Journal of Global Biosciences
ISSN 2320-1355
Volume 7, Number 1, 2018, pp. 5331-5341
Website: www.mutagens.co.in

Research Paper

BIODEGRADATION OF ORGANOPHOSPHOROUS PESTICIDE USING IMMOBILIZED ESTERASE AND TOXICITY ASSESSMENT

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Abstract

The prevalent use of organophosphorus pesticides poses a great threat to unintended environment and human health. On account of new arena bioremediation is an efficient techniques and cheap option for detoxification of such pollutants from the ecosystems. The present study is mainly focused on these aspects, isolated and screened Staphylococcus *spp* capable to degraded organophosphorus such as Quinalphos, Chlorpyriphos and monocrotophos pesticides. Accordingly quinalphos are efficiently degraded and the degradation is determined through intracellular esterase pathway. The stabilization of esterase using immobilization on sodium alginate beads and magnetic nanoparticles provides certain advantage as compared to normal enzyme. The present results indicate that the immobilized esterase seems to be a promising procedure for bioremediation, industrial and large scale applications.

Key words: Quinalphos**,** immobilization enzyme (esterase) and *Staphylococcus spp*.

INTRODUCTION

Biodegradation is a process of remove the contaminated pesticides using native degrading microbes system. Organophosphourus compounds such as chlorpyriphos, monocrotophos and quinalphos which are chemical pesticides commonly used to protect the crops from pest such as mites,ticks and spider . The long persistence of the quinalphos in the environment resulted in the bioaccumulation and potential toxicity towards non-target organisms due to which its use has been drastically reduced in the developed countries. This group of compounds has been replaced by the less persistent and more effective organophosphorus compounds. However, most of the organophosphorus compounds possess high mammalian toxicity. Certain concentrations of these substances have a mutagenic effect on organisms, which causes various degrees of genetic material damage and injuries. 0.1µg (0.1 ppb- parts per billion) of pesticide products have partial inhibitory effect on the growth of test plant roots causes chromatid aberrations in cells (Fibras, 2007).The symptom of quinalphos causes in human such as muscular weakness, blurred vision, profuse perspiration,

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confusion, vomiting, pain, and small pupils. There is a risk of death due to respiratory failure (Hayes et al., 1991; Senanayake and Karalliedde, 2012). The plant toxicity was tested by *Allium cepa* ,the test is used for establishing general toxicity and genotoxicity. The international program on plant bioassays (IPPB) has acknowledged *Allium* test for bio monitoring and testing of environment polluters (Jain Rachna et al*.,* 2012).

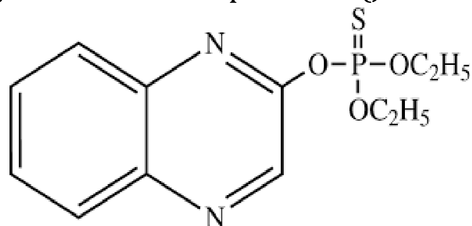

Figure 1.2 Structure of Quinalphos

Metabolism and degradation of Quinalphos

Metabolism of Quinalphos by microbial cultures and in soil includes metabolic reactions such as *N*-demethylation, *O*-demethylation, hydroxylation of *N*-methyl groups and cleavage of the phosphate–crotanamide linkage, with the formation of *O*-desmethylquinalphos monomethyl phosphate, dimethyl phosphate, *N*-methylacetoacetamide and *N*-methylbutyramide. Numerous studies have indicated that microorganisms can use an alternative electron acceptor and can even degrade synthetic organic compounds during anaerobic processes, such as anaerobic respiration. So far, there have been no reports on the anaerobic degradation of quinalphos (Vijay *et al.***,** 2006). The phosphorus is usually present either as a phosphate ester or as a phosphonate. Being esters they have many sites which are vulnerable to hydrolysis. The principal reactions involved are hydrolysis, oxidation, and alkylation and dealkylation (Singh et al., 2003). Microbial degradation through hydrolysis of P-O-alkyl and P-O-aryl bonds is considered the most significant step in detoxification. Both co-metabolic and bio-mineralization of organophosphorus compounds by isolated bacteria have been reported. Hydrolysis of organophosphorus compounds leads to a reduction in their mammalian toxicity by several orders of magnitude. Since most of the research has been directed towards detoxification, studies on the further metabolism of the phosphorus containing products have not been extensive. Hypothetical phospho-ester hydrolysis steps can be postulated, yielding mono-ester and finally inorganic phosphate, but this pathway has not been specifically studied. Analogous phospho-monoesterase and di-esterase, which degrade methyl and dimethyl phosphate, respectively, have been reported in *Klebsiella aerogenes* (Wolfenden and Spence, 1967) and are produced only in the absence of inorganic phosphate from the growth medium. Similarly, a variety of isolates that could use phosphorothionate and phosphorodithionate pesticides as a sole source of phosphorus were unable to utilize these compounds as a source of carbon (Rosenberg and Alexander, 1979).

The current studies reveals that quinalphos may be degraded by the isolated *Staphylococcus spp.* followed by determination of toxicity effect which was observed in Allium cepa root test and immobilization of esterase on magnetic nanoparticle

MATERIALS AND METHODS

Collection of soil sample

The soil samples were collected from various farming sites which were contaminated by commercially available pesticide such as **Quinalphos,** Chlorpyriphos and monocrotophos in agricultural field, Karnataka, India.

Isolation of pesticide degrading microbes

1 g of soil samples were serially diluted in 10ml of saline followed by dilution to appropriate concentration. 100µl of diluted samples were poured on nutrient agar and incubated at 37°C for 24hrs.The bacterial isolates were subjected to enrichment culture technique using Bushnell-Haas media (BH) containing Quinalphos as a sole carbon source and incubated at 37°C. Different concentrations of Quinalphos (1-4 %) were added to BH media for isolating potential pesticide degrading organism. likewise, followed this procedure is for the organophosphours pesticides Chlorpyriphos and monocrotophos.

Biochemical test

The selected potential bacterial isolate was identified by comparing the morphological and biochemical characteristics referred by Bergey's Manual (Buchanan and Gibbons, 1974).

Detection of the degrading enzyme (Esterase)

The 96 hrs cultures were subjected to centrifugation at 10,000 rpm for 15 mins at 4°C. The supernatant was discarded and the pellet was treated with lysis buffer for one hour. The treated sample was centrifuged at 10,000 rpm for 15 mins. 1ml of the supernatant was used as enzyme source. Esterase activity was monitored quantitatively according to the method of Gomori as modified by VanAsperen (1962). A typical assay mixture contained 5ml of 0.5mM substrate solution and 1ml of the enzyme extract. The reaction mixture was incubated for 15 mins at 27°C and the reaction was arrested by the addition of 1ml of DBLS reagent. The reaction mixture was allowed to stand for 30 mins and the intensity of the color formed was measured at 600nm. A standard calibration curve was constructed using 1-naphthol.

Estimation of pesticides (Quinalphos & Chlorpyriphos)

An aliquot of standard solution containing 0.2 to 1.0 ml of respective pesticides were taken separately in a 25 ml graduated tube and to it 1.0 ml of sodium hydroxide was added. The solution was kept for 30 mins at room temperature for complete hydrolysis. Then 1.0 ml of DPAAP was added and shaken thoroughly and kept at 0-5°C for 15 mins for reddish-violet color development from the initial yellow color. The solution was then diluted to the mark with water and absorbance was measured at 560nm against a reagent blank (Janghel *et.al,* 2006). The amount of pesticide was estimated from the standard calibration graph. Biodegradation experiment was carried out using different concentration (1 to 3%) of individual pesticides and their mixture by the bacterial isolate on BH media. The amount of pesticides degraded was estimated using standard calibration curve after 96 hrs.

Toxicity assay

The *Allium cepa* test provides a rapid screening procedure for chemicals and environmental agents which may represent environmental hazards. Root growth inhibition assay was performed as a 96hrs semi-static exposure test (Mamta Kumara et.al., 2009). Healthy equal sized common onion was obtained from the local market, Bangalore, Karnataka, India. The dried outer scales were carefully removed leaving the ring primordial intact. The base of each of the onion bulbs were grown on boiling tube containing 30ml of culture supernatant (4th day of biodegradation) and placed away

from sunlight for 96hrs after which the root length was measured. Results of the general toxicity are indicated by the length of test plants root. Tap water was used as negative control and 1 % pesticide was used as a positive control.

Enzyme Immobilization

Sodium alginate method

A 3% (w/v) sodium alginate solution in 50mM sodium phosphate buffer (pH 7.0) was prepared and warming at 50°C. After cooling down to room temperature, 1ml of culture filtrate was mixed with 9ml of sodium alginate solution. The mixture was taken into a syringe, and beads were formed by dropping the solution into 1M calcium chloride solution with gentle stirring at 4°C for 2 h. The formed beads were recovered by filtration and thoroughly washed with distilled water. The beads were dried using filter paper (Whatman no. 1) followed by exposure to the open air for 1 hour before use. The filtered calcium chloride solution was collected for enzyme activity determination (Jyothi et al., 2012).

Magnetic Nanoparticle (MNP) method

Immobilization of esterase coated on synthesized magnetic nanoparticles was carried out based on the protocol given by Khoshnevisan et al., (2011). 80 mg of magnetic nanoparticles were added to 5ml of the culture filtrate dissolved in phosphate buffer at pH 7.0. Reactions were carried out in shaker incubator for 7 hrs at room temperature. The amount of protein in the supernatant was determined by Lowry's method. (Lowry, 1951). The amount of bound enzymes was calculated by,

$$\text{Immobilization effiency (\%)} = \text{Ci} - \frac{\text{Cs}}{\text{Ci}} \text{X100}$$

Where, Ci is the initial activity of culture filtrate used for the reaction, Cs is the enzyme activity in the supernatant.

Reusability of immobilized esterase

To investigate the effect of immobilization on reusability of immobilized esterase was performed after30 min of each cycle. After each cycle, the beads were washed thoroughly with buffer and then suspended again in a fresh reaction mixture to measure enzyme activity. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

RESULTS AND DISCUSSION

The most active microbial strain was selected based on utilization of Quinalphos as a sole carbon source. The selected strain was characterized as a Gram +ve organism (Figure 1a &1b) and the biochemical characteristic of the strain is shown in Table 1 and figure 2. Based on Bergey's manual of bacteriology, the organism was found to be *Staphylococcus spp.*

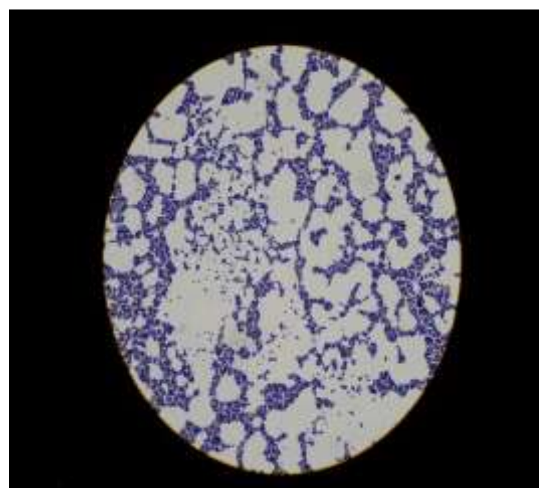

Figure 1a) Colony of the isolate **b)** Gram staining

Table 1: Biochemical characterization of the isolate

Test name	**Results**
Gram's nature	G+ ve
Cell shape	Cocci
Motility	Negative
Catalase	Positive
Mannitol fermentation	Negative
Yellow pigmentation	Negative

Figure 2 a) Mannitol fermentation **b)** Catalase positive

Likewise, Jain Rachna et al.,(2012) used gram's staining and biochemical characteristics for the identification and characterization of organisms capable of degrading Quinalphos. Rangaswamy and Venkateswaralu(1992) isolated 5 strains of *Bacillus spp*,of which Quinalphos-3 strain was capable of completely degrading 0.5% Quinalphos. In another study, Priyanka Singh Bhagel and Bhawana Pandey (2013) have studied the degradation efficiency by inoculating the *Monococcus* and *Diplococcus* bacteria using 1% Quinalphos. In the present study shows efficiency of the isolate for the degradation of Quinalphos upto 4%. Figure 3 shows the activity of intracellular esterase in the culture supernatant up to eight days. The maximum activity was observed at fourth day, decreasing activity which was observed after four days may due to decline phase of the organism as accumulation of toxic components, secretion of protease, changes in the physical conditions.

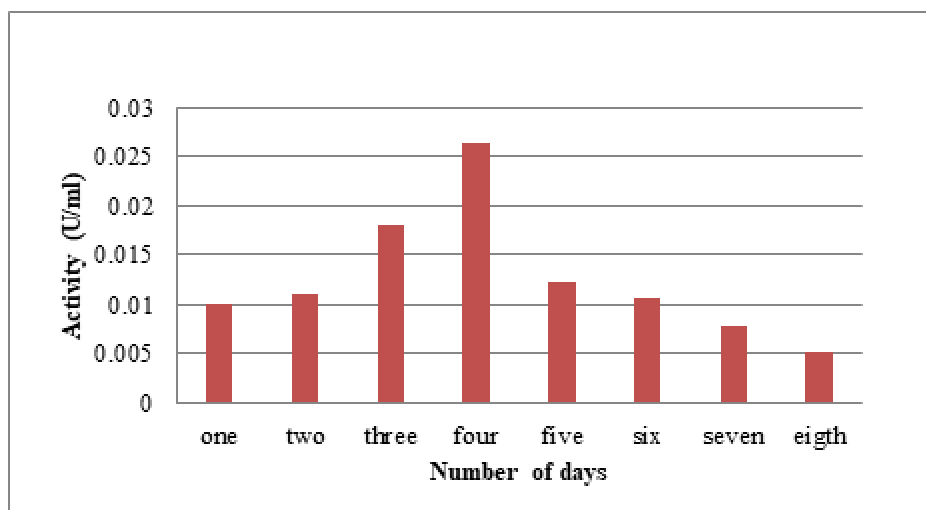

Figure 3 Activity of esterase at different days

The amount of Quinalphos degraded by the isolated *Staphylococcus spp*., were tabulated below (table 2) and the figure 4 shows the degradation pattern of Quinalphos after 96hrs of incubation.

Table 2: Biodegradation profile of Quinalphos after 96 hrs of incubation

Concentration of Quinalphos (%)	**Amount of Quinalphos (a = mg/100ml)**	**Recovered concentration of Quinalphos (b)**	**Recovery percentage (c = b/a x100)**	**Quinalphos degraded (%)**
Control	00	00	00	0.0
1	0.25	0	0	100
2	0.50	0.10	0.2	80
3	1.0	0.09	0.09	64
4	1.25	0.07	0.05	20

Figure 4 Degradation patterns of Quinalphos at various concentrations after 96 hrs incubation

In the present study, 80% biodegradation of Quinalphos was achieved in 4 days which is well correlated with growth kinetics study carried out by Jain Rachna et al.,

(2012).The observations of the present study show the degradation potential of the isolate over their result of 90% degradation after 10 days. Similar biodegradation research was carried out by Talwar et al., (2014) using *Ochrobactrum* spp on Quinalphos and it was found that organism degraded Quinalphos by hydrolysis to yield 2-hydroxyquinoxaline and diethyl phosphate which were further utilized as carbon sources.

Allium toxicity test was determined with the positive and negative control along with biodegraded culture supernatant. The maximum root length of *Allium cepa* measured was about 54 mm on 4th day culture supernatant which was higher than previous day culture supernatant. Comparably the result showed biodegrade culture grown plant has low toxicity thus it proves this isolated strain was capable of biodegradation of organophosphorus pesticides. The results were shown in table 3 & Figure7.

Table 3: Toxicity profile of *Allium cepa*

Incubation time (Hrs)	Sample	Average root length(mm)
24	Negative control	58
	Positive control	0.9
	Degraded sample	32
48	Negative control	58
	Positive control	0.9
	Degraded sample	39
72	Negative control	58
	Positive control	0.9
	Degraded sample	53
96	Negative control	58
	Positive control	0.9
	Degraded sample	54

Figure 7 *Allium cepa* root on different days of incubation.

Sarvesh et al., (2009) have given the maximum activity observed on 4th day by measurement of root lengths on *Allium cepa.* The immobilization of the enzyme was defined for esterase which was immobilized in the calcium alginate beads and expressed by the following equation. Figure 9 shows the enzyme immobilized sodium alginate beads with uniform size and 9b shows the reaction medium containing sodium alginate beads.

Figure 9 a) Immobilized sodium alginate beads **b)** Reaction mixture with beads

Immobilized yield (%) = (Activity of the immobilized enzyme)/(a-b) x 100
Immobilized yield (%) = (0.029)/(0.033– 0.002) x 100
Immobilized yield (%) = 93.5 %

Follows the highest percentage of immobilization efficiency of the enzyme esterase on magnetic nanoparticle was found to be 94.3%. Figure 10a shows MNP attracted by magnet in the production medium and 10b shows the activated MNPs.

Figure 10 a) MNP attracted by magnet **b)** Activated MNP

The reusability of esterase immobilized on sodium alginate beads and magnetic nanoparticles is shown in figure 11, it can be inferred that immobilized enzyme on MNP was found to be stable and reusable up to 7 cycles.

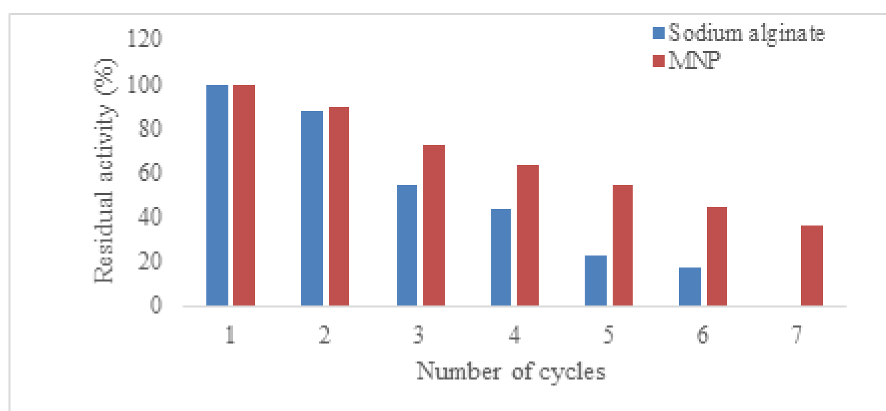

Figure11. Residual activity of immobilized esterase at different cycles.

The activity of entrapped assay showed 88%, 55%, 44% in reusable cycles with Quinalphos as a substrate, while complete loss in the activity of entrapped enzyme was observed during the sixth cycle. This decrease in activity was due to the leakage of enzyme from the beads, occurred due to the washing of beads at the end of each cycle. The reusability of MNP adsorbed enzyme at different cycles with Quinalphos as a substrate retains about 40% residual activity after 6 cycles. Results of the present study, shows the efficiency of MNP immobilization over sodium alginate entrapment method. The highest percentage of enzyme immobilization proves there are sufficient amount of nanoparticles available for enzyme immobilization. The maximum percentage of residual activity retained after 6 cycles indicated that the nanoparticle could provide a greater resistance towards change in the reaction medium.

Earlier, it was reported that alpha amylase entrapped in Ca-alginate beads was reused for 6 cycles with ~ 30% loss in activity (Kumar et al*.,* 1996). Similarly, another research was carried out on biodegradation of phenol compounds using immobilization of tyrosinase shows complete loss in activity after 4 cycles (Yoshino and Saito, 1994). In previous study, purified cellulase was immobilized on nanoparticle shows around 50 % residual activity after 6 cycles (Cheng and Chang, 2013).

SUMMARY AND CONCLUSION

The present study proves efficacy of the isolate in the biodegradation of pesticide at higher concentration, hence it can be used as an effective tool for bioremediation. A bacterium capable of degrading organophosphorus pesticide was isolated and identified from the pesticide contaminated soil samples. Based on the morphological, biochemical characteristics and Bergey's manual of determinative bacteriology the isolate was identified as *Staphylococcus* spp. The potential of the bacterial isolate to degrade Quinalphos was found to be 4% (1.25 mg) after 4 days of incubation. In order to determine the enzymatic pathway of degradation, the degraded sample was subjected to esterase assay and it was evident that Quinalphos was degraded via intracellular esterase pathway. To increase the reusability of esterase on biodegradation of pesticides, enzyme was immobilized using sodium alginate and magnetic nanoparticles. To best of our knowledge, the esterase immobilization on magnetic nanoparticle is new and used in bioremediation and is the first report in literature. Hence, the extensive study on enzyme-MNP interactions needs to be carried out for the optimal usage of nanoparticles in various fields.

ACKNOWLEDGEMENT
The authors are thankful to the Principal and Management of Arignar Anna College (Arts & Science) Krishnagiri, for their constant help and support in conducting this work to full satisfaction.

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