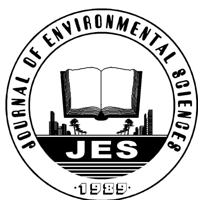

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Isolation and characterization of a profenofos degrading bacterium

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Abstract

Profenofos, a well known organophosphate pesticide, has been in agricultural use over the last two decades for controlling Lepidopteron pests of cotton and tobacco crops. In this study, a bacterial strain, OW, was isolated from a long term profenofos exposed soil by an enrichment technique, and its ability to degrade profenofos was determined using gas chromatography. The isolated strain OW was identified as *Pseudomonas aeruginosa* according to its physiological and biochemical properties, and the analysis of its 16S rRNA gene sequence. The strain grew well at pH 5.5–7.2 with a broad temperature profile. Bioremediation of profenofos-contaminated soil was examined using soil treated with 200 μg/g profenofos, which resulted in a higher degradation rate than control soils without inoculation. In a mineral salt medium (FTW), removal in the level of profenofos of 86.81% was obtained within 48 h of incubation. The intermediates of profenofos metabolism indicated that the degradation occurred through a hydrolysis mechanism, and one of the metabolites was found to be 4 bromo-2-cholorophenol (BCP) which in turn was also mineralized by the strain. The results of this study highlighted the potentiality of *P. aeruginosa* as a biodegrader which could be used for the bioremediation of profenofos contaminated soil.

Key words: profenofos; organophosphate pesticide; *Pseudomonas aeruginosa*

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Introduction

The development and use of organophosphate compounds (OPs) is greater than ever before, and it is predicted that this trend would most likely continue as new applications for these compounds are discovered (Frank *et al*., 1999). Their widespread use has caused severe environmental pollution, since OPs applied in agricultural areas do not remain at their target sites, but often enter aquatic environments via soil percolation, air drift or surface runoff (Andersona and Hunta, 2003; Rovedatti, 2001; Sawaya *et al*., 2000).

Bioremediation and enzyme detoxification are found as economical and reliable methods to resolve pesticide-related problems, having many advantages over alternative approaches such as incineration or pump-and-treat technology. The most significant step in detoxifying organophosphate compounds is hydrolysis as that makes the compounds more vulnerable to further degradation (Kumar *et al*., 1996). The enzyme responsible for catalyzing this reaction is referred as an esterase or phosphotriesterase. Research works have found a wide range of microorganisms possessing the organophosphate hydrolase enzyme (Horne *et al*., 2002; Dave *et al*., 1993; Mulbry *et al*., 1986). The most well-known examples of natural isolates being able to degrade organophosphates are *Pseudomonas diminuta* MG and *Flavobacterium* ATCC 27551, which have been shown to possess the organophosphate hydrolase (OPH) enzyme (Mulbry, 2000).

One of the organophosphorothiolate insecticides used in agriculture (especially in cotton) for pest control is profenofos (O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate). Profenofos has been classified as a moderately hazardous (toxicity class II) pesticide by the World Health Organiztion and it has a moderate order of acute toxicity following oral and dermal administration (WHO, 2004; USEPA, 2000). Profenofos is extremely toxic to fish and macro-invertebrates (Akerblom, 2004), and its acute toxic action is the inhibition of the acetylcholinesterase activity (Fukuto, 1990), also resulting in toxicity in humans (Costa *et al*., 2008). According to the reports from the US Environmental Protection Agency and previous research works, profenofos is a potential contaminant in a wide range of aquatic and terrestrial ecosystems (Jabbar *et al*., 1993; Safiatou *et al*., 2007; US EPA, 2006), and its residues have been found in foods and vegetables (Parekh *et al*., 1994; Radwan *et al*., 2005).

It was reported that profenofos is unstable in alkaline solutions with a 50% hydrolytic degradation time of 5.7 h at pH 9, compared with 14.6 d at pH 7. The logK_{ow} value for profenofos is 4.44 (Tomlin, 1994), indicating that profenofos would be expected to bind strongly to

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sediments. The effects of profenofos on total bacteria populations were supposed significant, however, studies showed that microbial populations decreased initially at concentrations of 100 to 300 μg/g and recovered rapidly to levels similar to those in the control (Martinez *et al*., 1992). The phenomenon indicated that profenofos can serve as carbon source.

In vitro biotransformation studies of profenofos found desthiopropylprofenofos and hydroxyprofenofos as metabolites (Abass *et al*., 2007). Phototransformation of profenofos studies showed cleavage of ester bond (Zamy *et al*., 2004). But no significant research has yet been conducted on the microbial degradation of profenofos.

The objective of the present study was to isolate, identify and characterize profenofos-degrading bacterial strains from soil exposed long-term to profenofos.

1 Materials and methods

1.1 Sample collection for enrichment studies

Two environmental samples were used in this study for the isolation of profenofos-degrading microorganisms. Soil samples were collected from different sites with a history of 6–7 years of profenofos applications in Hanchuan City, Hubei Province (30°40′59.7″N, 113°49′37.3″E), China. The collected samples were stored at 4°C. Soil properties are shown in Table 1.

Table 1 Properties of the soil samples

Sample No.	pH	Organic matter (g/kg)	Sand (%)	Silt (%)	Clay (%)
1	6.58	28.3	80.61	6.64	12.75
2	6.76	26.7	84.00	9.00	7.00

1.2 Medium for isolation

Salt medium FTW (Herman and Frankenberger, 1999) with the following composition was used (g/L deionized water): K_2HPO_4 0.225; KH_2PO_4 0.225; $(NH_4)_2SO_4$ 0.225; $MgSO_4·7H_2O$ 0.05; $CaCO_3$ 0.005; and $FeCl_2·4H_2O$ 0.005, and 1 mL of Focht trace elements solution (Focht, 1994). The Focht trace element solution contained (mg/L): $MnSO_4·H_2O$ 169; $ZnSO_4·7H_2O$ 288; $CuSO_4·5H_2O$ 250; $NiSO_4·6H_2O$ 26; $CoSO_4$ 28; and $Na_2MoO_4·2H_2O$ 24; and pH (7.2 ± 0.2).

The medium for isolation (FTW) was autoclaved and then supplemented with 100 mg/L profenofos. Plates for isolation were prepared by adding 20 g agar per liter of isolation medium.

Luria-Bertani (LB) medium composition was (g/L deionized water): NaCl 10.0; Tryptone 10.0; yeast extract 5.0; pH (7.2 ± 0.2) (Atlas, 2000) .

1.3 Isolation and identification of bacteria

The isolation method of Prescott *et al*. (2002) was followed with some modifications. Soil samples were sieved through a 90-mesh sieve to remove stones and plant material. Then, 10 g soil was placed in a 250-mL conical flask containing 100 mL FTW media and incubated at 30°C in a rotary shaker at 150 r/min for 2 d. The flasks were then left for a few hours to allow the soil particles to settle, and the suspension containing microorganisms was then used to inoculate fresh sterilized FTW media containing 100 mg/L profenofos and incubated for 2 d.

To obtain pure cultures of single strain, 5 mL aliquots of enrichment cultures were centrifuged at 3000 r/min for 5 min (TGL-16A centrifuge, Changsha Pingfan Instrument Ltd., China) and the cell pallets were resuspended in 2 mL sterile media. Aliquots of this suspension were streaked on FTW-profenofos agar plates. Inoculated plates were incubated under aerobic conditions at 30°C and discrete colonies were isolated.

The bacterium exhibiting the highest activity in profenofos degradation was identified according to Bergey's manual of systematic bacteriology (George, 2005) and further confirmation was made by sequencing of its 16S rRNA gene.

1.4 Genomic DNA isolation and sequencing of 16S rRNA gene

Genomic DNA was isolated using standard bacterial procedures (Goldberg and Ohman, 1984). The following primers were used for PCR amplification of the gene encoding 16S rRNA: 63f (5'-AGGCCTAACACATGCAAG TC-3'), 1387r(5'-GGGCGGAGTGTACAAGGC-3'). The PCR mixtures (50 μL) contained 10 μmol/L of each primer, PCR buffer, 5 U Taq DNA polymerase, BSA of 10 μmol/L and 2 μL DNA. The thermocycling conditions consisted of a denaturation step at 94°C for 3 min, 28 amplification cycles of 94°C for 20 s, 58°C for 40 s and 72°C for 1 min and a final polymerization for 3 min 30 s with a MJ Research Thermalcycler (PTC-100, MJ Research, USA). PCR products were visualized on 1.0% agarose gels with Gel Doc 2000 (Bio-Rad, USA). The purified PCR products were then cloned into the pMD-18 T vector (TaKaRa, Dalian, China) and sequenced in Sangon Shanghai, China. Nucleotide sequence similarities were determined using BLAST (National Center for Biotechnology Information databases).

1.5 Characterization of growth conditions

Three carbon sources (glucose, lactose, and methanol) were added to the FTW media at a concentration of 2 g/L to examine their effect on the isolate's growth. Glucose and lactose are typical carbon sources for bacteria. Methanol is efficient organic solvents for profenofos. The inoculation density was 20 mg dry weight (dw)/50 mL and incubation conditions were 30°C at 150 r/min. Triplicate analyses were conducted and mean values were adopted. Aliquots of 3 mL of culture were withdrawn at 12 h intervals and OD_{600} was measured in a spectrophotometer (T6 UV-Spectrophotometer, China) to monitor the strain growth.

To investigate the effect of pH on growth, strain OW was inoculated into FTW media supplemented with 100 mg/L profenofos at different pH values (5.5, 6.5, 7.2, 8.5). The pH was adjusted with 0.1 mol/L NaOH or H_2SO_4, and growth monitored by OD_{600} measurements.

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1.6 Biodegradation studies

The ability of the isolates to degrade profenofos was assessed in FTW medium containing 100 mg organophosphate per liter. Bacterial cells were pre-cultured in LB medium at 30°C with 150 r/min shaking, harvested by centrifugation at 6000 r/min for 10 min and washed three times with sterilized water. For all experiments, cells were used at a concentration of 20 mg dw/50 mL media. If not otherwise stated the incubation conditions were 30°C with 150 r/min shaking. Aliquots of 2 mL were taken at suitable intervals between 0 to 4 d and were subjected to gas chromatograph (GC) analysis after extraction of profenofos residues.

To examine the effect of nutrient composition on the degrading ability of the isolate the above medium was supplemented with an extra carbon source (0.5% glucose). Triplicate sets of each composition without inoculation were kept as controls.

1.7 Inoculation and degradation in soil

Air-dried sieved (< 2 mm) soil samples, used for isolation. One set of sterilized and non-sterilized soils in duplicates was inoculated with isolated cells and another set without inoculation was kept as controls amended with an identical amount of profenofos but without inoculation. Both sets were run simultaneously under identical conditions.

At different time points between 0 to 25 d, aliquots of 3 g soil were removed in duplicates from the microcosms and subjected to extracte profenofos residues in soil samples by an acetone-hexane (20:80, *V/V*) mixture (Horne *et al*., 2002) and then analyzed by gas chromatograph.

1.8 Gas chromatography analysis

Profenofos residues, extracted in hexane from salt media or from the soil samples, were analyzed using Agilent gas chromatograph (GC) (Model 6890 Series, USA) equipped with a Ni^{63} electron capture detector with HP-5MS column (length 30 m, diameter 0.25 mm). The operating conditions: initial temperature 120°C, then heated at 7°C/min to a final temperature of 250°C. The total run time was 33.07 min. The splitless mode was used for injection. The injector temperature was set at 240°C and the detector temperature was at 300°C. Nitrogen gas (99.999%) was used as the carrier gas with a gas flow at 23.3 cm/s linear velocity.

1.9 Detection of intermediate metabolites

The OW strain (20 mg in dw) was inoculated in 50 mL culture medium (FTW supplemented with 100 mg/L profenofos). After 24 and 48 h, the 2 mL aliquots were withdrawn and extracted. The solvent was evaporated and the residue obtained was dissolved in 5 mL of hexane and was injected in GC-MS (Agilent technologies model 6890, USA) with HP column (30 m length) containing inert mass selective detector with following working conditions: initial temperature 70°C for 2 min, raised at a rate of 25°C/min to 280°C with 25 min hold time.

2 Results and discussion

2.1 Identification of isolated strain

Four different microorganisms were isolated from the profenofos-exposed soil samples by the above mentioned method. All isolates were found to possess the ability to degrade the pesticide. Among them, strain OW showed the most efficient in degrading profenofos and is presented in this study.

The bacterial strain obtained was a Gram negative, motile and rod shaped that could grow on Simmons citrate agar and in mineral salts medium supplemented with glucose, sucrose, lactose, fructose, mannitol, methanol, ethanol, butanol, isobutanol, while xylose and maltose did not support the growth of this organism. Methyl Red, H_2S production and Urease tests were negative. Positive results were obtained in catalase, oxidase and indole production and denitrification tests.

Total genomic DNA was extracted and stored at –20°C. Afterward, this genomic DNA was used for 16S rRNA gene analysis. By sequencing the 16S rRNA gene of strain OW and comparing it with previously published 16S rRNA gene sequences, the strain was classified as a member of the genus *Pseudomonas*. The sequence displayed the highest similarity (98%) to that of *Pseudomonas aeruginosa* (genebank accession FJ763638).

2.2 Characterization of growth conditions with different carbon sources

Growth characteristics of the isolated strain were checked in different carbon sources along with profenofos. Glucose was a rich carbon source for growth (Fig. 1a). Growth was slower when profenofos was supplied as the sole carbon source. But strain OW could utilize methanol or lactose as carbon sources to limited extent. To further investigate characteristics of the strain OW, profenofos was selected as the carbon source for subsequent experiments.

In subsequent study, it reveals that in the presence of glucose, strain OW can only utilize glucose as carbon source and could not degrade profenofos anymore. There were no significant differences on growth when other carbon sources (methanol and lactose) were provided with profenofos (Fig. 1a).

2.3 Characterization of growth conditions at different pHs

The growth of isolated strain was monitored as the optical density at 600 nm in mineral salt media at pH 5.5, 6.5, 7.2, and 8.5, supplemented with 100 mg/L profenofos as sole carbon source. The most rapid growth was observed at pH 6.5 and 7.2 within 10 h of incubation, followed by pH 5.5. The growth was the lowest in the case of pH 8. After 60 h, the highest optical density was measured at pH 6.5, followed by pH 7.2 and 5.5. Possibly because of low solubility of pesticide in high pH, poor growth was observed at pH 8.5 (Fig. 1b).

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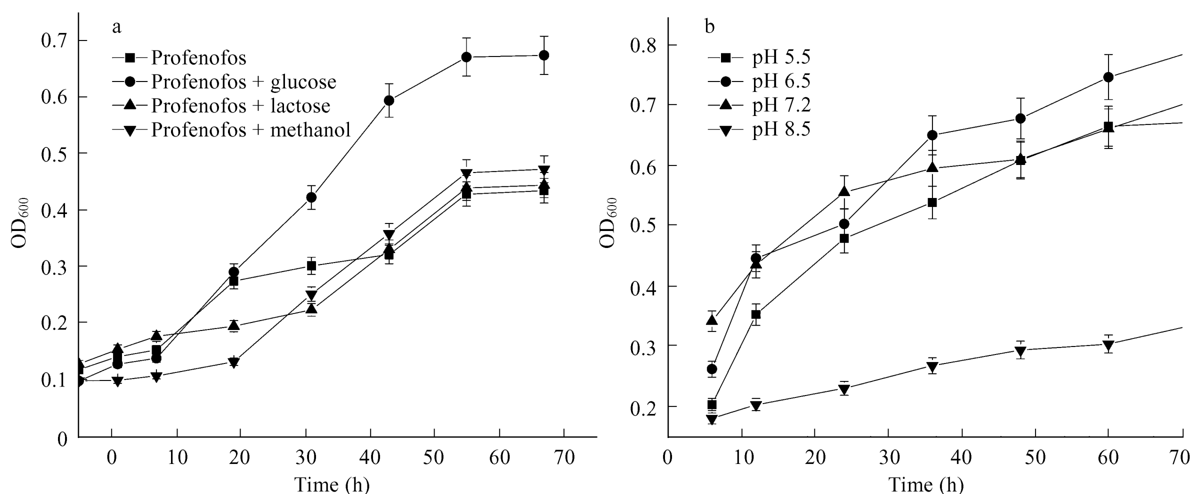

Fig. 1 Growth characteristics of OW strain in FTW media with extra carbon sources (a) and at different pH values (b). Conditions: (a) pH 7.2 and temperature 30°C; (b) profenofos 100 mg/L and temperature 30°C.

2.4 Biodegradation of profenofos

2.4.1 Degradation in liquid culture media

Under aerobic conditions, profenofos degradation by *Pseudomonas aeruginosa* OW was monitored by GC for a period of 96 h (Figs. 2a and 2b) in liquid culture media. After 48 h, 86.81% of pure profenofos disappeared, followed by a slower decrease of profenofos with longer incubation times. Rapid degradation began shortly after 6 h of incubation under aerobic conditions, suggesting that a constitutively expressed enzyme could be involved in the degradation of profenofos. The degradation of profenofos supported cell growth, indicating that isolated strain OW could utilize profenofos as a carbon source.

Previous reports concerning the isolation of organophos-

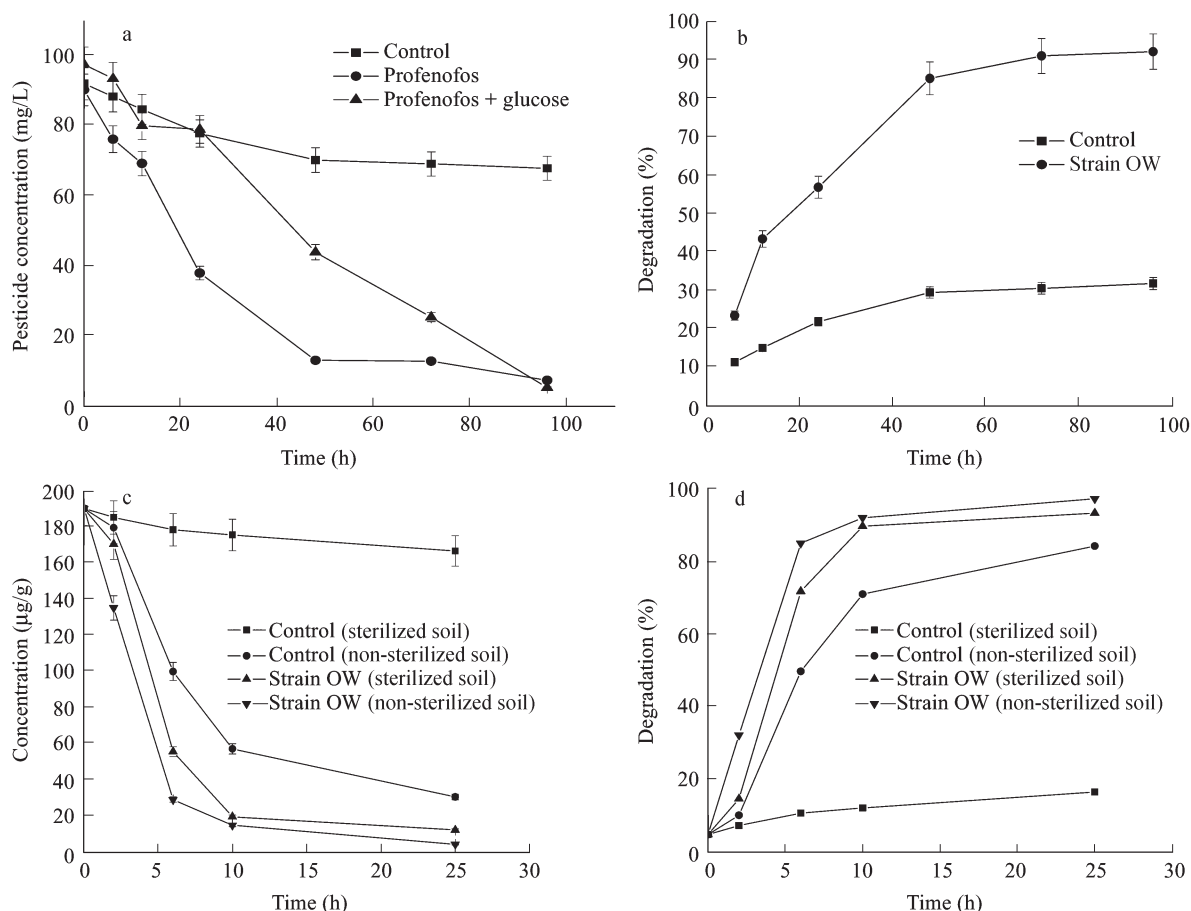

Fig. 2 Degradation kinetics of profenofos by OW strain. (a) in liquid batch culture media (FTW amended with profenofos); (b) % degradation in liquid batch culture; (c) in soil supplemented with profenofos at different days; (d) % degradation in soil.

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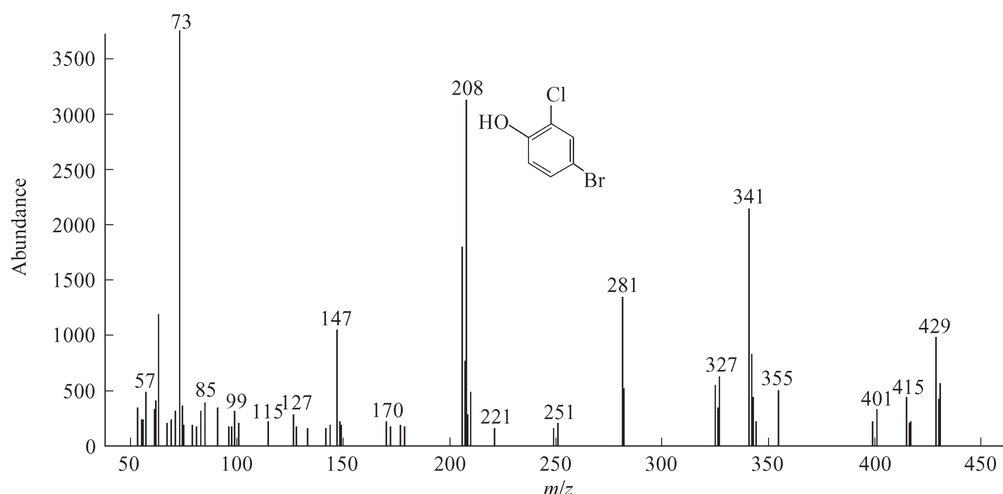

Fig. 3 Gas chromatography-mass spectrum of the metabolite produced during profenofos degradation by *Pseudomonas aeruginosa* (strain OW).

phorus degrading microorganisms suggest that the bacteria mainly degrade these compounds co-metabolically (Horne *et al*., 2002; Richard *et al*., 1997). Some reports showed that the isolated bacterium can utilize organophosphates as a source of carbon or phosphorus (Subhas and Dileep, 2003) from the hydrolysis products (Serdar and Gibson, 1985). The bacterium isolated in the present study had very strong phosphotriesterase (OPH) activity (Subhas and Dileep, 2006) and hydrolyzed 60 mg/L of profenofos within 24 h.

However, in the presence of other carbon sources (such as glucose), initially it stopped degrading profenofos but with the passage of time it degraded profenofos 97% within 96 h, indicating that when glucose was depleted, it started to utilize profenofos as a source of carbon, signifying the environmental adaptation of this bacterium (Fig. 2a). Similar results were reported by Brajesh *et al*. (2004).

In natural environments, the competition for carbon sources is immense and the utilization of profenofos as an energy source by this bacterium provides it with a substantial competitive advantage over other microorganisms. However, subculturing in nutrient-rich media several times led to the permanent loss of its profenofos-degrading capability. Parekh *et al*. (1994) found similar results for metamitron degradation by *Rhodococcus* sp. However, the present results do not agree with those from previous studies with carbofuran and ethoprophos (Bell *et al*., 1998; Karpouzas and Walker, 2004), in which the presence of glucose had no effect on the degrading ability of the bacteria. Successful removal of pesticides by the addition of bacteria (bioaugmentation) had been reported earlier for many compounds, including parathion (Zhongli *et al*., 2001), coumaphos (Kearney *et al*., 1986), ethoprop (Karpouzas and Walker, 2000), and atrazine (Struthers *et al*., 1998).

2.4.2 Degradation of profenofos in soils

The soil sample, previously used to isolate profenofos degrading bacteria, was used in this study.

The addition of OW strain to soils resulted in a more rapid rate of profenofos degradation than that by control (non-sterilized and sterilized) soils. Within 25-d incubation periord, the degradation of profenofos in control sterilized soils (without inoculation) was minimal where less than 16% of the applied concentration was degraded, whereas significant high degradation rate (84%) was recorded in control non-sterilized (without inoculation) soil (Fig. 2d).

These results suggested that the isolated *P. aeruginosa* worked well in the soil. The degradation kinetics pattern was almost the same in soil as that in liquid culture media, such as in first phase rapid steep decline in concentration of profenofos followed by a slower degradation.

2.5 Metabolic intermediates

Gas chromatography-mass spectrum (GC-MS) has been widely used in the identification of the degradation products of OPs (Wang *et al*., 2005). It was used in present study to identify the metabolic intermediates of profenofos degradation. Sample extracted in hexane from pesticide culture of bacterium after 24 and 48 h were injected in GC-MS. The comparison on the abundance and retention time of these peaks are presented in Fig. 3. The metabolite corresponding to the peak of 7.06 and 6.53 min retention time was identified as 4 bromo-2-cholorophenol (BCP).

The formation of 4-bromo-2-cholorophenol (molecular weight 206) proves the breaking of ester bond linkage of the parent compound by the bacterium. It suggests that the bacterium contains enzymes having esterase activity (Fig. 4). However, the full degradation pathway and mineralization of intermediates require further investigation.

3 Conclusions

In summary, *Pseudomonas* sp., ubiquitous in soil and water, is of considerable scientific and technological importance and comprises a taxon of metabolically versatile organisms capable of utilizing a wide range of simple and complex organic compounds. They are known to be involved in the biodegradation of natural or man-made toxic chemical compounds and the present study confirms

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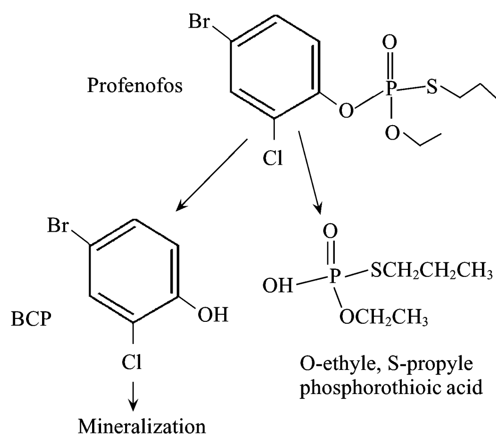

Fig. 4 Proposed pathway for profenofos degradation by *Pseudomonas aeruginosa* (strain OW).

that the isolated profenofos-degrading bacterium could be used successfully for the removal of profenofos from contaminated soils as the bacterial systems successfully degraded profenofos in soil and liquid media. It is well known that bioremediation is an attractive alternative to the classic treatment of OP pollution by taking advantage of microbial metabolism. This is the first study which showed that *P. aeruginosa* OW is capable of degrading profenofos therefore would be beneficial for the exploration of new bioremediation strategies for pesticides contaminated sites.

Acknowledgments

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