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Molecular Characterization of Monocrotophos Hydrolyzing Soil Fungal Strain *Penicillium aculeatum* ITCC 7980.10

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A B S T R A C T

Pesticides poses great threat to the environmental sustainability by affecting its microbial flora and fauna. Fungi are well known for the secretion of extracellular hydrolases for the degradation of these hazardous chemicals from its ambient environment. Hydrolase secreting soil fungi *Penicillium aculeatum* ITCC 7980.10 was selected for the study of its monocrotophos degrading efficiency under in vitro conditions in phosphorus free liquid culture medium. Fungal strain was molecularly characterized by 18S rDNA analysis as *Penicillium aculeatum* JQ660374. Monocrotophos degrading efficiency of the isolate was studied under optimum conditions each, at an interval of 5 days for 15 days incubation period. The isolated strain possessed meagre extracellular phosphatase activity 28.33 ± 0.40 U which resulted in the formation of 614.62 ± 0.38 µg ml^{-1} inorganic phosphates. Degradation process was studied by spectrophotometric analysis at 254nm, HPTLC and FTIR. Spectrophotometric analysis revealed 98.88% degradation of monocrotophos with a prominent decrease in the standard peak of monocrotophos (standard rf 0.19- 0.21) within 15 days of incubation as evident by HPTLC chromatograms. Molecular mechanism as studied by FTIR analysis revealed hydrolytic cleavage of vinyl bond with the formation of PO^{-4}. Degradation of monocrotophos followed first order kinetics with the rate constant (k_{deg}) of 0.0115 day^{-1} and half life of 2.53 days. The results of the study conclude that *Penicillium aculeatum* JQ660374 could be used as an efficient candidate for the detoxification of monocrotophos contaminated sites.

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INTRODUCTION

Monocrotophos (Dimethyl (E)-1-methyl-2 methyl carbamoyl vinyl phosphate) (MCP) is a broad spectrum organophosphate insecticide widely used for agricultural and household purposes. It works systemically and on contact [1]. Excessive use of MCP can cause cytotoxic, genotoxic and reproductive disorders in mammals and affects the central nervous system [2]. Foliar application of MCP led to its accumulation in the soil and thus affects the soil properties and microbial flora and fauna. Moreover, leaching of MCP pollutes the surface and/or groundwater, resulting in adverse effects on biological systems as conventional water treatments do not remove soluble pesticides [3]. Therefore, it is very essential to remove this hazardous chemical from the environment.

Biodegradation of MCP has been documented by several researchers as a major tool for its dexotification. Basic metabolic reactions involved in microbial degradation are N-demethylation, O-demethylation, hydroxylation of N-methyl groups and cleavage of the phosphate–crotanamide linkage, [4-6], with the formation of O-desmethyl monocrotophos monomethyl phosphate, dimethyl phosphate, N-methyl acetoacetamide and N-methylbutyramide.

Different microbial species such as algae, *Chlorella vulgaris Beijernik, Scenedesmus bijugates, Synechococcus elongates, Nostac linckia* and *Phormedium tanue* [7], several bacterial strains, *Azospirillum lipoferum* and *Bacillus* sp. [8], *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus*

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megaterium MCM B-423 [9], *Arthrobacter atrocyaneus*, *Bacillus megaterium*, and *Pseudomonas mendocina* [10], *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* subsp. *insidiosum* SBL 11 [11], *Paracoccus* sp. M-1[12], *Pseudomonas and Flavobacterium*[13] are well known for their MCP degradation potential.

Inspite of all these studies, bacteria possess the main disadvantage of their short life span and low tolerance capacity to different environmental stresses. Hence, new technique of using fungi as a tool for the degradation pathway came into existence.

Fungi generally biotransform pesticides and other xenobiotics by introducing minor structural changes to the molecule and rendering it nontoxic. This bio-transformed pesticide is released into the soil, where it is susceptible to further degradation by bacteria [14].

These filamentous organisms however, offer advantages over bacteria in the degradation of hazardous compounds by their oxidizing ability [15-17], tolerance [18], mycelial growth [19] and inexpensive nature [20].

In view of these advantages fungal degradation is considered as a powerful tool for the removal of pesticides from the ambient environment. Different fungal strains such as *Aspergillus* and *Penicillium* sp., [21], *Penicillium corrylophylum*[22], *Aspergillus oryzae* [23], *Aspergillus* sp. [24]*Aspergillus niger* JQ660373 [25], extracellular fungal hydrolases [26] has been proved efficient for the degradation of MCP. In addition to this other organophosphorus (OP) pesticides such as fenitrothion [phosphorothioic acid O,O-dimethyl-O-(3-methyl-4-nitrophenyl) ester] has also been, known to be degraded by *Trichoderma viri*de, *MortierelIa isabeIIina*, and *Saprolegnia parasitica* [27]. Bumpus *et al*., [28]had reported that *P. chryososporium is* able to degrade chlorpyrifos, fonofos, and terbufos to carbon dioxide.

These limited results suggest that the enormous potential of fungi to degrade organophosphorus insecticide is relatively common. Therefore, the present study has been designed to characterize the MCP degrading activity of a hydrolase secreting soil fungi *Penicillium aculeatum* ITCC 7980.10.

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.

Medium

Modified Czapek-Dox medium (CZM) was used as growth medium for the degradation of MCP by fungal strain, which contained 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.

Microbial Culture

Pure culture of *Penicillium aculeatum* ITCC 7980.10 was used for the study [26]. Its spore suspension was made in 0.85% saline to make a cell suspension of 1×10^8 cells per ml.

Molecular Characterization of Microbial Culture

For the molecular characterization, genomic DNA of *Penicillium aculeatum* ITCC 7980.10 was isolated by Cetyl Trimethyl Ammonium Bromide (CTAB) method [29] 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 TATGC–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 automated DNA sequencing facility provided by Banglore Genei, India. The obtained sequence was then compared to those in GenBank using Blast Alignment Tool and deposited to the GenBank, EMBL and DDBJ library.

Degradation of MCP by *Penicillium aculeatum* ITCC 7980.10

For the study of MCP degradation pure culture of *Penicillium aculeatum* ITCC 7980.10 was suspended in 1 ml of 0.85% saline to make a cell suspension of 1×10^8 cells per ml and 100µl of this suspension was inoculated in 125 ml of CZM medium containing 150 mg L^{-1} concentration of MCP and incubated at 28 ± 2˚C for 15 days in an orbital shaking incubator at 90 rpm under aerated culture conditions. Thereafter, MCP was extracted at an interval of 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 residual MCP was estimated at 254nm. The residual amount of MCP was calculated by molar absorption coefficient (calculated value $7.34x10^{-4}$ L $mg^{-1}cm^{-1}$).

Analytical Tools

The results were further confirmed by HPTLC containing dichloromethane: methanol (9:1) as the mobile phase 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 and FTIR analysis with a helium neon laser lamp as a source of infrared radiation.

Kinetics of MCP Degradation

Degradation kinetics of MCP was studied as per the method used by Jain *et al.*,[25].

Estimation of By- products of MCP degradation (alkaline phosphatase and inorganic phosphates)

The assay for enzyme alkaline phosphatase was performed with the mycelia-free CZM broth harvested at various time intervals i.e 2, 4, 6, 8, and 10 till 15 days of incubation. *p*-nitrophenyl phosphate was used as the substrate for enzyme activity as per Raghuramalu *et al*.,[30]. Released p-nitrophenol was determined at 410 nm using a UV–VIS spectrophotometer. Enzyme activity (U) was defined as the amount of enzyme required to form 1 µmole of p-nitrophenol per ml per minute under standard assay conditions.

The amount of soluble inorganic phosphate, a product of MCP degradation was estimated from mycelia free CZM medium by the ascorbomolybdate method of Murphy and Riley [31].

Statistical Analysis

All the experiments were carried out in triplicates and the results are presented as mean ± standard deviation. The statistical analysis was done by using Statistical Package for the Sciences System (SPSS). The variables were subjected to One Way ANOVA and Student t-test.

RESULTS

Molecular characterization of *Penicillium aculeatum* ITCC 7980.10

Genomic DNA of *Penicillium aculeatum* ITCC 7980.10 was isolated by CTAB method. PCR product of the amplified DNA appeared as intense bands on agarose gels. The approximate size of the amplified PCR product was 609 bp. The sequence was subjected to BLAST analysis to find out the nearest homology (Table 1). The sequence and comparative phylogenetic analysis of PCR amplified 18S rDNA gene fragment as shown in Figure 1 confirmed *Penicillium aculeatum* ITCC 7980.10 as closest homologue of *Penicillium aculeatum* (with a distance of 0.15). It was submitted to NCBI Genbank with an Accession No. JQ 660374.

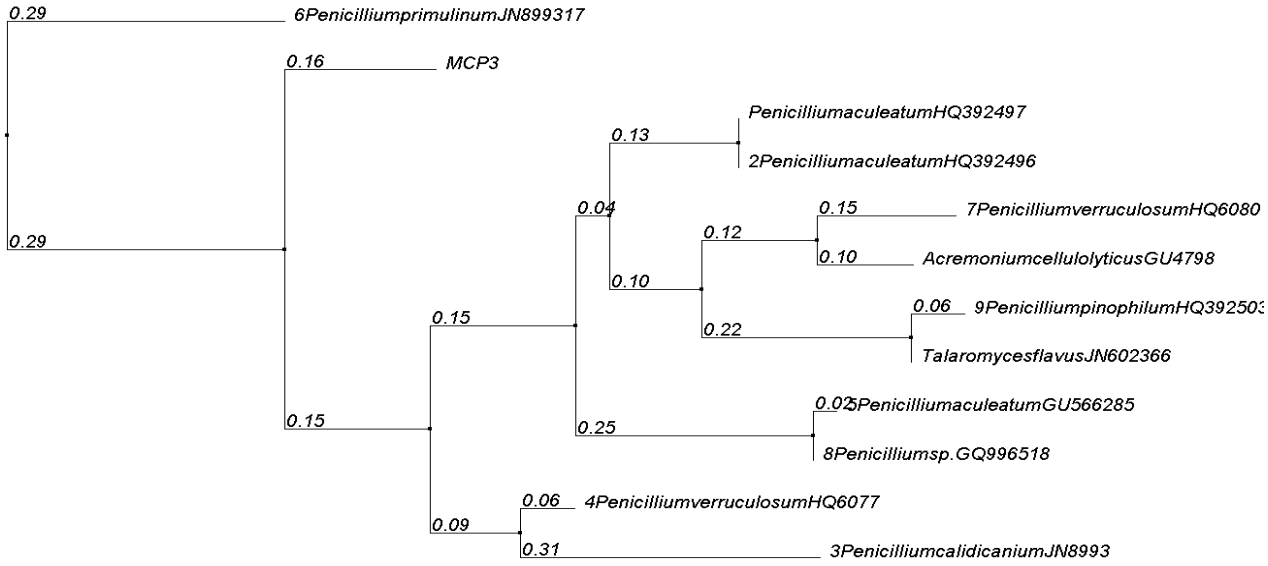

Figure 1. Neighbour joining Phylogenetic Tree constructed between ten closest homologues of *Penicillium aculeatum* ITCC 7980.10 by Clustal W program showing distance among different strains.

TABLE 1. Blast Analysis of *Penicillium aculeatum* 7980.10 (MCP3)

S. No.	Strain	Gen Bank Accession No.	Homologous sequence similarity (%)
1	*Penicillium aculeatum*	HQ392497	98
2	*Penicillium aculeatum*	HQ392496	100
3	*Penicillium pinophilum*	HQ392503	97
4	*Penicillium verruculosum*	HQ607791	95
5	*Penicillium primulinum*	JN899317	94
6	*Penicillium aculeatum*	GU566285	94

Degradation of MCP by *Penicillium aculeatum* JQ660374

The results of the degradation study confirmed that the fungal strain was capable of complete detoxification of MCP. *Penicillium aculeatum* JQ660374 degraded 98.8% of applied MCP concentration (150 mg L^{-1}) within 15 days of incubation under optimized culture conditions as shown in Figure 2. The percentage of MCP degradation increases linearly in the first 10 days with a maximum value of 90.6% at 10^{th} day of incubation. The residual MCP concentration was found to be 2.7 mg L^{-1} after 15 days time interval. Similar to the percentage of degradation, residual MCP concentration decreased rapidly in the first 10 days.

HPTLC

The results of spectrophotometric study were further confirmed by HPTLC. Figure 3 clearly indicate the visible significant decrease in the standard MCP peak with increasing incubation duration. Standard MCP peak was found at rf 0.19-0.21. No such peak was found after 15 days of incubation which clearly indicates the complete detoxification of MCP by *Penicillium aculeatum* JQ660374.

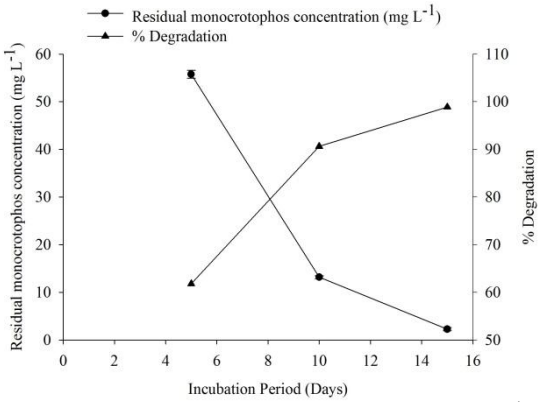

Figure 2. Residual monocrotophos concentration (mg L^{-1}) and percentage of monocrotophos degradation by *Penicillium aculeatum* JQ660374 at various time intervals (days). Error bars indicate standard deviation.

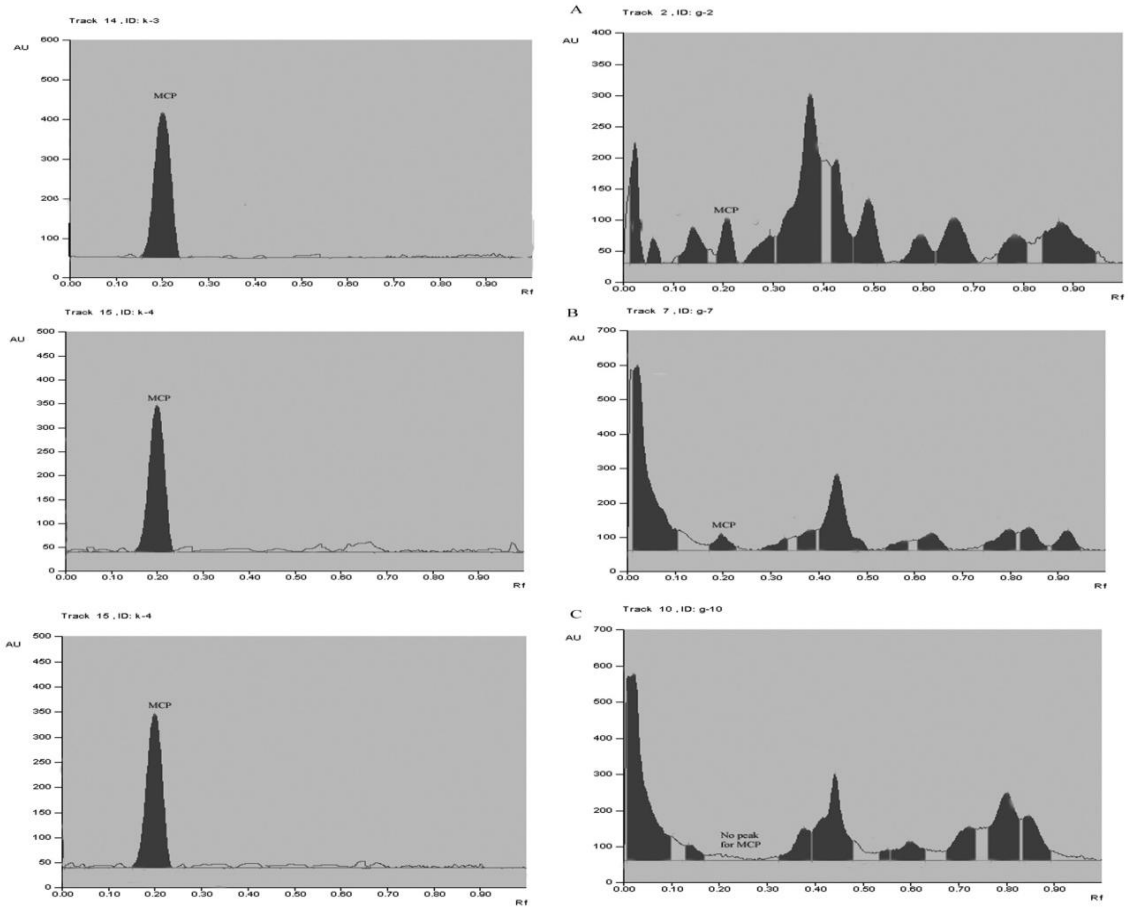

Figure 3. HPTLC chromatograms of residual MCP concentration in CZM medium by *Penicillium aculeatum* JQ660374 at (A) 5, (B) 10 and (C) 15 days of incubation with its respective control.

FTIR

Molecular insight of MCP degradation was studied by FTIR analysis. FTIR spectrum as shown in Figure 4 indicates hydrolytic cleavage of MCP with the formation of inorganic phosphates (–PO4). Peaks at 2925.35cm^{-1} and 2371.34 cm^{-1} characteristic for vinyl bonds were completely reduced in the spectrum after degradation. Whereas a new peak at 1441.12cm^{-1} was observed; characteristic of inorganic phosphates. Some other peaks at 1173.33, 1090.68 and 1046.66 cm^{-1} were also observed which were characteristic for aliphatic amines. A new peak at 1543.33 cm^{-1} characteristic for –NH or –NO was also pragmatic. This clearly indicated the hydrolytic cleavage of MCP by the fungal strain.

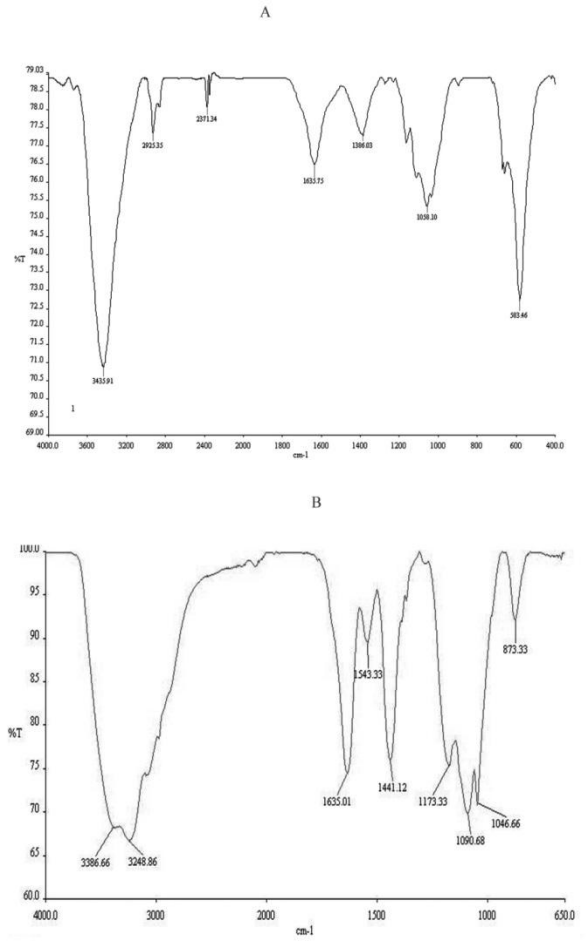

Figure 4. FTIR spectrum of MCP (a) before and (b) after degradation (15 days) by *Penicillium aculeatum* JQ660374.

Kinetics of MCP Degradation by *Penicillium aculeatum* JQ660374

Degradation kinetics of MCP was then studied by plotting the logarithmic concentration of MCP as a function of time. Figure 5 clearly indicates a linear relationship between logarithmic MCP concentration and incubation period. Therefore, degradation constant (k_{deg}) was calculated using first order equation. Half-life of MCP was found to be 2.53 days with k_{deg} of 0.0115 day^{-1}, which was significantly lower than that from control which was found to be infinite days.

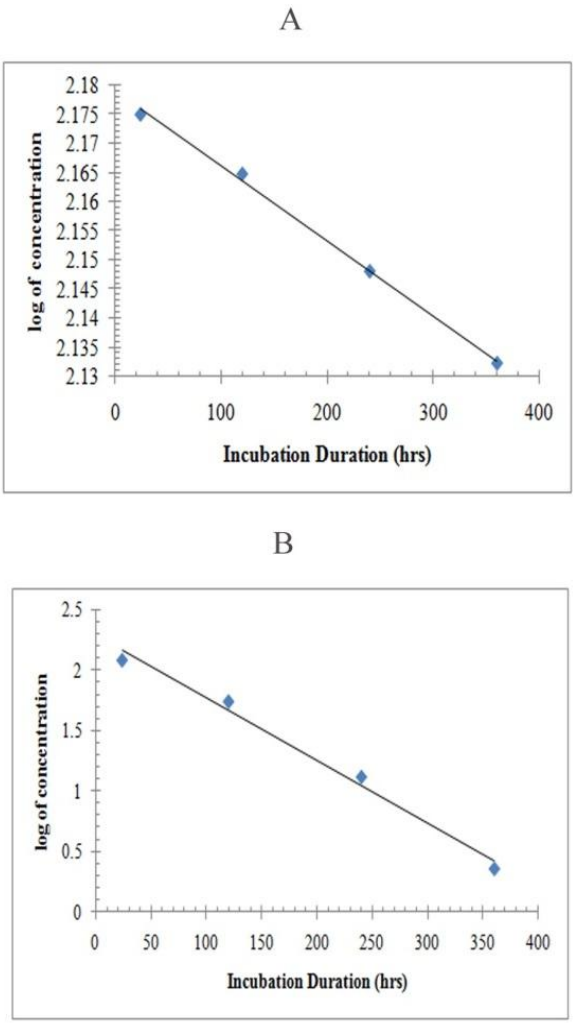

Figure 5. Degradation kinetics of MCP by *Penicillium aculeatum* JQ660374 $y = -0.005x + 2.294,\ R^2 = 0.987$ with respect to control. $y = -0.000x + 2.179,\ R^2 = 0.997$ Straight line equation shows that degradation of monocrotophos follows first order kinetics.

By-product estimation

MCP degradation by the fungal strain was accompanied by the formation of some by- products which were estimated at regular time interval. *Penicillium aculeatum* JQ660374 possessed moderate alkaline phosphatase activity which resulted in the formation of inorganic phosphates. Figure 6 clearly demonstrates that the

amount of both alkaline phosphatase activity and released inorganic phosphates increased till 14th day of incubation. Further increase in the incubation duration resulted in significant decrease in the enzyme activity. Whereas, the released inorganic phosphate content remained almost the same. Highest enzyme activity was found to be 28.33 ± 0.40 U which resulted in the formation of 614.62 ± 0.38µg m^{-1} of inorganic phosphates after 15 days of incubation. There was a linear increase in both the observed entities during first 10 days and the amount of both phosphatase and inorganic phosphates increased with a slow rate.

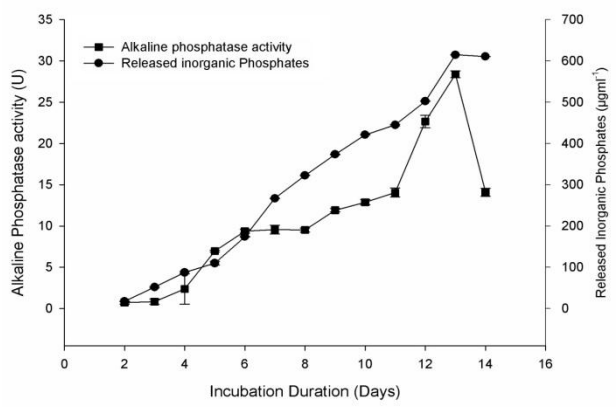

Figure 6 Alkaline phosphatase activity (U) and released inorganic phosphate content (µg ml^{-1}) by *Penicillium aculeatum* JQ660374 in CZM medium at various incubation durations. Error bars indicates standard deviation.

DISCUSSION

The study illustrates the potential of a hydrolase secreting soil fungi for the degradation of MCP in phosphorus free liquid medium under optimum in vitro aerated culture conditions. In our knowledge this is the first report demonstrating the potential of *Penicillium aculeatum* JQ660374 to degrade MCP within 15 days of incubation. *Penicillium aculeatum* JQ660374 degraded 98.8% of MCP within a very short period with the formation of inorganic phosphates resulted from extracellular alkaline phosphatase activity. HPTLC and FTIR analysis revealed the disappearance of MCP from the medium. Molecular insight of MCP degradation revealed hydrolytic cleavage of MCP by *Penicillium aculeatum* JQ660374.

Penicillium aculeatum ITCC 7980.10 was molecularly characterized by 18S rDNA analysis. BLAST analysis revealed 100% similarity with *Penicillium aculeatum* HQ392496. Therefore the identity of the isolate was confirmed as *Penicillium aculeatum* ITCC 7980.10 with an accession no. JQ660374.

Spectrophotometric analysis revealed that 98.8% of MCP was degraded by *Peniciillium aculeatum* JQ660374 within 15 days of incubation. Bhalerao and Puranik [23] had reported the ability of *Aspergillus oryzae* to completely mineralize MCP in M1 medium within 168 hours of incubation. Zidan and Ramadan [21] had reported 75% and 50% degradation of MCP within 4 days at the concentration 200 mg L^{-1} by *Aspergillus* and *Penicillium* sp., respectively.

The results were further confirmed by HPTLC which clearly demonstrate three fold significant reduction in the peak of MCP after 10 days of incubation. After 15 days no such peak was observed for MCP, which indicates complete degradation of MCP. In addition to the standard peak of MCP some extra peaks were also seen which might be due to its degradation products or metabolites. However, these were not identified as the emphasis was given on the disappearance of MCP by fungal isolate. The results were concurrent with the study of Bhalerao and Puranik, [23] and Jain *et al*., [32] which also correlated degradation of MCP by reduction in its peak in the chromatogram.

Molecular insight of MCP degradation was studied by FTIR analysis which clearly indicated the cleavage of vinyl bond and formation of $–PO_4$. This clearly demonstrates hydrolytic cleavage of MCP [23].

Degradation study of MCP by *Penicillium aculeatum* followed first order kinetics with K_{deg} $0.0115day^{-1}$ and half life of 2.53 days [6, 23, 33]. Whereas half life in control was found infinite and therefore clearly ruled out the possibility of auto degradation.

Degradation of MCP was accompanied by the formation of alkaline phosphatase and inorganic phosphates. The amount of both significantly increased up to 14th day of incubation and afterwards decreased significantly. Increase in the enzyme activity till 14th day might be due to the utilization of available MCP concentration as a substrate and the degradation of MCP may be the result of some enzymatic mechanism [34]. After 14 days almost 99% of the pesticide was degraded and might be the remaining 1% could not serve well as a substrate. Hence, the enzyme activity decreases on 15th day of incubation. Concurrent with the present findings previous studies of Bhadbhade *et al*., [9] and Bhalerao and Puranik,[23] had also suggested that the increase in phosphatase activity directly correlates with the release of inorganic phosphate content and decrease in MCP concentration. They had reported that MCP serves as a substrate for extracellular phosphatases and esterases with consequent formation of inorganic phosphates. Similarly Jain and Garg [26], and Jain *et al*.,[32] had also reported MCP as a substrate for extracellular fungal hydrolases.

In the very first days of incubation substrate is present in large amount and hence the enzyme activity increases exponentially. After long time (10-12 days) of incubation maximum amount of MCP (80-90%) degrades as a result of microbial process and now very small amount of MCP (10-20%) is present to be used as a substrate. Hence the enzyme activity decreases.

Therefore, the study concludes that *Penicillium aculeatum* JQ660374 proves itself to be the efficient tool for the degradation of MCP. Hence, it can be used for the degradation of this neurotoxin component from the environment.

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چکیده

حشره کش ها تهدید بزرگی برای پایداری محیط زیست با تاثیر بر گیاهان و جانوران به حساب می آید. قارچ ها به عنوان هیدرولیز برون سلولی برای تجزیه ی این ترکیبات شیمیایی خطرناک از یک محیط مناسب می باشند. قارچ *penecillium aculeatum* ITCC7980.1 برای مطالعات بازده تجزیه ی مونوکروتوفوس در شرایط آزمایشگاهی در محیط کشت مایع بدون فسفر انتخاب شد. گونه ی قارچی به صورت مولکولی با آنالیز 18s rDNA به عنوان *penecillium aculeatum* JQ660374 مشخص شد. بازده تجزیه از گونه ی جداسازی شده تحت شرایط بهینه در دوره های ۵ روزه با زمان انکوباسیون ۱۵ روز بررسی گردید. گونه ی جداسازی شده دارای فعالیت هیدرولیز کننده ی فسفات با مقدار ۲۸/۳۳±۰/۴ است که همراه با تشکیل $\mu g\ ml^{-1}$ ۶۱۴/۶۲±۰/۳۸ فسفات غیر آلی می باشد. فرایند تجزیه با آنالیز اسپکتروسکوپی در ۲۵۴ نانومتر، HPTLC، FTIR مطالعه شد. آنالیز اسپکتروسکوپی تجزیه یمونوکروتوفوس با کاهش چشم گیر در پیک استانداردمونوکروتوفوس (استاندارد مرجع ۰/۲۱-۰/۱۹) در زمان انکوباسیون ۱۵ روز نشان داد که با کروماتوگرافی HPTLC نیز اثبات گردید. مکانیسم مولکولی با آنالیز FTIR نشان داد که گسستگی پیوند وینیل با تشکیل po_4– همراه است. تجزیه ی مونوکروتوفوس دارای واکنش درجه اول با ثابت واکنش (k_{deg}) ۰/۰۱۱۵، و زمان نیمه عمر ۲/۵۳ روز می باشد. نتایج مطالعه نشان می دهد که می توان از قارچ *penecillium culaetum* JQ660374 به عنوان انتخابی مناسب برای دفع سمیت مونوکروتوفوس در مکان های آلوده به کار گرفت.