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Biodegradation of monocrotophos by bacteria isolated from soil

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Bacteria were isolated by enrichment culture technique from groundnut (*Arachis hypogaea* L.) soils and tested for their ability to degrade monocrotophos in mineral salts medium under aerobic conditions in the laboratory. Based on some of the morphological and 16S rRNA gene sequence analysis, the isolates were identified as *Rhodococcus phenolicus* strain MCP1 and *Rhodococcus ruber* strain MCP-2. The initial (0-day) recovery of monocrotophos in the culture medium was 94%; and by the end of 4th day, about 21% of added monocrotophos was lost from the uninoculated medium. By the end of 1st, 2nd, 3rd and 4th day 13, 20, 24, 30% and 18, 33, 37, 45% of monocrotophos was degraded, by *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, when the mineral salts medium was supplemented with monocrotophos as a C source. Simultaneously 12, 22, 26, 30% and 18, 26, 37, 40% of N-methylacetoacetamide a metabolite of monocrotophos was recovered in the media inoculated with the *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, during the same period. Decrease in the amount of monocrotophos with a concomitant increase in the level of N-methylacetoacetamide clearly indicates the degradation of parent compound.

Key words: Monocrotophos; N-methylacetoacetamide, biodegradation; isolation, identification.

INTRODUCTION

Monocrotophos ((3-hydroxy-N-methyl-cis-crotonamide) dimethyl phosphate) is an organophosphorus (OP) insecticide widely used to control aphids, leaf hoppers, mites and other foliage pests on crops, such as cotton,

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sugarcane, peanuts (groundnut) and tobacco because of its low cost and effectiveness. It was first produced by Ciba AG and Shell Development Co. in 1965 and has been classified as extremely hazardous, with an LD_{50} value of 20 mg kg^{-1} for mammals. Monocrotophos has contact, systemic and residual activities; the prolonged use of this compound has recently found its way into the water ways in the proximity of its application (Palmer et al., 2007). OP pesticides are widely used in India for protection of agricultural yields. However, these pesticides pose serious threats to organisms, including humans, and hamper soil microbial activity; thus, they are a cause for concern. Monocrotophos is a systemic pesticide and its action is mainly on organs such as skin, eyes and central nervous system. The half-life of monocrotophos in soil was reported to be 40 to 60 days (Sha, 1999; Tomlin, 2000; Bhadbhade et al., 2002a). Continuous and excessive use of OP compounds has led to the contamination of several ecosystems in different parts of the world (Cisar and Snyder, 2000; Tse et al., 2004). For example, surveys revealed that 100% of sampled catchments in Scotland and 75% of sampled aquatic sites in Wales were contaminated with OP compounds used in sheep dips (Boucard et al., 2004). OP compounds are highly toxic to mammals and are toxic to other non-target animals, and the toxic effects of OP compounds on invertebrates, vertebrates and wildlife are well documented (Galloway and Handy, 2003). The pesticides in aquatic environment are potentially toxic and are difficult to degrade by conventional treatment processes (Colin et al., 2004; Abdullah and Ling, 2010; Avasarala et al., 2011). Growing public concern on the contamination of drinking water supplies and the aquatic environment with organic pollutants has stimulated research activity for their treatment.

Monocrotophos insecticide during sexual development causes the feminization/demasculinization of the reproductive traits. Reproductive toxicity caused by organophosphates (monocrotophos) at cellular and molecular level in the ovaries of rat. The reproductive toxicity of monocrotophos has also been observed in bobwhite quail (Tian et al., 2012; Vijay et al., 2014). Organophosphorus pesticides have been extensively used in the area of agriculture to manage insect or pests of a number of economically important crops. Organophosphate pesticides are well known as the inhibitor of acetylcholinesterase activity, not only in insects, it can also affect central nervous system of other organisms (Vijay et al., 2013). Hence, the degradation of monocrotophos is imperative.

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 monocrotophos by microbial cultures and in soils (Guth, 1994; Bhadbhade et al., 2002a) with the formation of O-desmethylmonocrotophos monomethyl phosphate, dimethyl phosphate, N-methylacetoacetamide and N-methylbutyramide. The use of pesticide-degrading microbial systems for the removal of pollutants from contaminated systems requires an understanding of the ecological requirements of degrading organisms. Sunlight significantly influences the degradation of monocrotophos in soil but not in water, indicating the involvement of components of soil in photolytic degradation (Dureja, 1989; Lee et al., 1990). The rate of hydrolysis of monocrotophos in soil and aqueous environments is pH dependent, and the half-lives of monocrotophos are 131 days at pH 3, 26 days at pH 9 and 30 days at neutral pH at 25°C in the dark (Lee et al., 1990). Monocrotophos is weakly sorbed by soil particles because of its hydrophilic nature. Leaching of monocrotophos may pollute the groundwater, ultimately resulting in adverse effects on biological systems (Singh and Singh, 2003; Bhalerao and Puranik, 2009).

Together with its high mammalian toxicity, these characteristics make monocrotophos an ideal compound for decontamination and detoxification. Rangaswamy and Venkateswarlu (1992) isolated a monocrotophos degrading *Bacillus* sp. from previously treated soil. Several microorganisms have been isolated which are able to utilize pesticides as a source of energy. There are some reports of fungi including *Trametes hirsutus*, *Phanerochaete chrysosporium*, *Phanerochaete sordia* and *Cyathus bulleri* that are able to degrade lindane and other pesticides (Singh and Kuhad, 2000, 1999). However, most evidence suggests that soil bacteria are the principal agents responsible for enhanced biodegradation (Walker and Roberts, 1993). On numerous occasions, mixed bacterial cultures with pesticide degradation ability were isolated but their individual components are unable to utilize the chemical as an energy source when purified (Roberts et al., 1993); an example is the OP nematicide fenamiphos (Singh et al., 2003).

Bacteria capable of degrading several pesticides have been isolated from soil. *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* sp. insidiosum SBL 11 were found capable of utilizing monocrotophos as a source of phosphorus but not as a carbon source (Singh and Singh, 2003). *Rhodococcus phenolicus* strain $G2P^{T}$ utilize phenol, chlorobenzene and chlorobenzoic acid as source of carbon (Marc and James, 2005). As a measure of bioremediation, soil fungi capable of degrading monocrotophos were isolated from various geographical and ecological sites (Bhalerao and Puranik, 2009).

Although few microorganisms capable of degrading monocrotophos have been isolated, most of studies concerning on the degradation of monocrotophos with bacterial cultures focusing on a parent compound without prominence on its degradation products. In the present study we report the accumulation of N-methylacetoacetamide in significant proportions as degradation product in the mineral salts media inoculated with *Rhodococcus* sp. under aerobic conditions. Besides

there are no reports available on the degradation of monocrotophos by a novel bacterial strains, *Rhodococcus phenolicus* strain MCP1 and *Rhodococcus ruber* strain MCP-2.

MATERIALS AND METHODS

Chemicals

Monocrotophos with 99% purity was purchased from Sigma-Aldrich Company, Bangalore, India. All other chemicals and reagents used in this study were analytical grade.

Isolation and identification of bacteria

To isolate soil bacteria capable of degrading selected insecticide, monocrotophos enrichment culture technique was opted (Jayamadhuri and Rangaswamy, 2009). Commercial formulation of monocrotophos was added separately to 50 g portions of the soil samples to provide a final concentration of 50 µg g^{-1} soil. The soil samples were maintained at 60% water holding capacity (WHC) and incubated at room temperature (28 ± 4°C) under aerobic conditions. After five such additions with monocrotophos at 10-day intervals, triplicate soil samples were withdrawn for the isolation of soil bacteria by serial dilution agar plate method (Vijay et al., 2015).

Initially bacteria in the enrichment culture showing ability to degrade monocrotophos were grown on mineral salts media. After about 24 to 48 h of incubation at 37°C, several well-separated, individual colonies of different morphological types appeared and were further streaked onto fresh mineral salts media plates for further purification. Morphological features were studied using light microscope.

Degradation of monocrotophos residues by selected bacterial strains

The ability of the selected bacterial strains, *R. ruber* strain MCP-2 and *R. phenolicus* strain MCP1 to degrade the organophosphate insecticide, monocrotophos was tested under aerobic conditions in the laboratory. Aliquots from stock solutions, prepared in acetone, of the technical grade monocrotophos were added to 250 ml sterilized Erlenmeyer flasks to provide a final concentration of 50 µg ml^{-1} keeping in view of their toxic levels to the bacteria. The carrier solvent was completely evaporated to dryness and 50 ml portions of steam sterilized mineral salts medium (NH_4NO_3 1.5 g, $K_2HPO_4$1.5 g, KH_2PO_4 0.5 g, Mg SO_4 0.2 g, NaCl, 0.5 g, distilled water 1000 ml, pH 7.0) (Kai et al., 2006) was added into each flask under aseptic conditions.

The residues were then equilibrated for a day to obtain aqueous solutions of the monocrotophos and inoculated with an inoculum density of 1.0 OD cells measured at 600 nm using a Spectronic-20D spectrophotometer (Milton Roy) and incubated under continuous shake culture condition (150 rev min^{-1}) in an orbital shaking incubator at 37°C. Triplicate test samples were withdrawn from Erlenmeyer flaks after 1st, 2nd, 3rd and 4th day of incubation for solvent extraction and estimation of parent compound and metabolite by TLC and HPLC analysis.

Extraction of monocrotophos residues

The residues of monocrotophos from the triplicate samples of mineral salts media were extracted with equal volume of ethyl acetate (Bhadbhade et al., 2002b). At the desired intervals (1st, 2nd, 3rd and 4th day) the culture filtrate supernatant (CFS) was extracted with equal volume of ethyl acetate for the residues of monocrotophos**.** The solvent was evaporated and the residue obtained was redissolved in approximately 10 ml of methanol for the High performance liquid chromatography (HPLC) analysis. Organophosphate insecticide, monocrotophos was detected at 214 nm.

Quantification of monocrotophos residues by thin layer chromatopgraphy (TLC) and high performance liquid chromatography (HPLC)

For identification of degradation products of monocrotophos, the methanolic fraction was spotted along with authentic compounds N-methylacetoacetamide and monocrotophos on 300 µm thick silicagel-G plates. The plates were developed for a distance of 15 cm with chloroform-methanol-diethyl ether (10:1:1) and air-dried. The authentic compound and metaboloites were located by spraying the plates with ferric chloride reagent (27.0 g $FeCl_3.6H_2O$ in 1000 ml 96% ethanol). The silica gel of the samples alongside to the authentic compound N-methylacetoacetamide were scrapped off and carefully transferred to centrifuge tubes and 5 ml ethanol was added. After vigorous agitation for 2 min on a vortex mixture, contents were centrifuged at 4000 rpm for 15 min and the supernatant was collected for the estimation of N-methylacetoacetamide by a Spectrophotometer (Gundi and Reddy, 2006). Suitable aliquots of supernatant were treated with 0.5 ml ferric chloride reagent (as described above) and the volume was made up to 5 ml with distilled water, and the absorbance was measured at 510 nm in a Spectronic-20D spectrophotometer (Milton Roy). The quantity of metabolite, N-methylacetoacetamide was calculated by comparing the absorbance values with a standard curve prepared with authentic N-methylacetoacetamide.

The residues of the parent insecticide, monocrotophos have been determined by a slightly modified method of Rajendra et al. (2013) by HPLC analysis. The residues of the moncocrotophos redissolved in methanol was analysed in Agilent High Performance Liquid Chromatography (HPLC) system (1100 series), Ascentis® Express C18 HPLC Column (250 × 4.6 mm), Variable wavelength detector. The mobile phase was methanol: water (70:30, v/v), and the flow rate was 1 ml min^{-1}. A 20 µl of sample was injected into the column and the monocrotophos residues were monitored at 214 nm. Under these conditions the retention time of the parent insecticide, monocrotophos was 159 s.

Statistical analysis

All data are averages of three replicates. The data were analyzed for significant differences ($P \leq 0.05$) between inoculated and uninoculated pesticide samples in mineral salts media using Duncan's multiple range (DMR) test (Megharaj et al., 1993; Srinivasulu et al., 2012).

RESULTS AND DISCUSSION

Isolation and identification of monocrotophos degrading bacterial strains

Bacterial cultures were isolated by enrichment culture technique, after treating the soil samples five times with an insecticide, monocrotophos at 50 µg g^{-1} level. Primarily the bacteria designed as MCP1 and MCP2 and were tested for their ability to degrade the

(a)

TGGGCGAAGCCTTTTCCAGCGACGCCGCGTGAGGGATGACCGCCTTCGGGTTGTAAACCTCTTTCAG
CAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC
GTCGTCTGTGAAAACCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCA
GGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG
GCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAG
CTAACGCATTTAAGCGTCCCCGCCTGGGGAGTTACGGCCGCAAGGCTAAAAACTCGAAAGAGTTGAC
GGGGGGCCCCCCGCAAAACCGCGGGAGCATGTGGATTAATTCCNANGCCNAGNCGAAAGAACCTTA

(b)

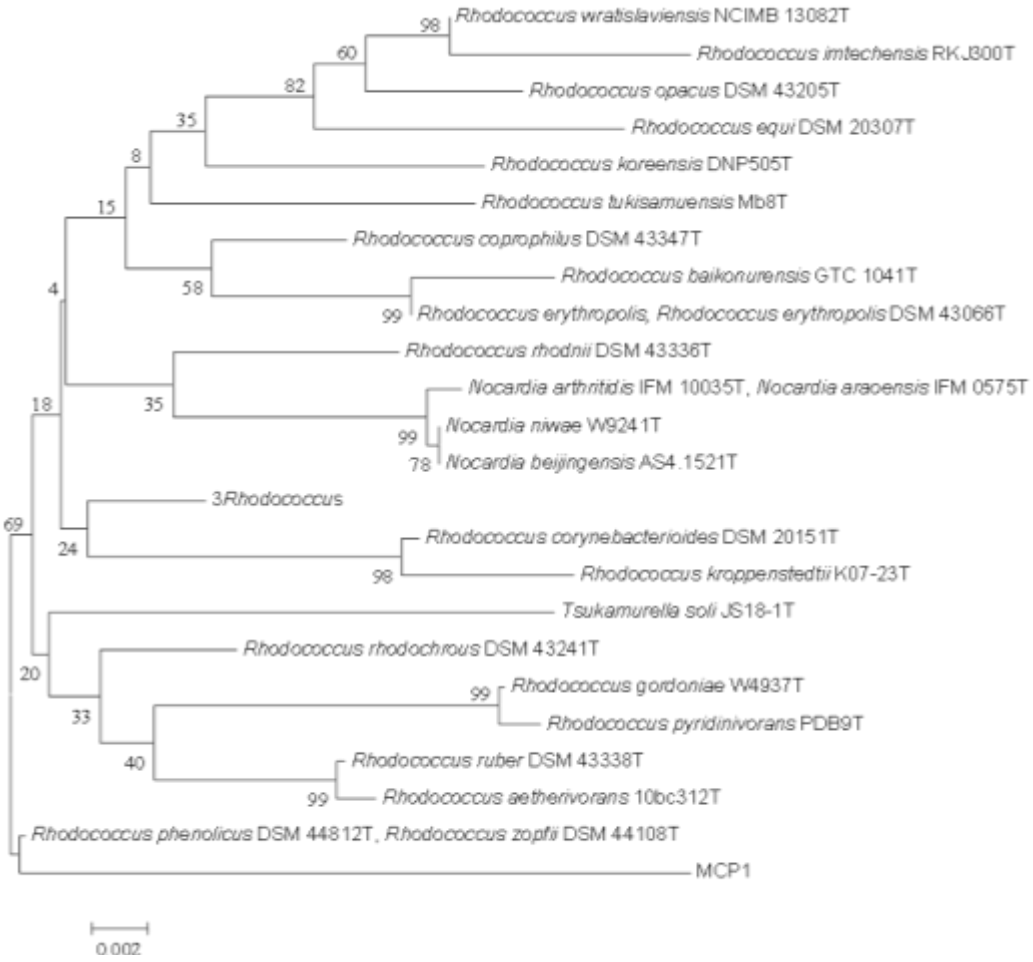

Figure 1. (a) 16S rRNA gene sequence; (b) Phylogram of *Rhodococcus phenolicus* strain MCP1.

monocrotophos in mineral salts medium. Both the bacterial strains, MCP-2 and MCP1 on mineral salts agar media after 24 hours showed opaque and round morphology, whereas MCP-2 colour has changed to salmon pink if incubation period was extended to 48 to 72 h. The bacterial strains grew well in mineral salts media supplemented with monocrotophos as carbon source at 37°C. The morphological features under light microscope were all Gram positive short rod shaped. Based on the partial 16S rRNA gene sequence, MCP1 belonged to *Rhodococcus phenolicus* sp. while MCP-2 belonged to *Rhodococcus ruber* sp The 16S rRNA gene sequences of the bacteria are represented in Figures 1a and 2a. Figures 1b and 2b illustrates the phylogenetic relationship between MCP-2, MCP 1 and their close relatives.

Degradation of monocrotophos by bacterial strains

After the incubation at desired intervals (1st, 2nd, 3rd and

(a)

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TGGGCGAAGCCTTTTCCAGCGACGCCGCGTGAGGGATGACCGCCTTCGGGTTGTAAACCTCTTTCAG
CAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC
GTCGTCTGTGAAAACCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCA
GGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG
GCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAG
CTAACGCATTTAAGCGTCCCCGCCTGGGGAGTTACGGCCGCAAGGCTAAAAACTCGAAAGAGTTGAC
GGGGGGCCCCCCGCAAAACCGCGGGAGCATGTGGATTAATTCCNANGCCNAGNCGAAAGAACCTTA
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(b)

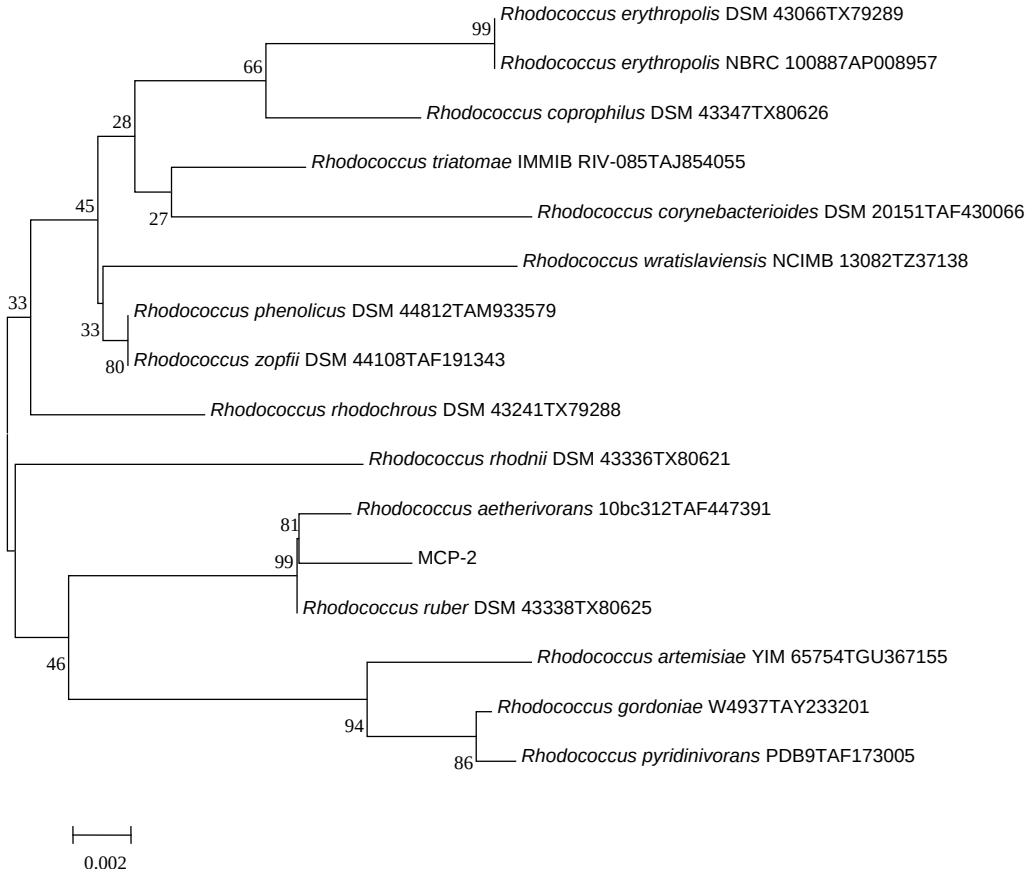

Figure 2. (a) 16S rRNA gene sequence; **(b)** Phylogram of *Rhodococcus ruber strain* MCP-2.

4th day), triplicates of the bacterial inaculated and uninoculated samples of mineral salts media were withdrawn from Erlenmayer flasks for the estimation of monocrotophos residues by TLC and HPLC analysis. The initial (0-day) recovery, with complex extraction and analytical procedures employed, of monocrotophos immediately after its application to the mineral salts medium was 94%. There was an appreciable decrease in the levels of monocrotophos during the incubation period even in uninoculated control. Thus, by the end of 4th day, about 21% of added monocrotophos was lost from the uninoculated medium and this observation reveals that abiotic degradation of monocrotophos also occurred (Figure 3). Conversely, Gundi and Reddy (2006) noticed significant decrease in the monocrotophos in sterile soils.

By the end of 1st, 2nd, 3rd and 4th day 13, 20, 24, 30% and 18, 33, 37, 45% of monocrotophos was degraded, by *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, when the mineral salts medium was supplemented with monocrotophos in comparison to uninoculated controls (Figure 3). Simultaneously, 12, 22, 26, 30% and 18, 26, 37, 40% of N-methylacetoacetamide was recovered from the media inoculated with the *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 respectively, at the same incubation period (Figure 4). Simultaneous decrease in the amount of monocrotophos

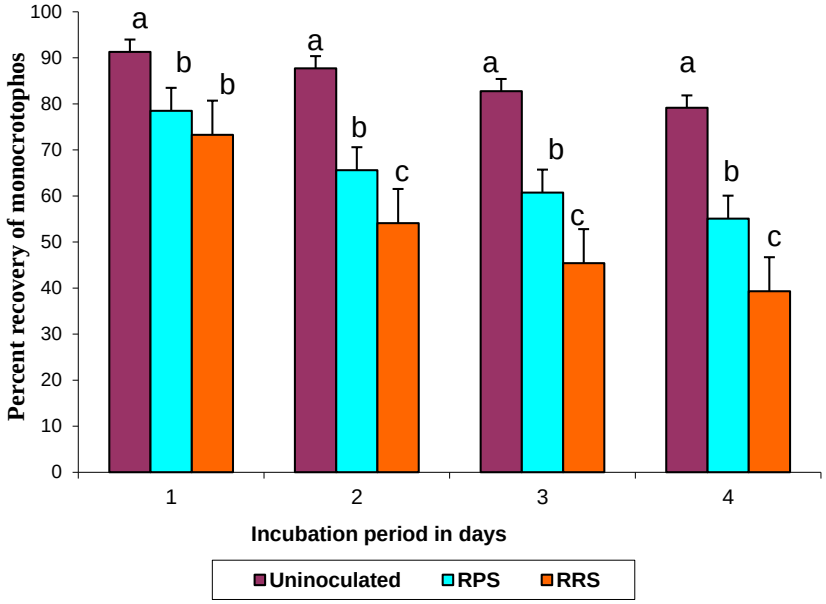

Figure 3. Degradation of monocrotophos by bacteria isolated from soil. Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test**.** Values represented in figure are means of three replicates. RPS = *R. phenolicus* strain MCP1; RRS = *R. ruber* strain MCP-2.

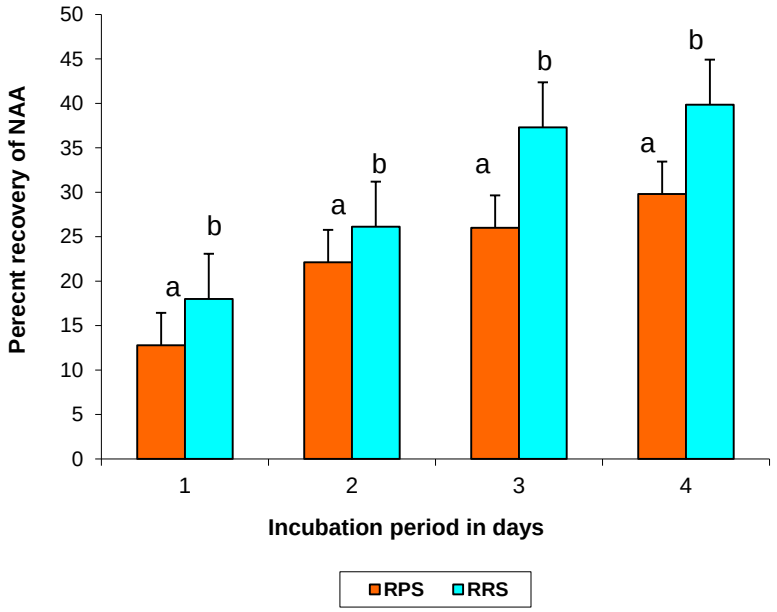

Figure 4. Percent recovery of N-methylacetoacetamide released from monocrotophos by solvent extraction from the culture media after incubation period. Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test**.** Values represented in figure are means of three replicates. RPS = *R. phenolicus* strain MCP1; RRS = *R. ruber* strain MCP-2.

with a concomitant increase in the level of its metabolite, N-methylacetoacetamide clearly indicates the degradation of parent compound. There was a significant decrease in the amount of monocrotophos in the media

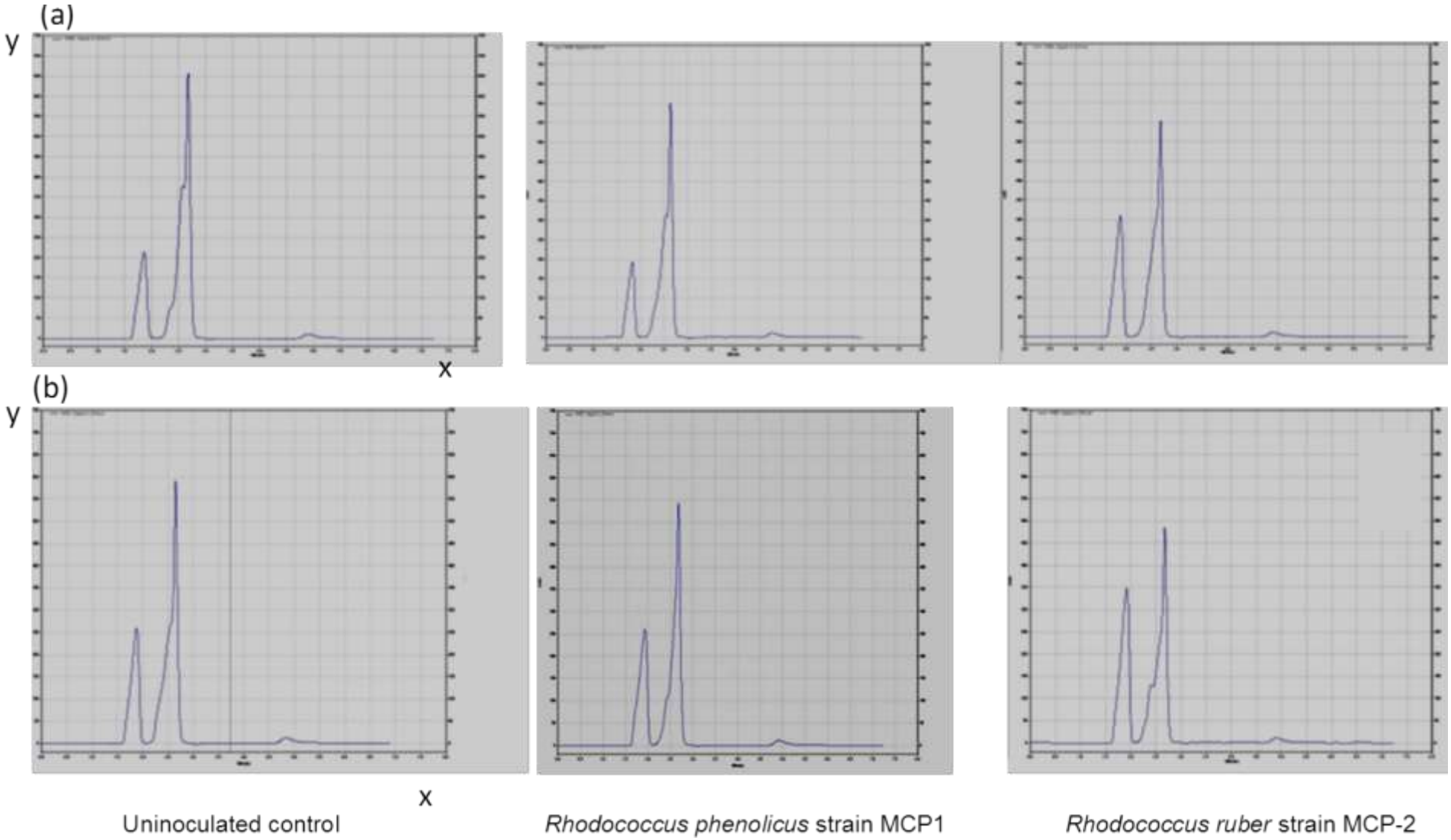

Figure 5. HPLC chromatograms of monocrotophos residues, obtained by solvent extraction from the culture media after (a) First day; (b) Second day of incubation. x = Retention time (RT), 2.5 - 2.7 min; y = Milli absorbance units (mAU).

inoculated with bacteria in comparison with uninoculated control as revealed by the HPLC chromatograms (Figures 5 and 6). Quantitative analysis of the residues of monocrotophos in organic solvent extraction of the medium by thin-layer chromatography showed the presence of

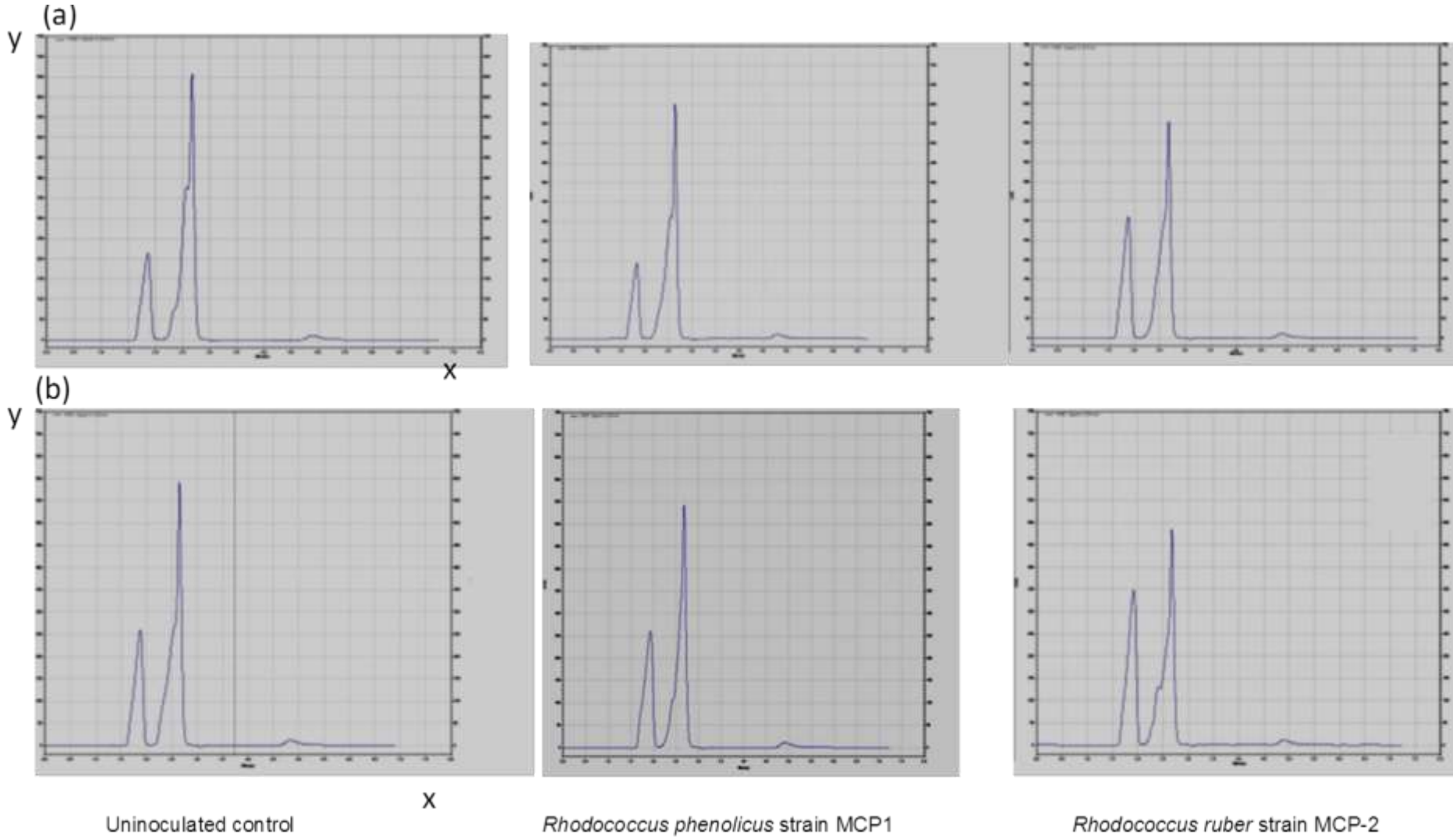

Figure 6. HPLC chromatograms of monocrotophos residues, obtained by solvent extraction from bacterial culture media after (a) third day; (b) Fourth day of incubation. x = Retention time (RT), 2.5 - 2.7 min; y = Milli absorbance units (mAU).

N-methylacetoacetamide in samples inoculated with the *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2. Formation of N-methylacetoacetamide by the hydrolysis of monocrotophos is the major route of degradation. Comparatively *R. ruber* strain MCP-2 showed

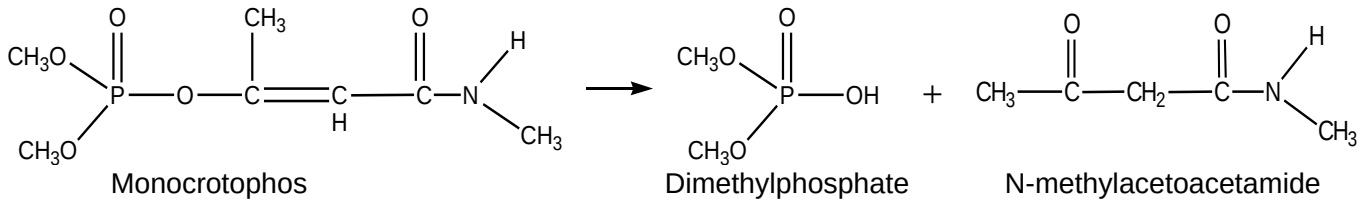

Figure 7. Hydrolysis of monocrotophos in mineral salts media.

highest degradation rate of monocrotophos than *R. phenolicus* strain MCP1 (Figure 3).

Metabolic reactions such as N-demethylation, O-demethylation, hydroxylation of N-methyl groups and cleavage of phosphate-crotanamide linkage occurred in the metabolism of monocrotophos by plants (Skripsky and Loosli, 1994), animals and in microbial cultures (Bhadbhade et al., 2002a, b) with formation of O-decimethyl monocrotophos monomethyl phosphate, dimethyl phosphate, N-methylacetoacetamide and N-methlybutyramide. In the current study, recovery of N-methylacetoacetamide in significant percentages (30-40%) from both the bacterial strains inoculated mineral salts media clearly indicates hydrolytic cleavage of bond between phosphate and crotonamide as shown in Figure 7. This metabolite was not toxic to animals in comparison with monocrotophos and O-desmethylmonocrotophos (Guth, 1994). However, the fate of N-methylacetoacetamide is exactly not known. This metabolite appears to be mineralized to CO_2 and NH_3 through the formation of methylamine and acetoacetate in the environment (Gundi and Reddy, 2006).

Studies on insecticide biodegradation revealed useful information for development of efficient technologies by manipulating suitable microbial strains for environmental development. Soil fungi capable of degrading monocrotophos were isolated from various geographical and environmental sites (Bhalerao and Puranik, 2009). *Rhodococcus* sp. are the potential degraders of persistent environmental pollutants/pesticides. Biotechnological application of this group of bacteria is based on the peculiaraties of their metabolism. Single strain can degrade multiple organic pollutants, for example, *Rhodococcus* strain DEE5151 utilize a broad range of alkyl ethers including diethyl ether, di-*n*-propyl ether, di-*n*-butyl ether, phenetole as sole carbon/energy sources (Kim and Engesser, 2004; Solyanikova and Golovleva, 2011). Annamaria et al. (2010) studied the degradation of carcinogenic/toxic compounds, RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine), by *Rhodococcus* sp. strain DN22.

Conclusions

The results of the present study indicate that the hydrolysis appeared to be a major pathway of degradation of monocrotophos with formation of N-methylacetoacetamide as degradation product. *R. ruber* strain MCP-2 showed degradation of 15% higher rate of monocrotophos than *R. phenolicus* strain MCP1. At the end of 4th day incubation around 30 to 45% of monocrotophos was mineralized by the isolates in comparison to uninoculated control. These data showed the efficiency of monocrotophos degradation by a bacterial isolates over a short time period. The results indicate the promise of the bacterial isolates *R. phenolicus* strain MCP1 and *R. ruber* strain MCP-2 in the bioremediation of organophosphorus pesticide-contaminated soils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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