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ORIGINAL ARTICLE

MICROBIAL DEGRADATION OF MONOCROTOPHOS BY PSEUDOMONAS STUTZERI

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Abstract:

Monocrotophos (MCP), an organophosphorus insecticide, is widely used to control insects on crops. Being readily water soluble and highly toxic, its removal from contaminated soil and water becomes inevitable. For the evaluation of biomineralization of monocrotophos, a bacterial strain, Pseudomonas stutzeri MTCC 2300 which is capable of degrading monocrotophos, was obtained from MTCC. The ability of the strain to mineralize monocrotophos was investigated under different culture conditions. The strain was able to degrade MCP to the extent of 96%, from the medium containing MCP at the concentration of 500mg/l-1, within 6 days at 30°C. The culture degrades MCP to carbon dioxide, ammonia and phosphates were determined. In the present study, the results revealed that, the microbial strain, Pseudomonas stutzeri MTCC 2300 could be used for the bioremediation of waste water and pesticide contaminated soil.

KEYWORDS:

Organophosphrous, Pseudomonas stutzeri, Monocrotophos, Biodegradation.

INTRODUCTION:

India is primarily an agro-based country with more than 60–70% of its population dependent on agriculture. However, 30% of its agricultural produce is lost owing to pest infestation. There is an urgent need for the development of an effective pest control strategy. In the absence of a better alternative, deployment of pesticides becomes inevitable despite their known hazardous effects. Utilization of pesticides in India is about 3% of the total world consumption and is increasing at the rate of 2–5% per annum.

Monocrotophos dimethyl-(E)-1-methyl-2-(methylcarbamoyl)vinyl phosphate] is an organophosphorus, nonspecific systemic insecticide and acaricide, used to control common mites, ticks, aphids, scale insects, leaf hoppers, mites ,spiders and foliage pests, by contact and stomach action. Its water-soluble nature helps to penetrate quickly into plant tissue (Tomlin, 1994). The pesticide monocrotophos is no longer under patent protection and is produced by at least 15 manufacturers, some of them producing an estimated 40% of total annual production (Anonymous, 1997). The total sales of this widely used pesticide are roughly 3% of all insecticide products. It is registered in approximately 60 countries. The product was withdrawn from use in the U.S. and the European Union, but is registered in Austria, France, Spain, Italy, and Greece (Watterson, 1988). MCP is still widely used in India for the protection of cash crops such as cotton, sugarcane, groundnut, tobacco, maize, rice, soybeans, and vegetables (Vig et al., 2001; Bhadbhade et al., 2002a).

Monocrotophos was amongst the top 15 pesticides used in the 20th century (Anonymous,2005). Monocrotophos is a water-soluble organophosphate insecticide with high oral and moderate dermal toxicity and a half-life of 14–21 days. The toxicologically relevant mode of action is the inhibition of

choline esterase activities (Skripsky and Loosli,1994). It is mobile in soil and hence has a potential for groundwater contamination. Monocrotophos is weakly sorbed by soil particles because of its hydrophilic nature, increasing a threat of groundwater contamination due to leaching (Singh and Singh, 2003). It is one of the most toxic substances ever developed and has been found to be highly toxic to birds with the LD50 in birds being 0.9–6.7 mg kg-1 (IPCS, 1993). It is highly irritating to the eyes and produces in coordination, slurred speech, loss of reflexes, weakness, involuntary muscle contractions, and paralysis of the body (Anonymous, 1997).

Biodegradation of monocrophos by soil bacteria was studied by Rangaswamy and Venkateshwaralu (1992).They isolated several strains of Bacillus and one isolate of Azospirillum lipoferum, which were capable of degrading the insecticides. Microbial degradation was more pronounced and rapid than chemical decomposition. Monocrotophos was the most susceptible to bacterial degradation.
Biodegradation is a reliable and cost-effective technique for pesticide abatement, and a major factor determining the fate of organophosphorus pesticides in the environment (Kerteszet al., 1994; Munnecke and Hsieh, 1974). Biodegradation of monocrotophos is dependent on the pH of the soil. At 25 °C, half-lives of 26, 134, and 131 days were calculated at pH 9, 6 and 3 respectively (Lee et al., 1990).

Bacterial transformations have been the main focus in research on organophosphate pesticide degradation. Pseudomonas aeruginosa, Clavibacter michiganense (Singh and Singh, 2003), Arthrobacter atrocyaneus, Bacillus megaterium and Pseudomonas mendocina (Bhadbhade et al., 2002b), Agrobacterium radiobacter (Horne et al., 2002), and other Pseudomonas species (Ramanathan and Lalithakumari, 1999) have been reported to degrade monocrotophos in solutions and soils.
The objective of the present study is to determine the potential of the Pseudomonas stutzeri to degrade the monocrotophos

MATERIALS AND METHODS:

Chemicals

Technical-grade monocrotophos (sigma-aldrich chemical Pvt Limited., Bangalore, India) was used in this study. A stock solution of monocrotophos at a concentration of 10,000 mg l-1 was prepared in distilled water. Working solutions were prepared from stock solutions. All other chemicals, reagents and solvents used in the present study were of analytical grade.

Microorganism and medium:

Pseudomonas stutzeri MTCC 2300 strain obtain from Microbial Type of Culture Collection, Chandigarh was used for the biodegradation of monocrotophos in the present study. Minimal salt medium (MSM) contained NH 4NO3 (1.5 g l-1), K2HPO4 (1.5 g l-1), KH2PO4 (0.5 g l-1), MgSO4 (0.2 g l-1) and NaCl (0.5 g l-1) (pH 7.0), supplemented with monocrotophos as a carbon source at a final concentration of 500 mg l-1. The culture was maintained on agar slant containing monocrotophos for the further use.

Inoculum preparation for monocrotophos degradation studies

A single pure colony of Pseudomonas stutzeri MTCC 2300 was streaked onto agar plates of MSM containing monocrotophos (500 mg/l-1) as the sole carbon source. After incubation at 30oC for 48 h the growth was washed by 1 ml sterile MSM, and 0.5 ml aliquots of the suspension were used to inoculate a series of liquid cultures (50 ml). Liquid cultures were incubated at 30oC on an orbital shaker at 120 rpm. At periodic time intervals constant aliquots were withdrawn and used to assess bacterial growth by measuring in UV-visible spectrophotometer (Shimadzu, Japan, Model UV-1601) at 600nm. At this point an aliquot of 0.5 ml was again transferred into 50 ml fresh liquid media. This procedure was repeated twice more to maximize the degrading potential of the isolate. The final liquid cultures obtained were used as an inoculum throughout the study. The controls were also run parallel aseptic condition, medium without monocrotophos which inoculated with Pseudomonas stutzeri MTCC 2300 and uninoculated medium with monocrotophos.

Degradation of monocrotophos in liquid media

The Pseudomonas stutzeri MTCC 2300 inoculum was transferred to MSM medium (1% v/v) containing monocrotophos (500 mg/l-1) as a carbon source. At the same time another flask containing glucose (0.05%), as an alternative carbon source, were also inoculated and incubated at 30oC on

an orbital shaker at 120 rpm. Non-inoculated media were also run in parallel as control.

Estimation of Co2

Mineralization of Monocrotophos by Pseudomonas sutzeri MTCC 2300 was checked by estimating the amount of CO2 evolved. A series of flask (250ml Erlenmeyer) containing 100 ml of mineral salt media with monocrotophos 500 mg l-1 was inoculated with Pseudomonas stutzeri MTCC 2300. The flask were incubated in different air tight jars containing glass beakers filled with standard sodium hydroxide solution to trap the evolved CO2. The flasks were removed after 48 hrs of incubation. The dissolved CO2 concentration in alkali solution was estimated by titrating it with 1N hydrochloric acid in the presence of phenolphthalein and barium chloride (Bhalerao and puranik, 2007).

Detection of Ammonia

The complete mineralization of monocrotophos was further checked by detection of ammonia. Methylamine is known intermediate product of the breakdown of monocrotophos (Bhadbhade et al., 2002 a). The mineral salt medium agar plates containing either methylamine (500mgl-1) or monocrotophos (500mgl-1) and phenol red indicator were prepared and inoculated with the culture and the plates were incubated at 30oC for 8 days. After the incubation period the plates were observed for the colour change from yellow to pink of the medium. The appearance of pink colour indicates the production of ammonia.

Enzyme assay for Phosphate Estimation

The assay for enzyme phosphatase was performed with the culture free broth harvested after 6 days of incubation. Monocrotophos and p-nitrophenol phosphate were used as the substrates for enzyme activity as per Raghuramalu et al. (1983). Released p-nitrophenol was estimated by using UV-visible spectrophotometer at 400 nm. Enzyme activity was defined as the amount of enzyme required to release 1µmole of p-nitrophenol per minute per millilitre under standard assay conditions.

The amount of soluble inorganic phosphate, a product of monocrotophos degradation by pseudomonasstutzeri MTCC 2300 was from culture free mineral salt medium by the molybdate method (Greenberg et al.,1992).

Detection of enzyme esterase.

For the detection of enzyme esterase, the isolates were spot inoculated on nutrient agar and MCP agar plates containing 1% (v/v) tributirin in water emulsion, respectively, and the plates were incubated at 30C for 48 h to observe esterase activity. To ensure that the isolates were not lipolytic, they were spot inoculated on nutrient and MCP agar plates containing 10% (v/v) groundnut oil in water emulsion and the plates were incubated at 30oC for 48 h.

RESULTS AND DISCUSSION:

Degradation of monocrotophos in liquid media by Pseudomonas stutzeri MTCC 2300

Figure1 reveals the GC analysis of the degradation of the monocrotophos. As per the Fig. 1 it indicates that there was a considerable removal of monocrotophos (500 mg/l-1) in Pseudomonas stutzeri MTCC 2300inoculated media, either in the presence or absence of glucose when compared to Pseudomonas stutzeri MTCC 2300 free media. The monocrotophos degradation in presence of glucose was relatively slow - 46% of the rate observed when monocrotophos was present as the sole carbon source. This can be explained by the preferential use of glucose by the Pseudomonas stutzeri MTCC 2300 as a carbon source instead of monocrotophos during the first 2 days. There are very few organophosphorus insecticides that could be used as the sole carbon source (Singh and Singh, 2003).

After four days, the degradation of monocrotophos, either in presence or absence of glucose, showed no significant difference. Thus, it may be that, monocrotophos consumption by Pseudomonas stutzeri MTCC 2300 commences after 48 h, when glucose levels have been depleted. Monocrotophos was completely degraded in the inoculated cultures after 6 days. during monocrotophos degradation an increase in optical density occurred, suggesting bacterial growth of P. stutzeri MTCC 2300 (Fig. 2 a, b). Zidan and Ramadan (1976) reported 75% and 50% degradation of MCP within 4 days at the concentration 200 mg l-1 by Aspergillus and Penicillium sp., respectively.

Mineralization of Monocrotophos:

Carbon dioxide, soluble inorganic phosphates, and ammonia are assumed to be the end products of Monocrotophos mineralization. When the chromatogram was sprayed with 1-chloro-2,4-dinitrobenzene, a yellow spot at RF value of 0. 45were observed in the aqueous phase of pseudomonas stutzeri MTCC 2300. This spot corresponded with that of standard methylamine. Uninoculated control medium did not show the spot of methylamine. (data not shown) The production of esterase enzyme by pseudomonas stutzeri MTCC 2300 with the release of methylamine as one of the metabolites of monocrotophos. Biomineralisation of MCP to phosphates, ammonia and carbon dioxide was brought about through the formation of intermediate compounds namely one unidentified metabolite, methylamine and volatile fatty acids like acetic acid or n- valeric acid (Bhadbhadeet al., 2002a).

Detection of ammonia:

Since methylamine is known to be toxic, ability of the organisms to breakdown methylamine further was checked. In the present study, when the pseudomonas stutzeri MTCC 2300 was inoculated on medium containing methylamine, change in colour of the medium was seen, indicating production of ammonia as an end product. It was seen that the initial pH of the medium (pH 6.8) was changed to alkaline (pH 7.8–8.0) due to the formation of ammonia from methylamine. This caused a change in the colour of the medium from yellow to pink. Such colour change was not observed in the control medium. The plates containing monocrotophos have demonstrated the production of urease and release of ammonia, as there was a change in colour of an indicator, phenol red, from yellow to pink. Methylamine, phosphates, ammonia, volatile fatty acids, carbon dioxide, and one unidentified compound were reported as the intermediates of MCP degradation by bacterial cultures (Bhadbhade et al., 2002a)

Detection of carbon dioxide

The respiration trend of Pseudomonas stutzeri MTCC 2300 obtained for utilization of monocrotophos is shown in Fig. 3. The rate of carbon dioxide evolution was found to be increased in the first 4 days of incubation and decreased in subsequent days. No significant change was noted after 8 days in carbon dioxide level. Since the production of carbon dioxide could be linked to the decline in monocrotophos concentrations under restricted conditions Fig.3. A microbial metabolic quotient based on estimation of released carbon dioxide has been used to measure the effect of pesticide on the soil microbial community. Mucke (1994) has reported similar mineralization of MCP to carbon dioxide in animal system. In the present study, the release of carbon dioxide during growth of culture in medium containing MCP indicated mineralization of monocrotophos. A positive correlation was observed between the pesticide transformation rate and microbial respiration activity in soil of different ecosystems (Jones and Ananyeva, 2001).

Detection of phosphates

The release of phosphates due to degradation of monocrotophos with respect to time showed an increase in phosphatase units with simultaneous increase in soluble phosphate are illustrated in Fig.4. The fig.4 revealed that the amount of phosphate released increases with increase in the incubation period. It is also associated with a corresponding decrease in the monocrotophos concentration, indicating that the phosphates are being released as a result of monocrotophos degradation. Degradation or detoxification of organophosphorus pesticides by the action of microorganisms is generally through the hydrolysis of P–O alkyl and P–O aryl bonds. This reaction is considered as the most significant step in the detoxification of organophosphorus compounds. The hydrolase enzyme responsible for catalysing this reaction is referred to as an esterase or phosphotriesterase (Kumar et al., 1996), or phosphatase (Rosenberg and Alexander 1979) and is considered to be the most important enzyme in the bacterial metabolism of organophosphates.

Detection of esterase:

A clear zone around the colonies on monocrotophos agar plates containing 1% (v/v) tributirin, indicated that pseudomonas stutzeri MTCC 2300 produced the enzyme esterase. No zone of clearance was observed around the colonies on nutrient and monocrotophos agar plates containing groundnut oil, indicated that the strain were not lipolytic. The cell free supernatant of the cultures grown in nutrient broth showed a zone of clearance around the agar wells in nutrient agar containing 1% (v/v) tributirin and

monocrotophos agar plates without ammonium sulphate, indicating that the enzyme produced by the strain was of quite constitutive nature. In the present study, Pseudomonas stutzeri MTCC 2300 were found to produce methylamine as the metabolite formed by the enzyme esterase, which could be an amidase capable of selecting amides as substrates, since esterases sometimes attack amide linkages (Hassal, 1990). Such metabolite has been reported in the degradation of many organophosphorus pesticides, such as dicrotophos, which is first demethylated to MCP and then is further degraded, with the formation of methylamine (Eto, 1974).

CONCLUSION:

In the present study, Pseudomonas stutzeri MTCC2300 showed a very high efficiency of monocrotophos degradation over a short time period. From the result observed in the present study, Pseudomonas stutzeri MTCC 2300 degraded of monocrotophos and formed metabolites, namely methylamine, phosphates, ammonia, and carbon dioxide. The results of the present study therefore, indicate that Pseudomonas stutzeri MTCC 2300 is a promising candidate for application in the removal of monocrotophos and other organophosphorous in contaminated soils, water and crops.

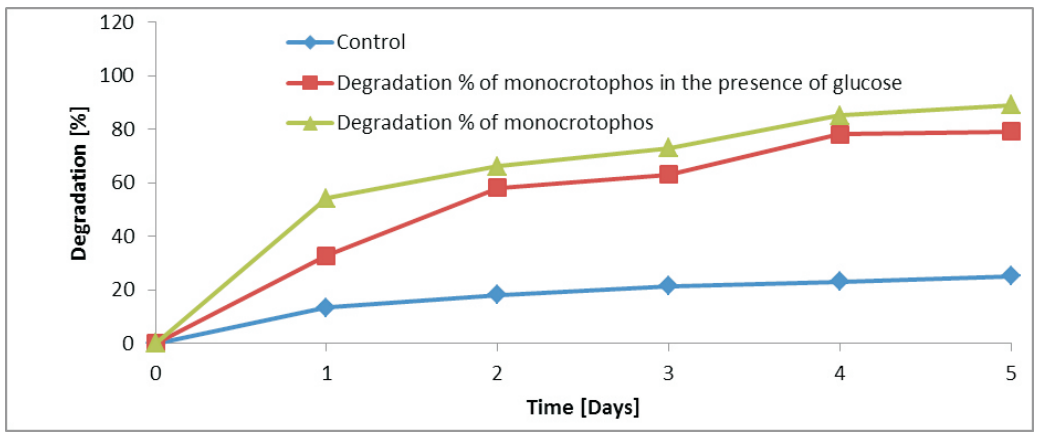

Fig 1. Degradation and % recovery of monocrotophos

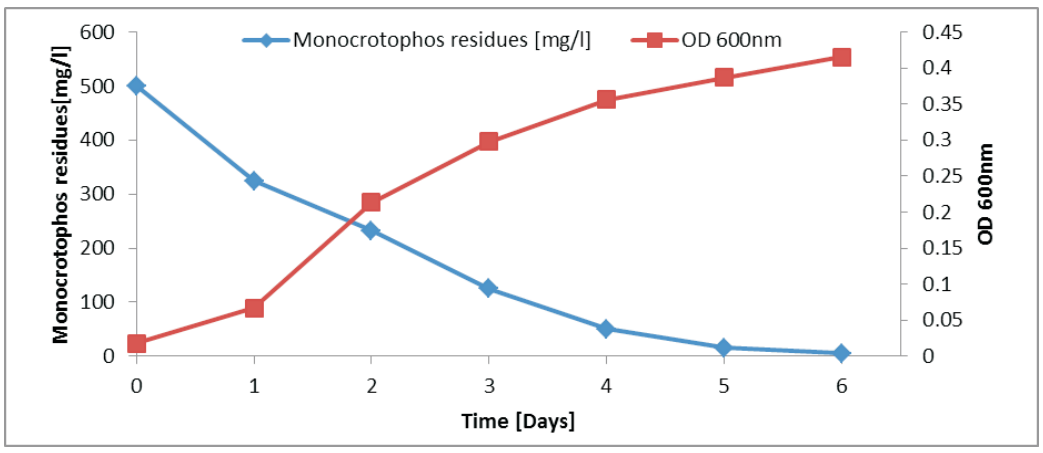

Fig 2a

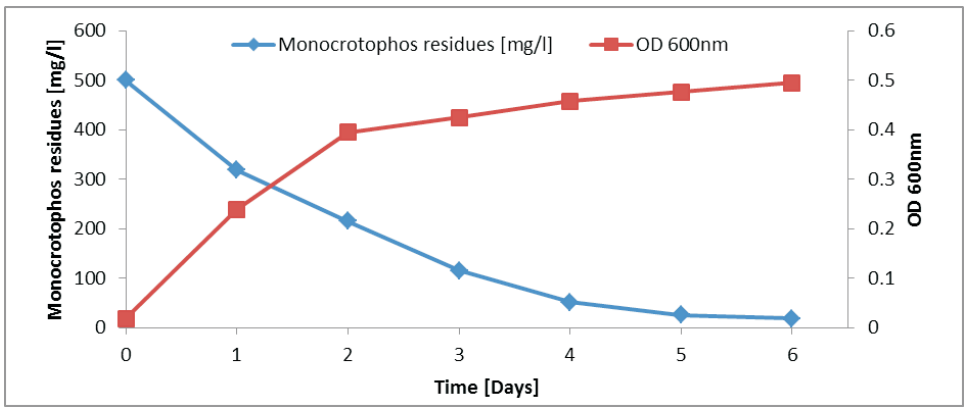

Fig 2 b

Fig 2. A. b optical density curve of Pseudomonas stutzeri MTCC 2300 on monocrotophos (500mg l-1) as a sole carbon source only or in the presence of glucose (0.05%) respectively and concentration of monocrotophos residues

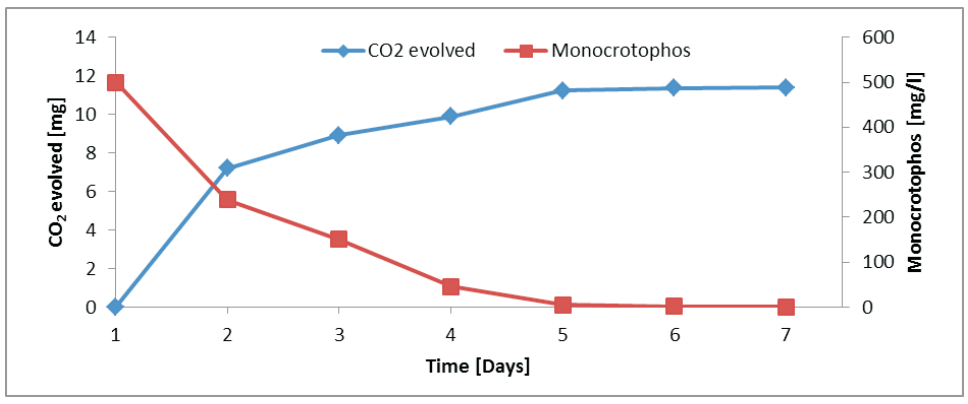

Fig 3.Evolution of carbon dioxide by Pseudomonas stutzeri MTCC 2300 in the presence of MCP (500 mg l-1) as a sole source of carbon.

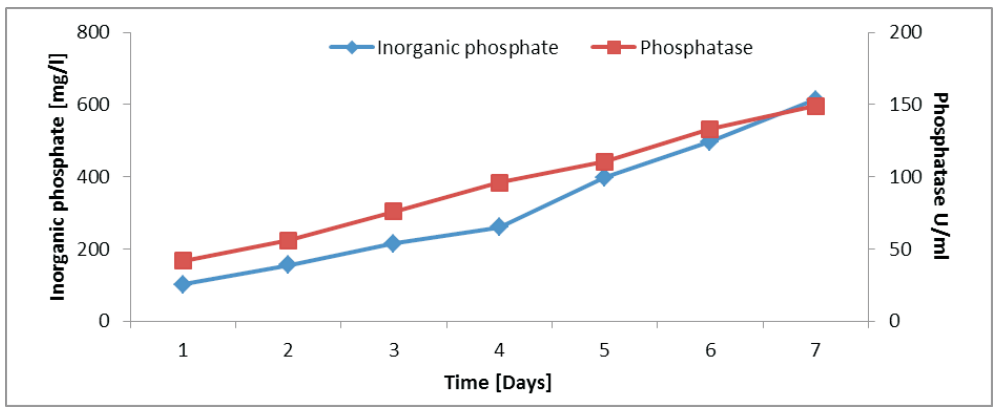

Fig 4. Amount of inorganic phosphate and phosphatise enzyme units released in the broth of Pseudomonas stutzeri MTCC 2300 grown in presence of monocrotophos

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