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Degradation of Monocrotophos by *Aspergillus oryzae* SJA1 isolated from *Oryza sativa* Field Soil

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Monocrotophos (MCP) is an organophosphours pesticide and controls the variety of pest in the agricultural fields. Fungal strain capable of degrading monocrotophos was isolated from *Oryza sativa* field soil. The SJA1 strain was identified based on 18S rRNA sequence analysis and it was designated as *Aspergillus oryzae* SJA1. The degradation process was studied by high performance liquid chromatography (HPLC). *Aspergillus oryzae* SJA1 degraded 500 mg/L monocrotophos in the aqueous medium as well as soil. The two metabolites such as diethylcyanamide and benzene 1,3-bis(1,1-dimethylethyl) were obtained during the degradation of monocrotophos. Besides, plant growth promoting traits such as phosphate solubilizing activity was also studied. The present study explores the potential of *Aspergillus oryzae* SJA1 in degrading monocrotophos which can be further used for remediating soil contaminated with MCP.

Key words: *Aspergillus oryzae* SJA1, Monocrotophos, Mycoremediation, Phosphate solubilization.

Monocrotophos [dimethyl-(*E*)-1-methyl-2(methylcarbamoyl) vinyl phosphate] is an organophosphorus insecticide, commonly used to control the mites, ticks and spiders, pest of cash crops such as cotton sugarcane, groundnut, maize, rice, soybeans and vegetables by contact and stomach action[1]. Monocrotophos (MCP) is a water soluble insecticide with moderate dermal toxicity[2]. It is mobile in soil and has a potential for ground water contamination.

Monocrotophos pesticide is used for agricultural fields in India and the degradation rate slightly affected by the initial pesticide, pH and catalyst concentration. The monocrotophos removal was in the range of 11 to 78% depending on the levels of the parameters studies. The degradation of monocrotophos based on the soil pH and the half-life of monocrotophos is being reported as 26 to 134 days at pH 9, 6, and 3 respectively[3].

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Microorganisms capable of degrading MCP were isolated from different soil samples and this revealed the predominance in isolating MCP degrading bacteria from exposed soils, belonging to the various genera like *Bacillus* sp.[4] *Pseudomonas aeruginosa*, *Clavibacter michiganensis* sp.[5,1] reported that isolates such as *Arthobacter atrocyaneusis*, *Bacillus megaterium*, *Pseudomonas mendocina*, were able to degrade 80 to 90% of MCP at maximum initial concentration of 500 mg/L in synthetic medium with in 48 h. The cultures were able to tolerate MCP upto concentration of 2500 mg/L and could utilize MCP as sole source of carbon in synthetic medium. MCP concentration in the range of 100 to 500 mg/L and the removal of MCP ranged between 77 to 88%. There are comparatively less work pertaining to degradation of MCP by fungus and some of the notable strains capable of successful degradation are White rot fungi, *Phanerochaete* sp. *Chrysosporium* sp. and *Trametes versicolor* has become the most commonly used model organisms in lignin degradation due to their good ligniolytic properties, fast growth and easy handling in culture[6]. In this study, a SJA1 strain with high

monocrotophos degrading capacity was isolated from monocrotophos spiked paddy field soil. The plant growth promoting activity, particularly the phosphate solubilization was also further studied.

MATERIALS AND METHODS

Soil sample

Soil sample was collected in sterile polythene bags from top layer of *Oryza sativa* field in Vellore district, Tamil Nadu, India. The collected soil sample was brought to the laboratory and air dried at room temperature. After sieving to a maximum particle size of <2 mm, the soil was immediately used for the experiment.

Chemicals

Certified analytical grade monocrotophos was purchased from Sigma-Aldrich. The technical grade monocrotophos, a 36% emulsifiable concentrate used in this study was procured from United Phosphorus Ltd, (Gujarat) India. All other reagents used in this study were of high purity and analytical grade.

Enrichment and isolation of fungi

Monocrotophos degrading fungi was isolated from a long-term monocrotophos applied *Oryza sativa* field soil. Approximately 20 g soil was inoculated into 100 ml Czapek Dox broth containing (in g/L) yeast extract, 3; peptone, 10; dextrose, 2; and monocrotophos, 100 mg/L was used for enrichment. The flask was incubated at room temperature on a rotary shaker at 120 rpm for 7d. After incubation, 10 fold dilutions of cultures were prepared and 100 µl of sample was spread on Czapek Dox agar medium containing 100 mg/L of monocrotophos. Isolated fungal culture was maintained on agar slopes of the same medium containing monocrotophos.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) and tolerance to MCP was determined for SJA1 isolate using broth assay. Erlenmeyer flasks containing 100 ml of M1 medium containing (in g/L) $NaNO_3$, 2; KCl, 0.5; $MgSO_4$, 0.5; glucose, 10; $FeCl_3$, 10 mg; $BaCl_2$, 0.2; and $CaCl_2$, 0.5; pH 6.8 and different concentration of pesticide was added. The flasks were inoculated with 1ml of fungal culture and kept on a rotary shaker at 120 rpm for 7 d at 30 ± 2 °C. After 7 d of incubation, the flasks were observed for mycelial growth and the mycelial mass was harvested by filtering through whatman filter paper no. 1. The dry weight of fungal biomass was determined by drying the sample at 80 °C. The MIC was noted as the concentration of MCP resulting in complete inhibition of fungal mycelial growth in flasks.

Growth Kinetics

In order to determine the growth pattern, 1 ml of fungal spore suspension was inoculated in 250 ml Erlenmeyer flasks containing 100 ml of Czapek Dox broth with and without monocrotophos (500 mg/L). The flasks were incubated at 30 ± 2 °C on rotary shaker at 120 rpm for 5 d. One flask from each series was periodically removed and the mycelial mass from each series was separated by filtration using Whatmann filter paper no. 1 and washed with deionized water. The dry weight of fungal biomass was determined by drying for constant weight at 80 °C in preweighed aluminum foil cups.

Taxonomic identification of fungal strain

The isolated fungal strain was identified by 18S rRNA sequence analysis. The fungal genomic DNA was isolated by using AMpurE Fungal gDNA Mini kit. In this kit detergent and other non-corrosive chemicals are used to break open the cellulosic cell wall and plasma membrane to extract DNA from fungal cells. The 18S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers CGW CGR AAN CCT TGT NAC GAS TTT TAC TN and AWG CTA CST GGT TGA TCC TSC CAG N. PCR reaction mix of 50 µl final volume contained: 50 ng sample gDNA, 100 ng forward primer, 100 ng reverse primer, 2 µl dNTP's mixture (10 mm), 5 µl 10X Taq polymerase buffer, 3 U Taq polymerase enzyme and PCR grade water to make up the volume. Amplified PCR product was sequenced by using ABI3730xl genetic analyser (Amnion Biosciences Pvt. Ltd. Bangalore, India). The sequencing result was submitted to the Gene bank National Centre for Biotechnology Information (NCBI) database.

Biodegradation of MCP in mineral medium and soil

To study the degradation of MCP in mineral medium, Erlenmeyer flask containing 100 ml of M1 medium spiked with 500 mg/L of MCP as the sole carbon source and inoculated with 1 ml of fungal spore suspension of SJA1 strain. The flasks were incubated at 30 °C on a rotary shaker at 120

rpm and samples were taken at regular time intervals from the culture flasks.

Degradation of MCP was carried out in the same soil sample from which fungal strain was isolated prior analysis; the soil was sterilized by threefold autoclaving for 30 min at 121 °C. Two trials were carried out: (1) Addition of pesticide (500 mg/kg), isolated fungal spore and nutrients (Carbon, Nitrogen, and Phosphorous), (2) Addition of pesticide (500 mg/kg) and isolated fungal spore without nutrients. The amount of Carbon, Nitrogen and Phosphorus were calculated using the relationship C/N/P 100:10:1. The sources of carbon, nitrogen and phosphorous were glucose, $(NH_4)_2SO_4$ and K_2HPO_4, respectively[7,8]. The removal of MCP was determined by High Performance Liquid Chromatography (HPLC).

Analytical methods

The 5-10 ml of aqueous samples from MCP degradation flasks were extracted with equal volume of ethyl acetate. 10 g of soil samples were collected from each treatment trails with and without amendment of nutrients for pesticide analysis. The soil samples were extracted with 20 ml of ethyl acetate used to determine the pesticide concentration by HPLC. The isocratic mobile phase was composed of methanol:water (70:30, V:V), which was pumped through the column at a flow rate of 1 ml/min. MCP and its metabolite were detected at 214 nm.

The metabolites produced during monocrotophos degradation were analysed by GC-MS (Gas Chromatography Mass Spectrometry). The sample from ethyl acetate extract in the M1 medium was injected in GC-MS. Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector (Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mm ID, 250 ìm df) column was used. The carrier gas used was helium at a flow rate of 1 ml/min. The following temperature program was used: initially the oven temperature was held at 60 °C for 2 min and then ramped from 10 °C/min to 300 °C withhold time for 4 min, total run time 30 min. The temperature of the injector was maintained at 300 °C. The ion trap was operated at 70 eV with a scan range of m/z from 50 to 600. A sample of 1 µl was injected in split mode (10:1). The intermediate and end product was identified based on the Wiley registry of mass spectral data.

Infrared spectra of the MCP parent compound and sample after fungal degradation were recorded at room temperature in frequency range 4000-400cm^{-1} with a FTIR. Spectrophotometer (8400 Shimadza, Japan with Hyper IR-1.7 Software for windows) with helium neon laser lamp as a source of IR radiations. Pressed pellet were prepared by grinding the extract samples with potassium bromide in motor with 1:100 ratio immediately analyses in the region of 4000-400cm^{-1} at a resolution of 4 cm^{-1}.

Data analysis

The experiments were performed in triplicates and the results are presented as avarage ± S.D. The data were subjected to two-way ANOVA and statistical significance was obtained.

Estimation of CO_2

The amount of CO_2 evolved during the degradation of monocrotophos by SJA1 strain was checked. A series of 250 ml Erlenmeyer flasks containing 100 ml of M1 medium supplemented with 500 mg/L of MCP. One ml of fungal spore suspension was added to the M1 medium and then the flasks were sealed with airtight jar containing glass beakers filled with standard sodium hydroxide solution to trap the evolved CO_2. The samples were taken at regular intervals upto 6 d. The dissolved CO_2 concentration in alkali solution was estimated by titrating with 1N hydrochloric acid in the presence of phenolphthalein and barium chloride[2].

Phosphate solubilization by SJA1 strain

The fungal strain was further used to determine the extent of phosphate solubilization in Pikovskaya broth by the chlorostannous reduced molybdophosphoric acid blue method. The 100 ml of pikovskaya broth was prepared in three different flasks at optimum pH 7.2 and then different phosphates (Tricalcium phosphate, Dicalcium phosphate and Zinc phosphate) were added to the flasks. After sterilization the broths were inoculated with 1 ml of fungal spore suspension. The flasks were then incubated at 30 ± 2 °C for 12 d under static condition and shaken at 12 h intervals. The 10 ml of samples were taken at every 72 h intervals and centrifuged at 4000 rpm for 20 min. 1 ml of supernatant was taken in sterile test tubes and equal volume of chloromolybdic acid and 1 drop of chlorostannous acid was added and volume was adjusted to 5 ml with distilled water. The absorbance of blue color developed

was measured at 600 nm. The amount of phosphate solubilized was calculated using the calibration curve of KH_2PO_4. The change in pH following tri calcium phosphate, dicalcium phosphate and zinc phosphate solubilization were also recorded.

RESULTS

Isolation and characterization of fungal strain SJA1

In the present study, a fungal strain SJA1 was isolated from *Oryza sativa* field soil through enrichment culture technique on Czapek Dox agar supplemented with monocrotophos. The minimum inhibitory concentration of monocrotophos was obtained for the fungal strain SJA1. The SJA1 strain could tolerate upto 700 mg/L of monocrotophos and had a confluent growth at 500 mg/L of monocrotophos. The molecular characterization based on 18S rRNA sequence analysis was used to identify SJA1 strain. BLAST results of the 18S rRNA gene sequence of SJA1 strain exhibiting close relationship with 99% similarity to that of the 18S rRNA gene of *Aspergillus oryzae*. Multiple sequence alignments and phylogenetic tree (Figure 1) revealed that the strain SJA1 cluster with *Aspergillus* sp. Therefore, the SJA1 isolate was designated as *Aspergillus oryzae* SJA1 and the sequence result was submitted to GenBank NCBI database and accession number KC842216 was obtained.

Growth kinetics

Growth kinetics of *Aspergillus oryzae* SJA1 in the presence and absence of monocrotophos as a function of time were presented in Figure 2. The visible increase in mycelial mass with time was obtained during the metabolism of monocrotophos by SJA1 strain. Initially the growth of SJA1 strain was suppressed in the presence of pesticide, but after adaptation to pesticide the culture showed higher growth. The amount of biomass produced during the growth study with pesticide was much higher when compared to the growth in absence of pesticide. This could be mainly due to availability of extra carbon source upon degradation of pesticide in the medium.

Biodegradation of monocrotophos in aqueous medium and soil

The results showed that monocrotophos degradation by SJA1 significantly different (*P* Â 0.05) were evaluated (Figure 3). The SJA1 strain in M1 medium containing monocrotophos as the sole organic compound revealed that fungal strain was capable of utilizing applied pesticide as a source of carbon and energy to grow, and confirmed the degradation of monocrotophos*Aspergillus oryzae* SJA1 degraded monocrotophos in the aqueous medium to an undetectable level in 72 h (Figure 4). The degradation dynamics of monocrotophos in the soil are presented in Figure 5. The SJA1 strain was inoculated into soil with 500 mg/kg of monocrotophos and nutrients, such as carbon, nitrogen, and phosphorous, after 120 h of incubation showed 100% degradation of monocrotophos. There is no appreciable difference in the soil inoculated with SJA1 strain and 500 mg/kg monocrotophos without addition of nutrients, the 100% degradation was obtained within 168 h. The degradation products of monocrotophos in the culture extracts were analysed by GC-MS. Based on GC-MS analysis, it was confirmed that two metabolites were obtained during the monocrotophos degradation by SJA1 strain. After 72 h of incubation, final metabolites of monocrotophos was identified with mass ion at m/z of 98 and 190 which corresponds to (i)

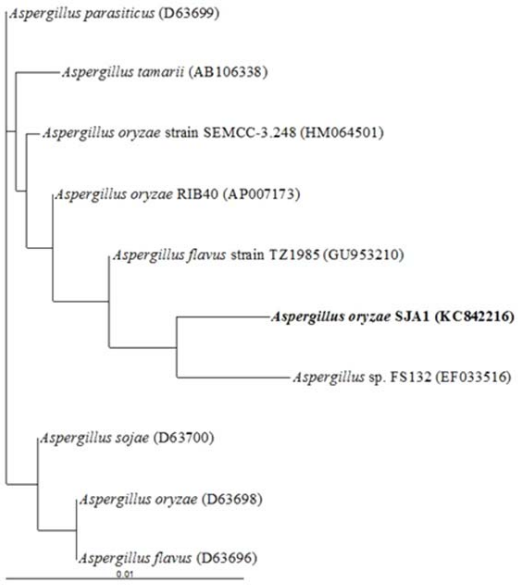

Fig. 1. Phylogenetic relationship of *Aspergillus oryzae* SJA1 based on 18S rRNA gene nucleotide sequences

Diethylcyanamide and (ii) benzene, 1,3-bis(1,1-dimethylethyl) by Mass Spectrometry, respectively (Figure 6).

The FTIR studies indicated the various structural changes of monocrotophos. Comparison of FTIR spectrum of control (Figure 7a) with extracted metabolites after complete degradation of monocrotophos by *Aspergillus oryzae* SJA1 (Figure 7b) clearly confirmed the degradation of monocrotophos. The infrared spectrum of monocrotophos degraded sample showed a band at 3452 cm^{-1} corresponds to N-H stretch. The two bands were presented at 1639 and 1631 cm^{-1}, which are the characteristics of C=C stretch. The peak positions at 1384 cm^{-1}, 675 cm^{-1} which are C-C stretching and C-H bending, respectively.

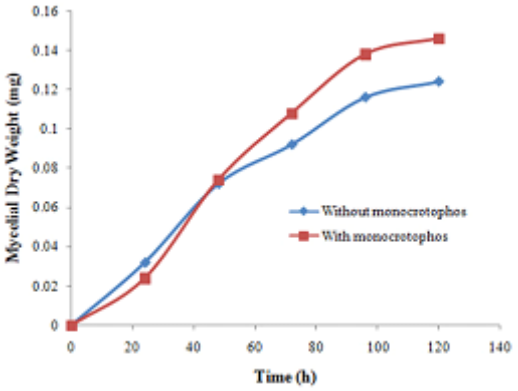

Fig. 2. Growth performance of *Aspergillus oryzae* SJA1 in the presence and absence of monocrotophos (500 mg/L)

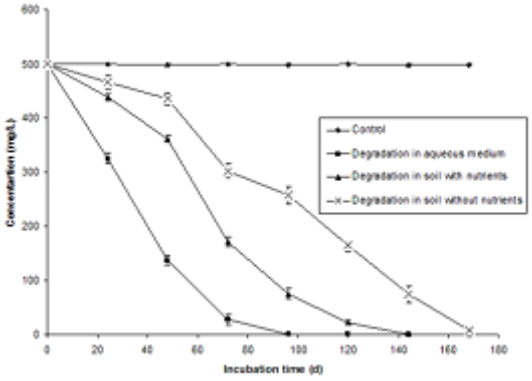

Fig. 3. Degradation dynamics of monocrotophos by SJA1. Symbols are the means of three replicates and error bars represent the standard deviation which was within 5% of the mean

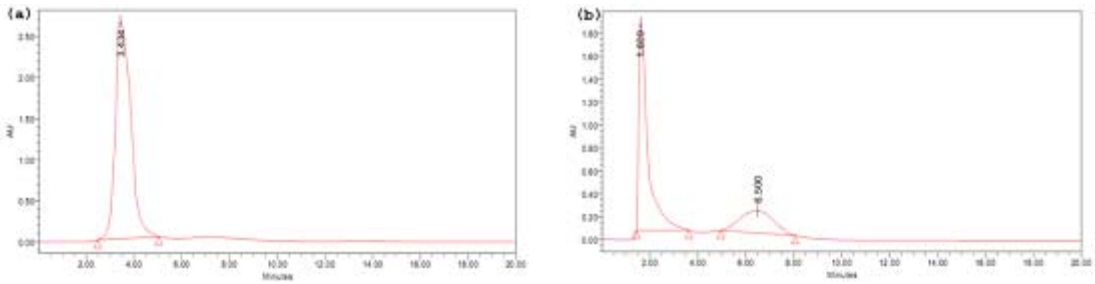

Fig. 4. (a) The HPLC chromatogram of monocrotophos at standard condition (b) HPLC chromatogram of biodegradation of monocrotophos in aqueous medium by *Aspergillus oryzae* SJA1.

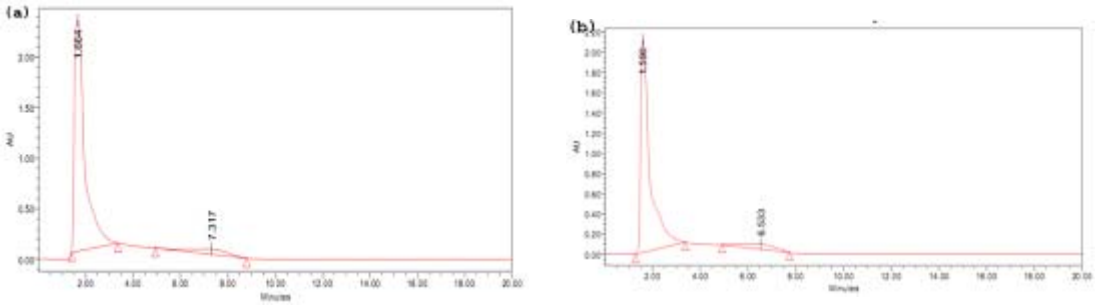

Fig. 5. (a) HPLC chromatogram of biodegradation of monocrotophos in soil with nutrients; (b) HPLC chromatogram of biodegradation of monocrotophos in soil without nutrients.

The data obtained for CO_2 estimation are shown in Figure 8. The rate of CO_2 evolution was found to be increased till 6 d of incubation. The level of CO_2 production could be linked to the decline in monocrotophos concentration under degradation condition.

Phosphate solubilization

Phosphate solubilization was estimated by using different inorganic phosphate substrate in Pikovskaya broth. Varying levels of phosphate solubilization were recorded with different substances such 253 µg/ml (pH 3.6) with tricalcium phosphate, 317 µg/ml (pH 3.8) with dicalcium phosphate and 492 µg/ml (3.7) with zinc phosphate after 12 d of incubation.

DISCUSSION

Many studies for the degradation of pesticides by other fungi such as *Aspergillus niger*

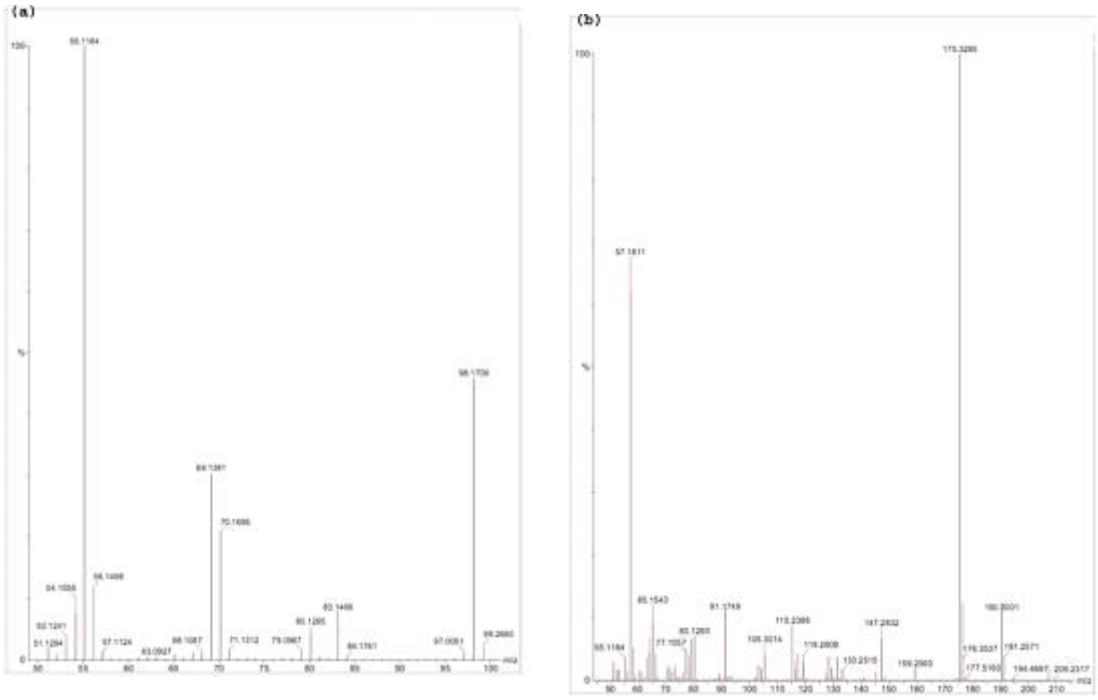

Fig. 6. Mass Spectrometry detection of metabolites appeared during the degradation of monocrotophos in the aqueous medium by *Aspergillus oryzae* SJA1 (a) diethylcyanamide (b) benzene 1,3-bis(1,1-dimethylethyl)

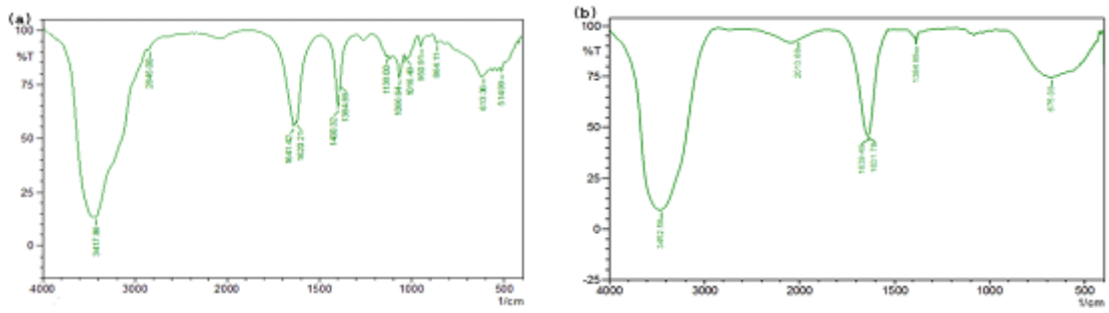

Fig. 7. (a) Control (b) FTIR spectrum of biodegradation of monocrotophos in aqueous medium

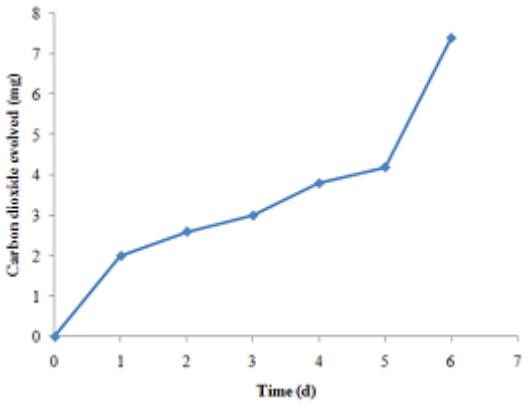

Fig. 8. Evolution of carbon dioxide by *Aspergillus oryzae* SJA1 in the presence of monocrotophos as a sole source of carbon

degrading pyrethroid[9], fenitrothion[10], carbaryl[11], dimethoate[12] and *Aspergillus flavus* for metolachlor[13] were reported. There are few reports on degradation of other organophosphate pesticides such as pyrazophos, dimethoate, malathion, lancer, profenfos derivatives by fungi like *Aspergillus flavus* and *Aspergillus sydowii*[14]. However, in our study *Aspergillus oryzae* SJA1 was isolated and used for the degradation of monocrotophos.

In general, metabolic reactions such as *N*-demethylation, *O*-demethylation, hydroxylation of *N*-methyl groups and cleavage of the phosphate-crotanamide linkage, occur during the degradation of monocrotophos by microorganisms with the formation of *O*-desmethylmonocrotophos monomethyl phosphate, dimethyl phosphate, N-methylacetoacetamide and N-methylbutyramide[15-17]. In the present study, Diethylcyanamide and benzene 1,3-bis(1,1-dimethylethyl) reported to be involved in the biodegradation of monocrotophos were detected in *Aspergillus oryzae* SJA1.

Zidan and Ramadan[18] reported the biodegradation of monocrotophos at duration of four days at a concentration of 200 mg/L to 50 to 70% by *Aspergillus* sp. and *Penicillium* sp. respectively. Bacterial and fungal strain decreased the pH of the culture media after 15 day of incubation from 7.2 to 3.2 [19]. Not all the organophosphrous pesticide undergoes hydrolysis in water solution under acidic condition[20]. Degradation of different pesticide present at high concentration of microorganism has been reported in previous works[21]. Our findings revealed that *Aspergillus oryzae* SJA1 preferred to utilize monocrotophos even without addition of nutrients in the soil and its degrading ability was positively influenced by the presence and absence of nutrient sources. This might be because, potentially, the monocrotophos degrading enzymes in *Aspergillus oryzae* SJA1 are expressed even in the absence of readily available nutrient sources.

Bhalerao and Puranik[2] reported that the production of CO_2 was increased in the first 5 d of incubation and decreased in subsequent days. A positive correlation was observed between the pesticide transformation rate and microbial respiration activity in soil of different ecosystems[22]. In the present study, the release of carbon dioxide during growth of culture in aqueous medium containing monocrotophos indicated the mineralization of monocrotophos.

Phosphorus is one of the major essential mineral fertilizers and is world's second largest agricultural chemical required by plant growth[23]. Soil microbes have the ability to convert fixed form of phosphorus in soil to soluble forms that can be easily taken up by plants[24]. Previously, the beneficial effects of phosphate solubilizing fungi on various crops has been demonstrated with *Aspergillus* sp.[25], *Penicillium* sp.[26] and *Trichoderma* sp.[27,28]. There are also a few reports on the effect of phosphate solubilizing fungi on chickpea plants. Dudeja *et al*.[29] reported stimulatory effect of *Aspergillus awamori* strain on chickpea variety H208 in terms of nodules number, nodule dry weight, nitrogenase activity and grain yield. However, in the present study *Aspergillus oryzae* SJA1 was efficiently degraded monocrotophos and solubilized the inorganic phosphate.

CONCLUSIONS

In this report, the fungal strain SJA1 was isolated from long term monocrotophos applied *Oryza sativa* field soil. The biodegradation of monocrotophos by this strain was simple, rapid and highly effective. The SJA1 strain efficiently degraded the monocrotphos without addition of nutrients in the soil. To our knowledge, this is the first report in the degradation of monocrotophos produced new metabolites such as

Diethylcyanamide and benzene 1,3-bis(1,1-dimethylethyl). Apart from degradation, a SJA1 strain has an added advantage of promoting plant growth by solubilizing phosphate. The SJA1 strain is attractive candidate and useful for environmental remediation of monocrotophos and agronomic applications.

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