellular enzyme. Intracellular hydrolase activity was also checked. It was found that A. *niger* possessed potent extracellular hydrolase activity (10.34 ± 0.2 U and Sp. activity 3.42 U/mg) in comparison to intracellular activity (2.31 ± 0.01 U and Sp. Activity 1.12 U/mg). Therefore, the secreted fraction was collected and was partially purified by ammonium sulfate precipitation and gel filtration on sephadex G- 100. The data for the purification of the extracellular OPH was summarized in Table 1.

It shows that the purified enzyme showed a purification fold of 37.04 ± 3.07 with a percent yield of 55.37 ± 0.16. The enzyme activity was found to be 450.02 ± 2.64 U with the protein content of 5.26 ± 0.35 μg/ml. Hence, the calculated specific activity of purified enzyme was 85.69 ± 6.29 U/mg. Purified OPH composed of two different subunits. The molecular mass of each of the subunits as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were found to be approximately 33 Kda and 67 Kda [Figure 1].

OPH Concentration

As depicted in the previous section hydrolase enzyme composed of two different subunits of different molecular weights i.e. 33 and 67 Kd, respectively. Therefore both these purified subunits fractions (individual as well as combined) were tested for their optimum concentration for the efficient degradation of MCP. The enzyme activity of all the three different protein fractions followed an L-shaped pattern. It is evident from Figure 2 that 150 μg/ml is the optimum concentration among all the tested enzyme concentrations. Therefore, this selected concentration was used for further degradation study. Further it was also

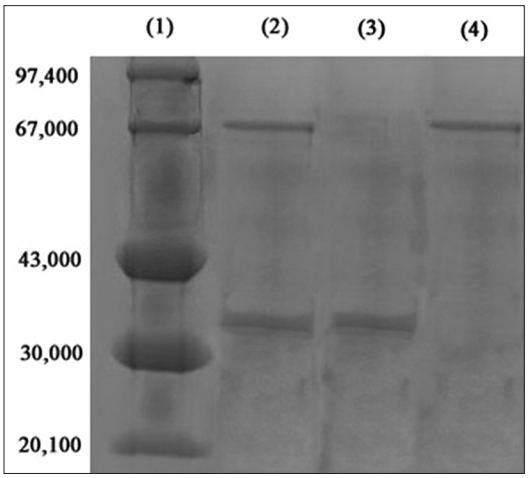

Figure 1: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the partially purified extracellular enzyme from five isolated strains. Lane 1, marker proteins (from top to bottom) phosphorylase *b* (Mr, 97,400), bovine serum albumin (Mr, 67,000), ovalbumin (Mr, 43,000), carbonic anhydrase (Mr, 30,000) and Soyabean Trypsin Inhibitor (Mr, 20,100), respectively; lane 2, purified enzyme G-100, lane 3, low molecular weight protein diethylaminoethanol (DEAE) CL6B, lane 4, high molecular weight protein DEAE CL6B. The gel was stained for protein with Coomassie brilliant blue R-250 and destained in methanol-acetic acid-water (7:2:1)

Table 1: Purification profile of extracellular fungal hydrolases from *A. niger* JQ660373

	Total activity (U)	Total protein (mg)	Sp. Activity (U/mg)	Purification fold	Yield %
Crude					
OPH	812.63±2.81	351.33±5.97	2.31±0.034	1	100
Extracted (PPT)					
OPH	152.13±3.36	112.21±3.79	1.35±0.01	0.58±0.04	18.72±0.2
Supernatant					
OPH	553.73±3.52	221.9±8.45	2.49±0.11	1.07±0.03	68.14±0.67
G-100					
OPH 33	320.12±4.46	5.26±0.35	60.85±3.46	26.34±2.34	39.39±1.16
OPH 67	450.02±2.64	5.26±0.35	85.69±6.29	37.04±3.07	55.37±0.16
DEAE CL6B					
OPH 33	297.14±5.52	2.15±0.01	138.2±2.42	59.82±1.14	40.32±1.34
OPH 67	425.11±4.46	2.26±0.04	188.1±3.36	81.42±2.34	52.31±1.13

A. niger: Aspergillus niger, DEAE: Diethylaminoethanol, OPH: Organophosphate hydrolases

observed that combined fraction of both subunits showed high efficiency towards MCP degradation and hence showed high enzyme activity. Therefore, this fraction was selected for the degradation of MCP.

Degradation of MCP in Soil

MCP degraded rapidly in the soil by both the applied methods *viz.* microbial as well as enzymatic as shown in Figure 3-5 for spectrophotometric, HPTLC and FTIR respectively.

Spectrophotometric analysis

Microbial method followed a long log phase whereas in case of enzymatic method a long exponential phase of 25 days was followed by a short log phase. Residual MCP concentration left after 30 days of incubation by microbial and enzymatic methods were found to be 19.35 ± 0.13 and 0.26 ± 0.03 μg/ml of respectively. The percentage of degradation in the control sample was found negligible (16.7%) which exclude the possibility of auto degradation of MCP under controlled conditions.

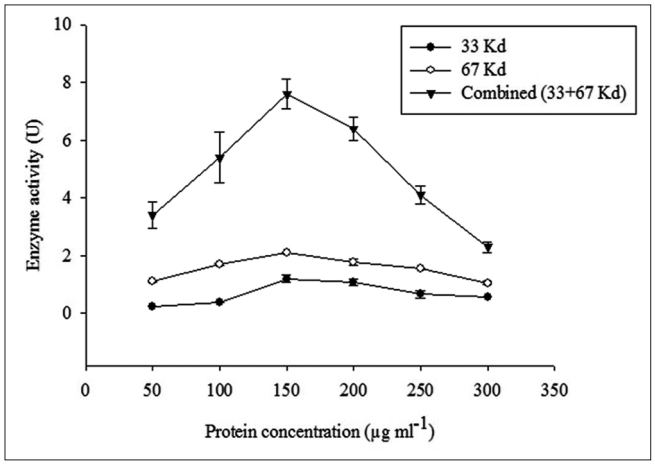

Figure 2: Optimization of hydrolase activity at different protein concentrations viz. 50, 100, 150, 200, 250, 300 µg/ml of protein for different subunits of purified M1 viz. 33, 67 and both combined. Error bars indicates standard deviation

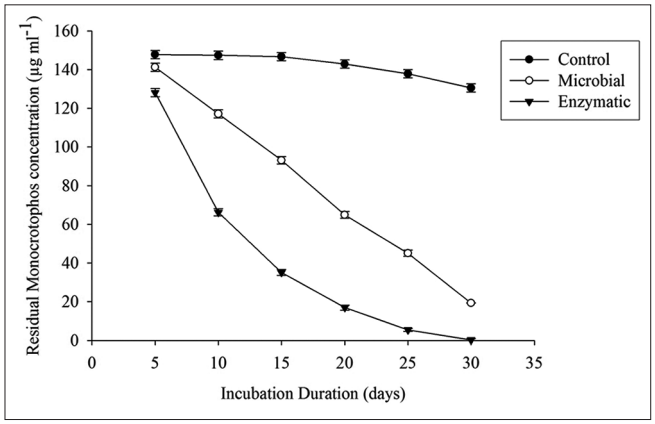

Figure 3: Residual monocrotophos concentrations (µg/ml) in control, microbial and enzymatic methods in soil at different time intervals. Error bars indicates standard deviation

HPTLC

The results of the spectrophotometric determination of residual MCP concentration were further confirmed by HPTLC analysis. The results depicted in Figure 4 clearly indicated that increasing the incubation period decreases the peak of MCP. Standard peak of MCP was observed at 0.19-0.21 rf value. After 25 days of incubation no peak for MCP was seen in enzymatic sample which indicates complete degradation of MCP from that sample. Though, a short peak was seen in case of microbial method. In addition to the standard MCP peak some other peaks were also observed which were characteristic of the by-products of MCP degradation. However, these were no identified as much emphasis was given on parent compound degradation.

FTIR

FTIR spectrum of MCP degradation as shown in Figure 5 clearly indicates hydrolytic cleavage of MCP with the formation of inorganic phosphates ($-PO_4$). Peaks at 3406 cm^{-1} and 3263 cm^{-1} as visible in control samples were characteristic for vinyl bonds. These peaks were completely reduced in the spectrum after degradation. Whereas new peaks at 1386.03 and 1443.95 cm^{-1} observed were characteristic of inorganic phosphates. Some other peaks at 1058.30 and 1046.66 cm^{-1} were also observed which were characteristic for aliphatic amines. Some new peaks at a range of 1543.33-1706.06 cm^{-1} characteristic for −NH or −NO were also pragmatic. It is also depicted from the figure that the number of peaks increased with enzymatic method when compared to fungal as employed in the present study. It also signifies the efficiency of enzymatic method towards degradation of MCP in sandy loam soil.

Degradation Kinetics

Degradation kinetics of MCP followed straight line equation [Figure 6]. Hence the rate constant and half-life was calculated by using first order kinetics. The kinetic rate constants were observed to be 0.002 and 0.136 day^{-1} and hence the calculated half-life of MCP was 12.64 and 5.14 days respectively for microbial and enzymatic methods, respectively. The half-life of MCP was significantly high (infinite) in control sample.

DISCUSSION

Pesticides pose great threat on environment. The first and foremost target of pesticide usage is, soil microbial flora and fauna. Though they are targeted, but only a few studies have been conducted to explore the pattern of degradation of these hazardous chemicals from soil [29,53]. Most soils are contaminated by naturally occurring harmful or toxic elements, pesticides, to some or more extent [54]. Paddy soil exhibited more persistence of MCP, followed by rice, leaves and water [55,56].

Therefore, this study is a comparative effort to find the degradation pattern of MCP in soil by microbial and enzymatic methods.

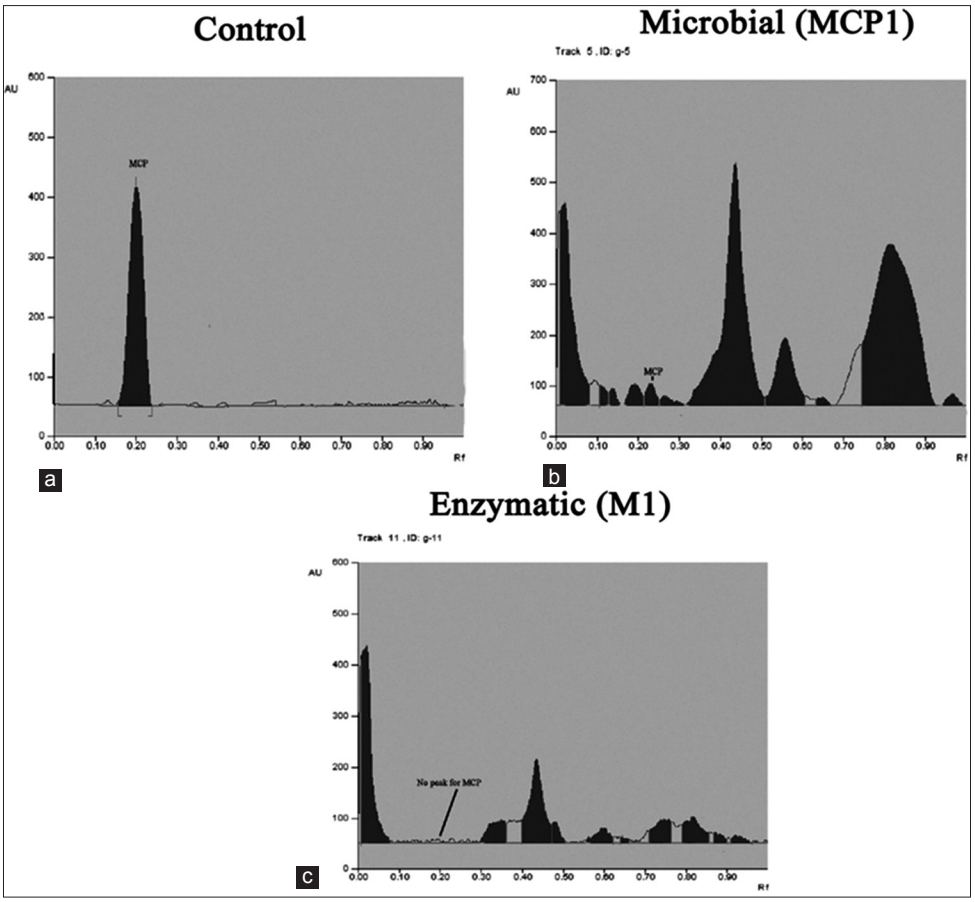

Figure 4: High pressure thin layer chromatography chromatograms of residual monocrotophos concentration in soil by (a) control, (b) microbial and (c) enzymatic after 25 days of incubation

Molecularly identified fungal strain A. *niger* JQ660373 was used as a candidate microorganism in the present study [50]. 1 ml each of spore suspension as well as purified hydrolase fraction was inoculated in soil in dark. At regular interval of 5 days residual MCP was extracted and quantified. It was observed that MCP rapidly degraded from soil samples and the rate of MCP degradation was higher for enzymatic method than the microbial method.

First, the fungal strain was tested for its intra as well as extracellular enzyme content. It was found that the strain possessed high extracellular hydrolase activity. The high hydrolase activity fraction was then purified by G-100 chromatography and SDS-PAGE analysis depicted that the enzyme was composed of two subunits of different molecular weight [52]. Both hydrolase fractions were then separated by ion exchange chromatography.

Both fractions, individually as well as combined were tested for their MCP degradation efficiency. It was observed that the combined fraction degrade MCP very effectively. There may be some synergistic association between these two fractions for the degradation of MCP.

To the best of our knowledge, it is the first study comparing the degradation rate of MCP by microbial and enzymatic methods, but there a number of individual studies of MCP degradation by microorganisms.

In our study, 87.1 and 99.85% MCP was degraded in sterile soil within 30 days of incubation by microbial and enzymatic methods respectively. Gundi and Reddy [29] reported 96-98% degradation of MCP in black versitol and red alfinsol soil at 10 and 100 $\mu g/g$ concentration of pesticide.

However, Jia *et al.*, [53] had reported that the addition of *Paracoccus* sp. M-1 (10^6 CFU/g) to fluvo-aquic soil and a high-sand soil containing MCP (50 mg/kg) resulted in a higher degradation rate than that obtained from non-inoculated soil.

Lee *et al.*, [57], had demonstrated that MCP decayed rapidly within 3 days in non-sterilized (by mixture of microorganism) in hanford sandy loam soil against 30 days in sterilized soil. Faster disappearance of MCP from soil sample (by mixture of microorganism) can be attributed to the participation of soil microorganism in the degradation of MCP as demonstrated by other studies [28,44,47,58]. The amount of MCP dissipated from control soils in the present study was not considerable in this study, which excludes the possibility of auto degradation of MCP. Longer persistence of MCP in fumigated and sterile soils/aqueous systems than

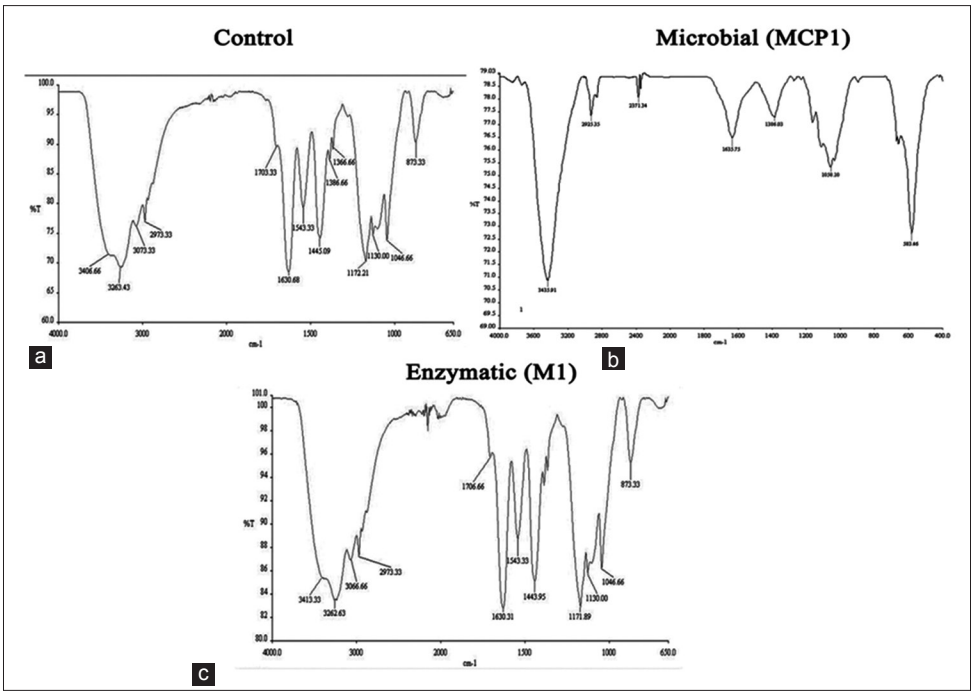

Figure 5: Fourier transform infrared spectroscopy spectrum of monocrotophos degradation by (a) control, (b) microbial and (c) enzymatic after 30 days of incubation

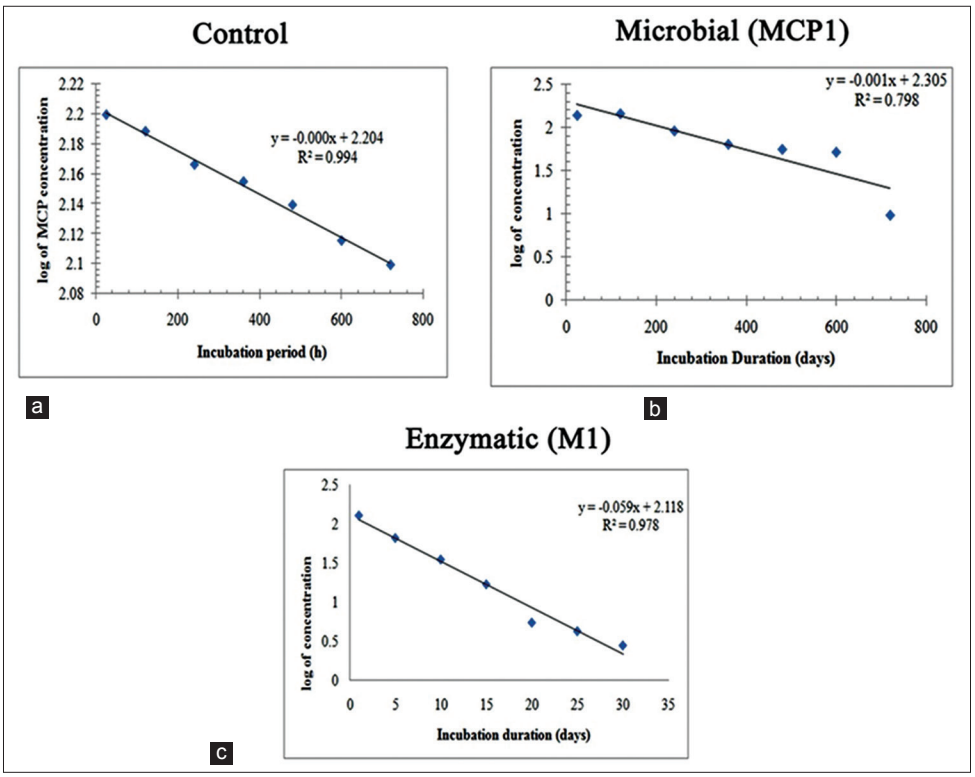

Figure 6: Degradation kinetics of monocrotophos (MCP) by (a) control, (b) microbial (MCP1) and (c) enzymatic (M1) methods in soil at different incubation periods. Straight line equation shows that degradation of monocrotophos follows first order kinetics

in corresponding non-sterile and non-fumigated systems was also reported by other researchers [59-63]. Gundi and Reddy, [29] had reported that repeated applications of MCP to soils failed to accelerate the rate of degradation under *in vitro* conditions.

MCP readily undergo photo degradation in soils causing approximately 40-50% of total degradation [57,61]. Therefore, in the present study samples are incubated in dark, which ruled out the possibility of photo degradation, resulting in larger half-life in control.

Glenn and Gold [30] had also suggested that extracellular protein plays a major role in the biodegradation of pesticides. Concurrent with our study other researchers had also reported the routinely use of, culture filtrates of fungi as crude enzyme preparations for enzymatic degradation of organic compounds including OP compounds [31-34]. On the other hand, Tarafdar *et al.*, [31] had also reported no significant correlation between biomass production and organic P mineralization by fungi isolated from desert soils.

It was found that the rate of MCP degradation was higher in the initial period and then there was observed a lag phase in which the degradation seemed to stop. This might be due to the utilization of MCP as a substrate by released extracellular enzymes, higher the substrate, more is its utilization or might be the accumulation of toxic metabolite of MCP inhibited the degradation. HPTLC results also demonstrated that degradation of MCP is followed by production of different metabolites as evident by different peaks in the chromatograms. These metabolites were found to be stable as the peak height does not decrease.

Gundi and Reddy, [29] had reported that degradation of MCP resulted in the formation of N-methyl acetoacetamide, which is quite toxic and accumulates in soil for longer period.

Lamar and Dietrich, [64] had conducted a field study to examine the ability of two white rot fungi, *Phanerochaete chrysosporium* and *Phanerochaete sordida*, to degrade pentachlorophenol (PCP). These were able to degrade 88-91% of the PCP in 6.5 weeks. But, instead of complete mineralization it was transformed to its intermediate products which remained tightly bound to soil particles and hence, were not available for further degradation. Ricotta *et al.*, [65] had also reported slow mineralization of PCP by a white rot fungus under laboratory conditions. This condition is quite serious because these intermediates may be more toxic than the original compound and must also be degradable using a feasible option. Oxygen restriction (anaerobic conditions) may also be the possible reason for the partial degradation of pesticides with the formation of toxic intermediates [66].

In concurrence to the results Kearney, [22] had stated that fungal bioremediation depends upon the environmental conditions as temperature pH, nutritional status oxygen levels and moisture in soil. These conditions vary and may not always be optimal for the growth of microorganism or for the production of extracellular enzyme for transformation of pollutants [67]. Therefore, the kinetics of pesticide degradation is commonly biphasic with a very rapid degradation rate in the beginning followed by a very slow prolonged dissipation with formation of quite stable residues. This might be due to strong sorption of pesticide to soil, which decreased its bioavailability or may be due to low temperature inefficient for microbial degradation of contaminants, particularly in Northern parts of Europe and North America. Leaching of pesticide to deeper layers of soil may also decrease its rate of degradation [66,68].

Degradation kinetics of MCP had been mostly studied in naturally occurring soil. In the present study it is for the first time that the degradation kinetics is being studied by means of fungal suspension or by fungal hydrolases. Moreover, it is the first study regarding the enzymatic degradation of MCP in soil.

Degradation of MCP followed first order kinetics [51]. The kinetic rate constants were observed to be 0.002 and 0.136/day and hence the calculated half-life of MCP was 12.64 and 5.14 days respectively for microbial and enzymatic methods respectively.

The degradation of MCP at both concentrations in black vertisol and red alfinsol soils was rapid accounting for 96-98% of the applied quantity and followed the first-order kinetics with rate constants (k) of 0.0753 and 0.0606/day and half-lives ($t_{1/2}$) of 9.2 and 11.4 days, respectively [29].

Bhalerao and Puranik, [48] had reported the half-life of MCP in both fluvo-aquic soil and high-sand soil, for about 10 days respectively, which indicated it's less persistent nature. Similarly, Lee *et al.*, [57] had recovered <6% and 21% of the applied MCP concentrations from the Hanford soil after 16 days of aerobic and anaerobic incubation with the rate constants of 0.17 and 0.09/day, with the half-lives of 4 and 8 days, respectively.

A granular formulation (5%) of MCP, showed a half-life of 10 days in an Indian clay soil when applied at a rate of 1.5 g a.i. ha^{-1} [69]. The same was reported by other scientists with a half-life of 3-4 days in horticultural and vegetable fruits [70,71].

Molecular insight of MCP degradation clearly indicated the cleavage of vinyl bond with the formation of new peaks characteristic of $-PO_4$ in the FTIR spectrum. The results were concurrent with the study of eminent scholars [48,51,52] which also correlated degradation of MCP by reduction of its peak when compared to that in the standard chromatogram by hydrolytic cleavage.

Enzymatic degradation of MCP from sandy loam soil was found to be very fast. Therefore, the study concludes that extracellular purified hydrolases proved to be the most efficient tool for the degradation of MCP from soil.

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Source of Support: Nil, Conflict of Interest: None declared.