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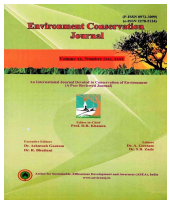

Biodegradation of quinalphos by gram negative bacteria *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A

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ABSTRACT

Five bacterial strains for biodegradation study were initially isolated from quinalphos exposed soil from cotton field of Indian Agricultural Research Institute, India by Serial dilution and enrichment technique. Amongst them, two strains *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A were exposed to different concentrations (50-250 ppm, parts per million) of quinalphos to evaluate their tolerance and the optimum concentration of quinalphos supported growth. Growth kinetics of the isolates was studied by means of optical density of the culture media (150 rpm, rotation per minute) at 30ºC for 15 days. The optimal concentration of quinalphos for the growth of *Pantoea agglomerans* was 100 ppm, while for *Acinetobacter sp.* dcm5A was 200 ppm. Bacterial strains *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A degraded quinalphos 60% and 79.7% respectively in 7 days incubation and 100% degradation by both strains was observed after 13 days of incubation.

Introduction

Quinalphos (O,O-Diethyl O-quinoxalin-2-yl phosphorothioate), a potent organophosphorus insecticide was introduced into the market by Bayer AG under the trade name Bayrusil. Owing to its high insecticidal properties, this chemical is widely used to suppress and control the pest attack in a variety of crops such as rice, cotton and groundnut (Armes *et al.,* 1992). It is an acetylcholinesterase inhibitor and acts through direct contact and stomach action. The toxic effects of quinalphos in animals have been reported (Bokonjie *et al.,* 1987; Srivastava, 1989). In soil, rapid degradation of quinalphos into quinoxalin-2-ol has been observed under aerobic conditions with a DT_{50} of about three weeks. Further breakdown of quinalphos into CO_2 and polar metabolites was also reported (Schimdt, 1972). Quinalphos degraded in soil under anaerobic conditions in a red sandy loam and sandy clay soil with a half-life of 13 days and in a black clay soil with half-life of 15 days have been reported. The metabolite 2-hydroxyquinoxaline was identified and quantified and could be detected in soil after complete dissipation of parent compound after 20 days (Babu *et al.,* 1998). The hydrophilic nature of quinalphos is weakly sorbed by soil particles. Hence, there is a threat that it can be seen in wastewater arising from quinalphos manufacturing units or it may enter into the rainwater and groundwater when sprayed on crops because of leaching. The leaching upto 0.025 ± 0.0013 µg quinalphos g-1 d. wt. soil was observed in 60 days (Mayanglambam *et al.,* 2005).

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The leaching of insecticide quinalphos was observed up to a depth of 9 inch in soil (Awasthi *et al.,* 1984). The translocation of quinalphos in soil was also reported (Karoly *et al.,* 1988; Bhavani, 2020). In soil thin layer and column chromatography, quinalphos shows lower mobility. Due to leaching of this insecticide quinalphos, groundwater may be contaminated and it also affect the diversity of non-target soil organisms.

Decomposition of toxic organics by soil microorganisms is the most important and safe and natural technology and a good alternative to chemical and physical methods such as incineration and solvent extraction (Comeau *et al.,* 1993). Bacteria which has the capacity of degrading several pesticides, were isolated from soil. They include diazinon-degrading *Flavobacterium sp.* (Sethunathan and Yoshida, 1973), chlorpyriphos-degrading *Flavobacterium sp*. (Mallick *et al.,* 1999), monocrotophos-degrading *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423 (Bhadbhade *et al.,* 2002), metametron-degrading *Rhodococcus sp*. (Parekh *et al.,* 1994), ethoprophos-degrading *Pseudomononas putida* (Karpouzas and Walker, 2000), monocrotophos-degrading *Pseudomononas aeruginosa* F10B and *Clavibacter michiganense* subsp. *inidiosum* SBL 11 (Subhas and Singh, 2003; Kaur *et al.,* 2021; Garg *et al.,* 2022). The role of microbes in biodegradation is well known in all over the world. However, a scanty information is available on biodegradation of quinalphos by bacteria in soil. Therefore, this study has been conducted to evaluate the capability of the bacterial strains isolated from soil exposed to quinalphos by enrichment and adaptation culture technique in degradation of quinalphos.

Material and Methods

Isolation, screening and growth conditions of the microbial isolates

Five bacterial strains for biodegradation study were isolated from quinalphos exposed soil (25% EC@4L/ha, sprayed at regular intervals) from cotton field of IARI, India by Serial dilution and enrichment technique. Enrichment was done by adding 5.0 g of soil sample in 100 ml basal medium BM and then incubating at 37°C for 15 days. The basal medium consists of Glucose (5.0 g), K_2HPO_4 (0.2 g), KH_2PO_4 (0.2 g), $(NH_4)_2SO4$ (1.0 g), NaCl (0.1 g), $MgSO_4.7H_2O$ (0.2 g), KCl (0.2 g), $FeCl_3$ (0.003 g), $CaCl_2$ (0.02 g). The medium was prepared by dissolving all the chemicals in 998 ml double distilled water followed by maintaining the pH at 7.2 using 1N NaOH / 1N HCL. The medium was autoclaved at 121°C temperature and 1.5 kg /cm^2 for 15 minutes. After autoclaving, 2.0 ml of sterile Trace Element Solution was added to the medium *(6)*. The composition of the Trace Solution was as follows: H_3BO_3 (2.80 g), $MnCl_2.4H_2O$ (1.86 g), $CuSO_4.5H_2O$ (0.20 g), Na_2MoO_4 (0.75 g), $CoSO_4.7H_2O$ (0.20 g), $ZnSO_4.7H_2O$ (0.25 g), 8 M HCl (2.0 ml) in 998 ml double distilled water. After 15 days of incubation, different dilutions of medium were prepared in sterilized distilled water, and then 100 µL of this solution was spread evenly using a sterilized glass spreader on Nutrient agar plates which composed of Peptone (10 g), Yeast Extract (5 g), NaCl (5 g), Agar (16 g) in 1000 ml double distilled water at 6.5 pH. The plates were then incubated at 30°C for 24 hrs. Each bacteria colony was treated as a different strain and pure homogenous strains were acquired by repeated streaking for more than 20 times.

The dependence of the bacterial strains on quinalphos (technical grade obtained from Montari Industries Limited, Nehru Place, New Delhi, India) was determined by variably modifying the composition of Basal Medium BM (the medium with both carbon and phosphorous sources) with a known concentration of quinalphos into Modified Basal Medium One MBM1 (the inorganic phosphorus sources, K_2HPO_4 and KH_2PO_4 in BM were replaced with quinalphos) and Modified Basal Medium Two MBM2(both the inorganic phosphorus and carbon sources, K_2HPO_4, KH_2PO_4 and glucose in BM were replaced with quinalphos). The isolates were initially screened for their efficiency in degradation of quinalphos by growing them in Basal medium BM with quinalphos (100 ppm) as additional source of carbon and phosphorus. The cultures were kept on the shaker with 150 rpm (revolutions per minute) speed at 30°C.

Soil characteristics

Soil was analyzed for physico-chemical parameters using standard methods. Soil pH was calculated by using saturated soil paste (1:2.5 :: soil : water). Organic carbon was calculated by standard method (Walkley and Black, 1934).

Identification of the bacterial strains

Two best growing bacterial strains were selected out of the initially screened five isolates. DNA isolation was done using DNeasy Tissue Kit (50, Qiagen) followed by 16S rDNA sequence analysis.

. Optimization of quinalphos concentration

Optimum concentration of the pesticide at which there is the maximum growth of two selected strains of the bacteria was studied by taking 25 ml each of MBM1 and MBM2 broth mediums supplemented with various concentrations of quinalphos ranging from 50 ppm-250 ppm in 100 ml flasks. These broths were inoculated with two cultures separately (1.0 ml each of inoculum) and incubated under constant shake condition (150 rpm) in a shaker incubator at 30°C for 15days. Bacterial growth was measured at regular intervals at 576 nm using a spectrophotometer.

Growth pattern of the isolates

To check the growth pattern of bacterial strains, the isolates (1.0 ml each of inoculum) were inoculated in 50 ml each of broth BM, broth MBM1 and broth MBM2 supplemented with optimum concentration of quinalphos (100 ppm for *Pantoea agglomerans* and 200 ppm for *Acinetobacter sp.* dcm5A*)* in 100 ml conical flasks. The flasks were then incubated on rotary shaker (150 rpm) at 30°C. At regular intervals, 1.0 ml of the sample was taken out in an Eppendorf tube for growth curve study. The experiment was conducted in triplicate and growth was measured at 576 nm spectrophotometrically.

Extraction and estimation of residual quinalphos by Gas Liquid Chromatography (GLC)

3 ml aliquots of the inoculated culture mediums were withdrawn on alternate days for 15 days from the flasks where bacterial strains were grown in MBM2 broth and incubated (150 rpm at 30°C). Quinalphos was extracted by partitioning 3 ml of the culture medium containing the pesticide with equal amount of ethyl acetate. This process was repeated for three more times to ensure the complete transfer of Quinalphos from the medium to ethyl acetate. The extracts were evaporated to near dryness (Univapo vacuum evaporator) and dissolved in 100 µL of ethyl acetate. The extracts were then analyzed by GLC.

The extracted samples were redissolved in 5 ml Ethyl acetate, and analyzed on Shimadzu chromatograph model GC-17 AAF, V3, 230 V LV model equipped with electron capture detector (ECD). SGE column; 30 x 0.25 mm i.d., I D-DPI 0.25 UM (Made in Australia). The flow rate of carrier gas used (Nitrogen) was 20 ml min^{-1}, the temperature of Injector, Column and Detector was 300°C, 200°C and 250°C, respectively. One microlitre of sample was injected into the GLC with a 10 µL Hamilton micro syringe. The retention time for Quinalphos was 12.1 minutes. Quinalphos as low as 0.01 □g L^{-1} could be detected accurately. The recoveries of quinalphos were routine more than 90%.

Results and Discussion

Results of present study of preliminary screening of bacteria, which were isolated from soil samples for biodegradation studies are given **(Table 1).** Among the five isolates (T1, T2, T3, T4 and T5),T1 and T2 showed maximum growth in broth supplemented with quinalphos as additional source of carbon and phosphorus. Hence, the two strains of bacteria were selected for further studies.

Table 1: Growth of Five Bacterial Strains[a] in Terms of Optical Density (λ 576nm) in Basal Medium with Quinalphos as Additional Source of Carbon and Phosphorus

Days	T1	T2	T3	T4	T5
0	0.201	0.219	0.183	0.187	0.182
3	0.399	0.310	0.294	0.301	0.212
5	0.602	0.699	0.425	0.330	0.250
7	0.814	0.900	0.614	0.411	0.479
9	0.985	1.086	0.745	0.617	0.682
11	1.193	1.302	0.998	0.823	0.820
13	1.269	1.375	0.914	0.831	0.801
15	1.397	1.414	0.904	0.800	0.711

[a]T1, T2, T3, T4 and T5 are the bacterial strains which were initially isolated for biodegradation study, and two best growing strains T1 and T2 were identified as *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A respectively.

Both the bacterial strains isolated have the capacity that they can survive in the soil either due to tolerance or due to their ability to degrade it, as the soil used in the present investigation was quinalphos exposed (25%EC@4L/ha, sprayedat regular intervals). The role of those organisms capable of responding to pesticides as a substrate and thereby derives energy and utilizable nutrients

for their metabolism is significant in bioremediation study. Availability, low microbial toxicity and high nutritive value of the pesticide seem to be the properties that enhance the biodegradation of pesticides in the soil.

Soil characteristics

Soil used in the current investigations was sandy loam (71% sand, 21% silt and 8% clay) with pH 7.02, organic matter 0.92% and 9.8% moisture.

Identification of bacterial strains

Based on the DNA sequencing results, the bacterial strains T1 and T2 were identified as *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A respectively.

Optimum concentration of quinalphos for bacterial growth

Optimum concentration with quinalphos as a sole carbon and phosphorus sources for the growth of *Pantoea agglomerans* was 100 ppm while for *Acinetobacter sp.* dcm5Awas 200 ppm **(Figure 1).** Rests of the experiments with these two bacterial strains were performed at its optimum concentration of quinalphos.

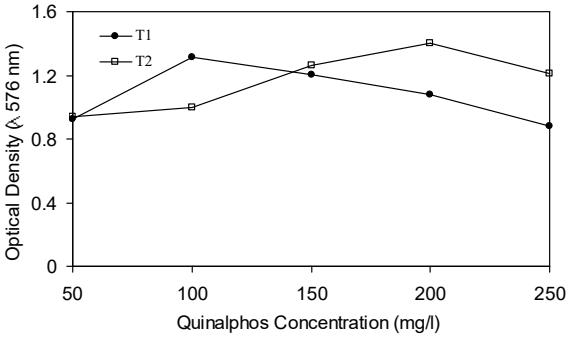

Figure 1: Optimum concentration of quinalphos utilized by *Pantoea agglomerans* T1 and *Acinetobacter sp.* dcm5A T2 as sole carbon and phosphorus sources

Growth pattern of the isolates

Growth kinetics of the isolates was studied by means of optical density of the culture media as well as the bacterial biomass, in 15 days culture. The growth behaviour in terms of optical density as obtained from the present study is given in Figure 2.a for *Pantoea agglomerans* and in Figure 2.b for *Acinetobacter sp.* dcm5A.It was apparent from the figures that when both the bacterial strains were grown in BM broth with normal carbon and phosphorus sources, followed almost linear growth pattern, in which growth increased regularly with days of incubation till 15 days. This was due to the presence of optimum conditions with sufficient and easily available carbon and phosphorus sources in the medium. Bacterial strains while grown in MBM1 broth and MBM2 broth, sigmoid growth pattern was observed in which lag phase followed by log phase and then a period of stability. This is because bacteria initially take more time to adapt themselves in the medium. Once adapted, they produce the enzymes that degrade quinalphos and utilized it as a sole carbon and phosphorus source. No further increase in growth rate was observed after 7 and 9 days for *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A, respectively which may be due to depletion of food sources. This shows that quinalphos concentration might be acting as a limiting factor.

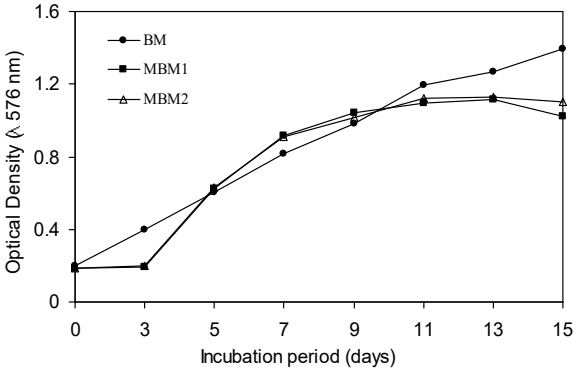

Figure 2a: Growth pattern of *Pantoea agglomerans* in terms of optical density in Basal Medium BM, Modified Basal Medium One MBM1 and Modified Basal Medium Two MBM2

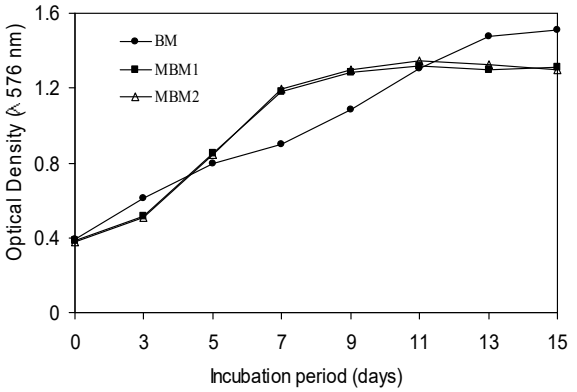

Figure 2b: Growth pattern of *Acinetobacter sp.* dcm5Ain terms of optical density in Basal Medium BM, Modified Basal Medium One MBM1 and Modified Basal Medium Two MBM2

Biodegradation of quinalphos in the medium

GLC analysis confirmed that both the bacterial strains degraded quinalphos and derived energy and utilizable nutrients for their metabolism in the medium supplemented with quinalphos as sole carbon and phosphorus sources. Quinalphos residues by bacterial strains *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A are shown in Table 2. On zero day just after inoculation of bacteria into the growth medium, 96.4 ± 0.11 µg/ml and 187 ± 0.52 µg/ml of quinalphos residues were detected for *Pantoea agglomerans* and *Acinetobacter sp.* dcm5A, respectively in the medium.

Table 2: Quinalphos residues (µg ml^{-1}) after the inoculation of *Pantoea agglomerans* and *Acinetobacter sp.* dcm*5A* in the Medium with Quinalphos as Sole Carbon and Phosphorus Source

Days	T1[a]	T2[b]
0	96.4 ± 0.11	187.0 ± 0.52
3	90.6 ± 0.02	162.4 ± 0.18
5	55.0 ± 0.26	99.5 ± 0.02
7	38.6 ± 0.04	38.0 ± 0.56
9	13.3 ± 0.03	22.7 ± 0.02
11	0.07 ± 0.01	11.4 ± 0.06
13	N D[c]	0.04 ± 0.01
15	N D	N D

[a]**T1 - *Pantoea agglomerans***
[b]**T2 - *Acinetobacter sp. dcm5A***
[c]**N D means that concentrations were below the detection limit**

In case of bacterial strain *Pantoea agglomerans* only 6.02 % of quinalphos was observed to be degraded till 3rd day. Thereafter quinalphos was degraded at a faster rate almost 60% on 7th day which further degraded to 99% by 11th day, but rate of degradation was proceed slower in comparison to degradation rate from 3-5th day. Most significant rate of degradation was observed between 3-5th day which was found to be 60.7%. These may be probably due to nutrient depletion during the log phase of degradation from 3-5th day. 100% degradation of quinalphos by this strain was observed after 11 days of incubation.Similarly, bacterial strain *Acinetobacter sp.* dcm5A degraded quinalphos upto 13% by 3rd day followed by sharp increase in growth rate resulting 80% degradation of quinalphos on 7th day. Most significant degradation rate of quinalphos was observed between 3-5th day that was 61.2% which showed that *Acinetobacter sp.* dcm5A is more efficient in utilizing quinalphos at higher concentration as their nutritive materials for their metabolism in comparison to *Pantoea agglomerans* (Figure 3). After this, degradation rate became slower and 100% degradation of quinalphos by this bacterial was observed after 13 days of incubation.

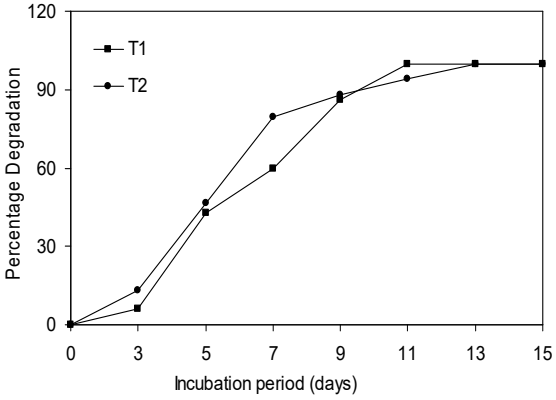

Figure 3: Percentage degradation of quinalphos by *Pantoea agglomerans* T1 and *Acinetobacter sp.*

dcm5A T2 in the medium supplemented with pesticideas a sole carbon and phosphorus sources

Degradation or detoxification of organophosphorus pesticides takes place by the action of microbes. It is generally through the hydrolysis of P-O alkyl and P-O aryl bonds. This reaction is termed as the important step in the detoxification and bacterial metabolism of organophosphorus compounds. The hydrolyses enzyme responsible for catalyzing this reaction is called as an esterase or phosphotriesterase (Brown, 1980; Kumar *et al.,* 1996) or Phosphatases (Rosenberg and Alexander, 1979). As a result of hydrolysis of quinalphos, there is a formation of 2-hydroxyquinoxaline as a primary metabolite like other organophosphorus insecticides, (Babu *et al.,* 1998). However, during the present study, no experiment was done for identification of the degradation products due to the nonavailability of authentic compounds of the metabolism. Similar report on the formation of two unidentified metabolites in culture media that were inoculated with *Chlorella vulgaris, Scenedesmus bijugatus, Synechococcus elongates, Phormedium tenue* and *Nostoc linckia,* isolated from a black cotton soil has been observed (Megharaj *et al.,* 1987). GLC analysis thus indicates that both the

bacterial strains were capable of degrading quinalphos and derives nutritive materials from it when they were grown in the medium without any additional carbon and phosphorus sources.

The parent compound (Rt 12.1 min) was degraded into two unidentified metabolites at Rt 5.6 and 14.4 min by 7th day onwards. Figure 4(A) & 4(B).

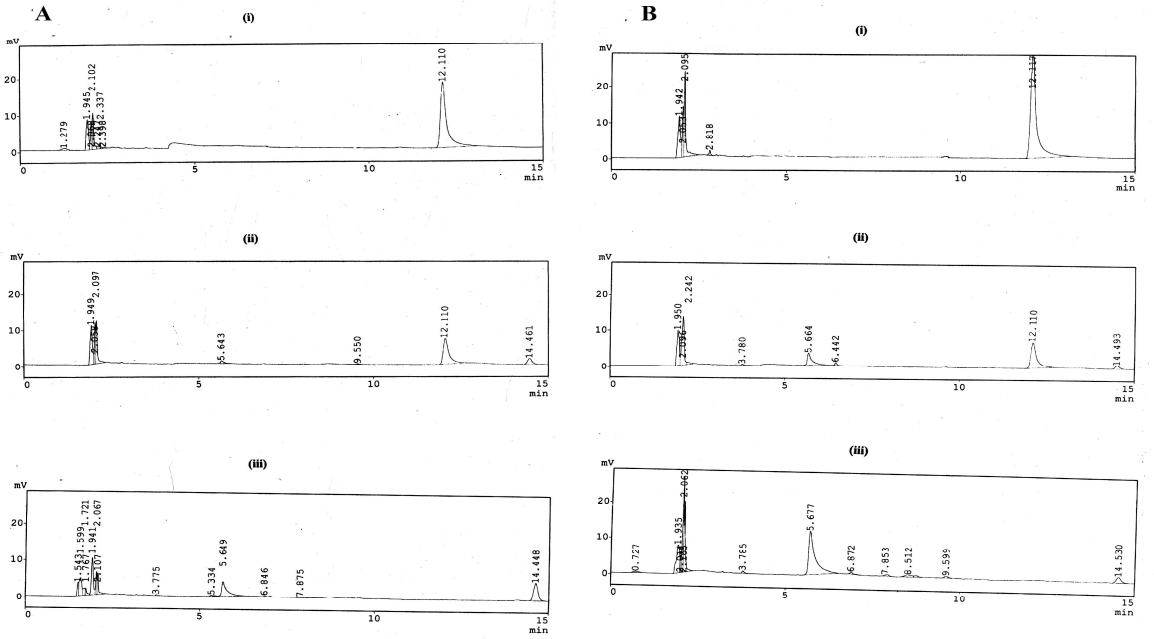

Figure 4: (A) Chromatogram of quinalphos (Rt 12.1 min) degradation by *Pantoea agglomerans* on: (i) Zero day (ii) 7th day (iii) 13th day. (B) Chromatogram of quinalphos (Rt 12.1 min) degradation by *Acinetobacter sp.* dcm5A on: (i) Zero day (ii) 7th day (iii) 15th day

Bacterial degradation of quinalphos has also reported earlier (Rangaswamy and Venkateswarlu, 1992). Both *A. lipoferum* and *Bacillus sp*., were isolated from quinalphos treated soil, degraded quinalphos 32% and 44% respectively by the end of 7 days of incubation. Nearly 56% and 76% were degraded by both bacterial strains *A. lipoferum* and *Bacillus sp*., respectively on 14 days. There was a notable decrease in the concentration of both monocrotophos and quinalphos during the incubation period even in un inoculated controls. From the uninoculated medium, about 40% of added monocrotophos was loss as compared to quinlphos as 16% loss of quinalphos from the corresponding uninoculated samples during the period of 11 days (Rangaswamy and Venkateswarlu, 1992). The results of this study clearly suggested that quinalphos is highly susceptible to microbial metabolism, thus supporting and conforming to the result of earlier reports (Megharaj *et al.,* 1987;Rangaswamy and Venkateswarlu, 1992).

Conclusion

The pattern and behavior of quinalphos in environment and the impacts of its residues on the population dynamics of soil microbes need further investigation in different fields having different crops. The results of current study provide important information about the microbial degradation of quinalphos. The study indicates that soil bacteria are good degraders of quinalphos and therefore could be a reason for its low persistence in the soil. Furthermore, progress in selecting suitable strains of bacteria, which can detoxify or convert the complex pesticide molecules into simple non-toxic residual molecules and use them as a source of nutrition, should lead to the development of field technology for site specific bioremediation for the treatment of soil or ground water which is contaminated by quinalphos.

Conflict of interest

The authors declare that they have no conflict of interest.

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