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Degradation of acephate by *Enterobacter asburiae, Bacillus cereus* and *Pantoea agglomerans* isolated from diamondback moth *Plutella xylostella* (L), a pest of cruciferous crops

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Abstract

Acephate-degrading bacterial isolates were isolated from the larval gut of diamondback moth *Plutella xylostella*, a notorious pest of cruciferous crops worldwide that has developed resistance to insecticides. Partial 16S rRNA gene sequencing identified the isolates as *Bacillus cereus* (PX-B.C.Or), *Enterobacter asburiae* (PXE), and *Pantoae agglomerans* (PX-Pt.ag.Jor). All isolates grew on minimal media (MM) in the presence of acephate at 100 and 200 ppm, with maximum growth at 200 ppm. LC-MS analyses of spent medium showed that *E. asburiae* degraded acephate to methamidophos and *O, O*-dimethyl phosporamidate and *B. cereus O,S*-dimethyl to phosphorothioate but *P. agglomerans* to an unnamed compound. All three isolates used acephate as a source of carbon and energy for growth; however, *P. agglomerans* used it also as source of sulphur. Strong evidence revealed that the bacterial communities present in the gut of diamondback moth might aid in acephate degradation and play a role in the development of insecticide resistance.

Key words

Acephate, *Bacillus cereus*, *Enterobacter asburiae, Pantoea agglomerans*, Microbial degradation, *Plutella xylostella*

Introduction

Diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a major insect pest of cruciferous crops worldwide. It has developed resistance to about 82 compounds belonging to different classes of insecticides and in 17 countries (Furlong *et al*., 2013). The overall management cost for diamondback moth is estimated at US$ 4-5 billion annually (Zalucki *et al*., 2012), and the pest is ranked second in the Arthropod Pesticide Resistance Database (APRD, 2012). Insecticide resistance due to different physiological mechanisms has been reported and includes reduced cuticular penetration (Liu and Shen, 2003), altered midgut site for *Bacillus thuringiensis* toxins (Tabashnik and Carriere, 2010), altered target site for carbamates and organophosphates (Yu and Nguyen, 1992; Temeyer *et al*., 2008), metabolic enzyme-mediated resistance such as glutathione (GSH) *S*-transferase for parathion (Enayati *et al*., 2005), carboxylesterase for malathion (Maa *et al*., 2000), microsomal P-450 monooxygenase for pyrethroids (Kasai *et al*., 2000) and decreased nerve sensitivity (Hama *et al*., 1987).

Acephate is an organophosphorus insecticide that inhibits acetyl cholinesterase activity and is extensively used to control several insect pests, particularly sucking pests. When acephate reaches soil medium, it can be mineralized to CO_2, methyl mercaptan and phosphoric acid *via*

methamidophos or *O*-methyl N-acetylphosphoramidate, or *O,S*-dimethyl phosphorothioate (Yen *et al.,* 2000). Several microorganisms, which are natural inhabitants of soils, can degrade organophosphorus chemical substances (Megharaj *et al*., 2011; Singh *et al*., 2012). These insecticide-degrading soil bacteria can metabolize and use these compounds as source of carbon, nitrogen and sulphur (Aleem *et al*., 2012; Zhao *et al*., 2010). Studies have investigated degradation of parathion, acephate, methyl parathion, malathion, monocrotophos and dimethoate in widely used organophosphorous insecticides by examining diverse groups of microorganisms, formation of different metabolites, enzymology and genetic basis of degradation (Kanekar *et al*., 2004).

The gut of diamondback moth, like that of many other insects, harbours a plethora of microbiota that plays an essential role in growth and development, pathogenesis, and environmental adaptation of host insects (Douglas, 2011). Nevertheless, potential for these gut microbiota in detoxification or degradation of insecticides is little known. Both for insect pests and non-targeted insects, symbiont/ microflora–mediated detoxification can represent a previously unappreciated mechanism for the development of insecticide resistance (Whalon *et al*., 2008). The gut microbiotics, which can degrade insecticide compounds, could provide a new means for rapid acquisition of insecticide resistance by their hosts.

The objectives of the present study were to isolate and identify culturable bacteria from the gut of diamondback moth larvae that can degrade acephate in insect system and to identify the degraded products.

Materials and Methods

Collection of diamondback moth : Population of diamondback moth larvae (~200 larvae from each location) were collected from cabbage crops in different locations: Balasore, Oddanchatram and Jorhat of India, during 2012-14. Cabbage is cultivated as a major vegetable in the above-mentioned locations and all collections were performed during morning hours (7-9 a.m.).

Isolation, identification and phylogenetic position of acephate-degrading bacteria : Third-instar larvae collected from cabbage were surface-sterilized with sodium hypochlorite (0.1%) and ethanol (70%) for 5 sec to remove adhering contaminants, especially external microflora, and underwent homogenization (Gebbardi *et al*., 2001). Homogenate was placed in sterile Lubria-Bertani (LB) agar media (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar) in triplicate and incubated at 30˚C for 48 hrs. Several bacterial colonies were chosen from the isolated consortium and were streaked separately onto agar plates containing 100 and 200 µg ml^{-1} acephate (M/S Bayer Crop Science, Mumbai, India). Colonies that grew on agar plates with acephate were selected. Later, these cultures were streaked onto the broth of LB agar media to obtain pure cultures. This procedure was repeated several times to ensure the purity of isolated colonies. Then, pure cultures were inoculated into LB broth and incubated for 48-72 hrs at 30°C for maximum growth.

Genomic DNA was isolated from bacteria by SDS-lysis protocol (Syn *et al*., 2000). PCR amplification of 16S rRNA genes and its complete sequence was obtained by following procedure of Petti *et al.* (2005). The CLUSTAL_W algorithm of MEGA 6 was used for sequence alignment and MEGA 6 was used for phylogenetic analysis of sequences. Phylogenetic tree was constructed by using the character-based maximum-likelihood method (Tamura and Nei, 1993).

Minimal media study: After obtaining pure culture, bacterial isolates' ability to use acephate as source of carbon, nitrogen or sulphur was assessed by growth in minimal medium lacking alternative carbon (MM), carbon and sulphur (MM1) and carbon and nitrogen (MM2) (Table 1). All salts were GR grade, with nitrogen and sulphur content < 0.002%. A 250 ml conical flask containing 50 ml MM (MM, MM1, or MM2) supplemented with acephate (100 and 200 µg ml^{-1}) and 1 ml bacterial inocula was incubated on shaker at 120 rpm for 7 days at 37˚C (Table 2). The MM with bacterial inocula served as control for growth studies (control 1) and MM with acephate without inocula served as a control for liquid chromatography mass spectrometry (LC-MS; control 2). pH for all media used for growth studies was adjusted to 7.0.

Growth studies with acephate as a sole source of carbon, sulphur and nitrogen: The molar ratio of C: N: S per mole of acephate was 4:1:1, so acephate can be a source of sulphur, nitrogen and carbon. The bacterial ability to use acephate as a

Table 1 : The following composition of minimal media used for the acephate degradation studies

INGREDIENT	MM (C)	MM1 (C-S-)	MM2 (C-N-)
		g l^{-1}	
$KH2PO_4$	1	1	1
K_2HPO_4	1	1	1
NH_4NO_3	1	1	-
$MgSO_4 7H_2O$	0.2	-	0.2
$CaCO_3$	0.02	0.02	0.02
$FeSO_4 . 7H_2O$	0.01	-	0.01

(C)- carbon source; C-S) carbon and sulphur source; (C-N) carbon and nitrogen source

Table 2 : Details of minimal media along with different concentrations of acephate and bacterial inocula used during the study

Control	Test sample 1*	Test sample 2**
MM + *Enterobacter asburiae*	MM + acephate + *E. asburiae*	MM+ acephate + *E. asburiae*
MM + *Bacillus cereus*	MM + acephate + *B. cereus*	MM+ acephate + *B. cereus*
MM + *Pantoea agglomerans*	MM + acephate + *P. agglomerans*	MM+ acephate + *P. agglomerans*
MM1 + *E. asburiae*	MM1 + acephate + *E. asburiae*	MM1+ acephate + *E. asburiae*
MM1 + *B. cereus*	MM1 + acephate + *B. cereus*	MM1+ acephate + *B. cereus*
MM1 + *P. agglomerans*	MM1 + acephate + *P. agglomerans*	MM1+ acephate + *P. agglomerans*
MM2 + *E. asburiae*	MM2 + acephate + *E. asburiae*	MM2+ acephate + *E. asburiae*
MM2 + *B. cereus*	MM2 + acephate + *B. cereus*	MM2+ acephate + *B. cereus*
MM2 + *P. agglomerans*	MM2 + acephate + *P. agglomerans*	MM2+ acephate + *P. agglomerans*

*, acephate (100 ppm)l **, acephate (200 ppm)

Table 3 : Colony characteristics and biochemical characteristics of acephate degrading bacteria

Sample No.	Bacteria	Strain	Accession No.	Shape	Colour	Elevation	Gramnature	MR test	VP test
1	*E. asburiae*	PXE	KC410777	Circular	White	Raised	+ cocci	-	+
2	*B. cereus*	PX-B.C.Or	KC985225	Round	White	Raised	+ rods	-	+
3	*P. agglomerans*	PX-Pt.ag.Jor	KC985229	Round	Creamy	Convex	- rods	+	-

-, negative; +, positive; MR- Methyl Red; VP- Voges Proskauer

source of carbon, sulphur and nitrogen was assessed in with growth in three different MM lacking alternative sources for each essential nutrient as described previously. At defined time points, aliquot samples were withdrawn and bacterial growth was measured spectrophotometrically at OD 660 nm and compared against control sample (control 1). Growth rate was estimated by the slope of line representing linear fit of increase in OD over time during the exponential phase of culture growth.

Metabolite analysis by LC-MS : Samples containing acephate were extracted initially with methylene chloride (100 ml) as solvent. The pooled extract was collected in a round-bottom flask after passing through anhydrous sodium sulphate (mg). The content was evaporated to dryness using vacuum rotary evaporator, and high-performance LC–grade acetonitrile (2 ml) was added to the residue. Each of these extracts was later analysed by LC-MS (Thermo, LCQ Deca XP MAX) (Anwar *et al*., 2009). In LC profile, gradient mobile phase consisted of formic acid (solvent A) and methanol (solvent B) at 80:20 proportion. Samples (5 µl) were injected with a total run time of 15 min by electrospray ionization. The MS operating condition was optimized with capillary temperature of 300 ºC, capillary voltage 18V, spray voltage 5KV and polarity at positive mode.

Results and Discussion

Previous studies have reported that microorganisms can metabolize several chemical substances including those of insecticides. Some of them can mineralise many aliphatic, aromatic and heterocyclic compounds. Soil microorganisms such as *Saccharomyces rouxii* WY-3 (Liu *et al*., 2001), *Aspergillus orantus* (Liu and Zhong, 1999), and *Pseudomonas* sp. Ind01 (Aleem *et al*., 2012), *Pseudomonas aeruginosa* IS6 (Ramu and Seetharaman, 2014), *Pseudomonas acephalitica* Ind (Pinjari *et al*., 2012) could degrade acephate. In the present study, acephate degradation by *E. asburiae*, *B. cereus* and *P. agglomerans* isolated from the gut of diamondback moth larvae, an important pest of cruciferous crops worldwide that has developed resistance to insecticides, was reported.

Three best isolates were selected from the gut of diamondback moth that could utilize acephate for their growth. The molecular characterization of 16S rRNA gene for degrading acephate for three isolates, *E. asburiae*, *B. cereus* and *P. agglomerans*, was determined and their phylogenetic position was assigned. Furthermore, identification was confirmed by biochemical characterisation (Table 3). Phylogenetic analysis of isolate in genus *Enterobacter* showed strain PXE was closest match to *E. asburiae* AAB08 and *E. asburiae* YMC/KNO7/05 (Fig. 1). For the isolate in genus *Bacillus*, phylogenetic analysis revealed two clades, with strain *B. cereus* (PX-B.C.Or) in second clade with 100% bootstrap value (Fig. 2) and *P. agglomerans* (PX-Pt.ag.Jor) in the first clade, with closest match to *P. agglomerans* 312 and *P. agglomerans* 10n (Fig. 3). Thus, these isolates belonged to three different genera and

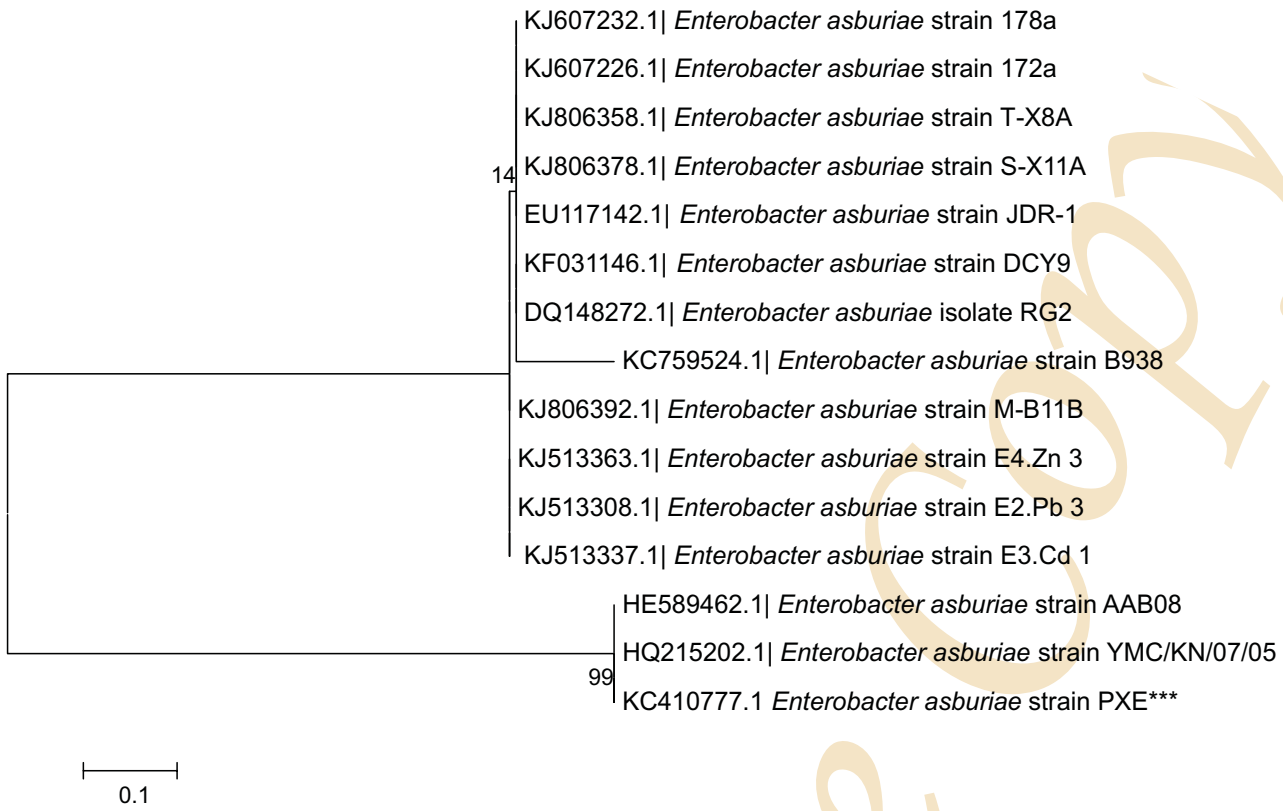

Fig. 1 : Phylogenetic analysis of 16S rRNA gene sequences for the bacterial strain *Enterobacter asburiae* PXE.

were termed as *B. cereus* (accesssion no. KC985225), *P. agglomerans* (KC985229) and *E. asburiae* (KC410777), respectively.

Growth study with different MM showed the potential for *E. asburiae*, *B. cereus* and *P. agglomerans* to utilize acephate as a sole carbon source. *E. asburiae* and *B. cereus* grown on MM medium lacking an alternative source of carbon but supplemented with acephate, showed enhanced growth rates at 660 nm, therefore growth was due to addition of acephate. For *P. agglomerans*, growth rate was satisfactory. The control samples showed poorer growth rates: maximum growth in *E. asburiae* and *B. cereus* was found in 200 µg ml^{-1} acephate. Growth of *P. agglomerans* was satisfactory, but poor for *E. asburiae* and *B. cereus* in MM1 with acephate (100 µg ml^{-1}) as carbon and sulphur source relative to control.

LC-MS was used to identify the metabolites formed after acephate degradation. With maximum growth of *E. asburiae* (PXE) in MM with acephate at 200 µg ml^{-1}, LC_MS revealed four spectral peaks, with retention time of 2.65, 2.55, 3.25 and 6.83 min, revealed as methamidophos (O,S-dimethyl phosphoramidothioate; 143.16 m z^{-1}), unnamed compounds (122.14 m z^{-1} and 65.19 m z^{-1}) and trace amount of O,O-dimethyl phosporamidate (126.93 m z^{-1}), respectively. Other compounds with molecular mass 92.20, 97.19, 119.28 and 121.21 m/z were detected (Fig. 4). These compounds were not detected in the control sample. With maximum growth of *B. cereus* (PX-B.C.Or) in MM with acephate (200 µg ml^{-1}) as sole carbon source, a single peak was observed at retention time of 6.96 min. Two peaks were revealed, one with identical spectra to O,S-dimethyl phosphoramidothioate (142.96 m z^{-1}) and a trace quantity of acephate was not utilized (183.81 m z^{-1}) (Fig. 5). LC-MS confirmed that the concentration of acephate decreased and that of O, S-dimethyl phosphorothioate increased.

Growth of *P. agglomerans* (PX-Pt.ag.jor) on MM1 supplemented with acephate (100 and 200 µg ml^{-1}) was satisfactory as compared with control. Two main peaks with retention time of 5.22 and 5.57 min were detected and differed from that for standard acephate (7.35 min). The masses were estimated at 64.99, 91.93, 192.99 and 96.72 m/z (Fig. 6), with these compounds unidentified. Previously, Verma *et al*. (2006) reported degradation of endosulfan by a

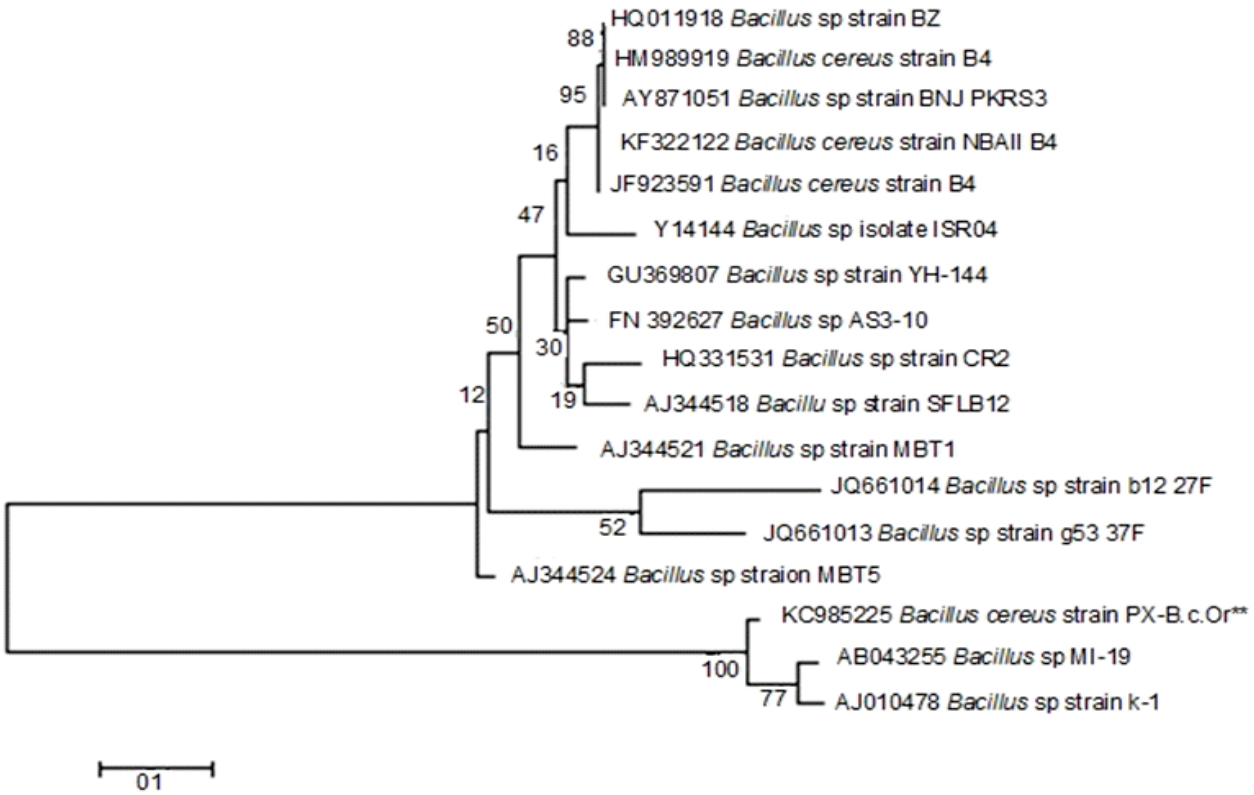

Fig. 2 : Phylogenetic analysis of 16S rRNA gene sequences for the bacterial strain *B. cereus* PX-B.C.Or

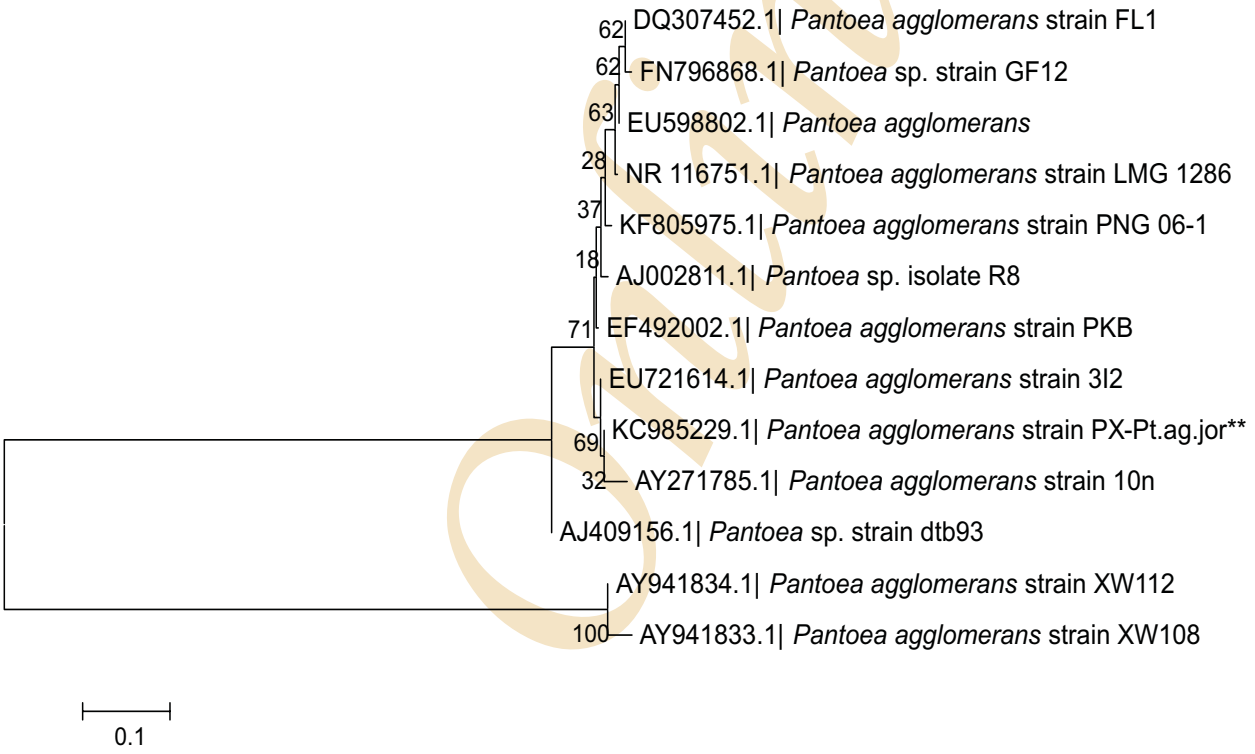

Fig. 3 : Phylogenetic analysis of 16S rRNA gene sequences for the bacterial strain *P. agglomerans* PX-Pt.ag.jor

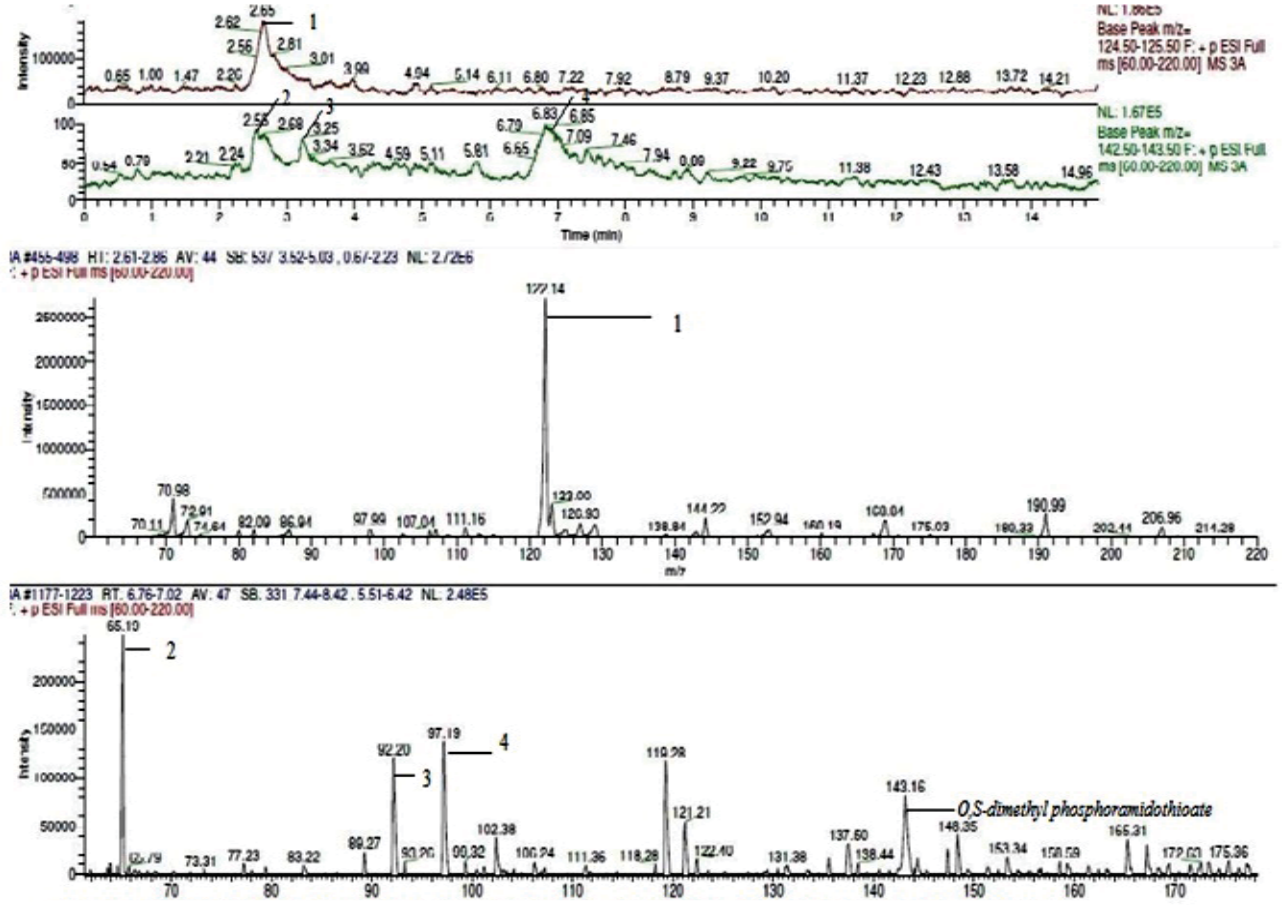

Fig. 4 : Chromatogram and mass spectra showing degradation of acephate into O,S-dimethyl phosphoramidothioate by *E. asburiae* (PXE)

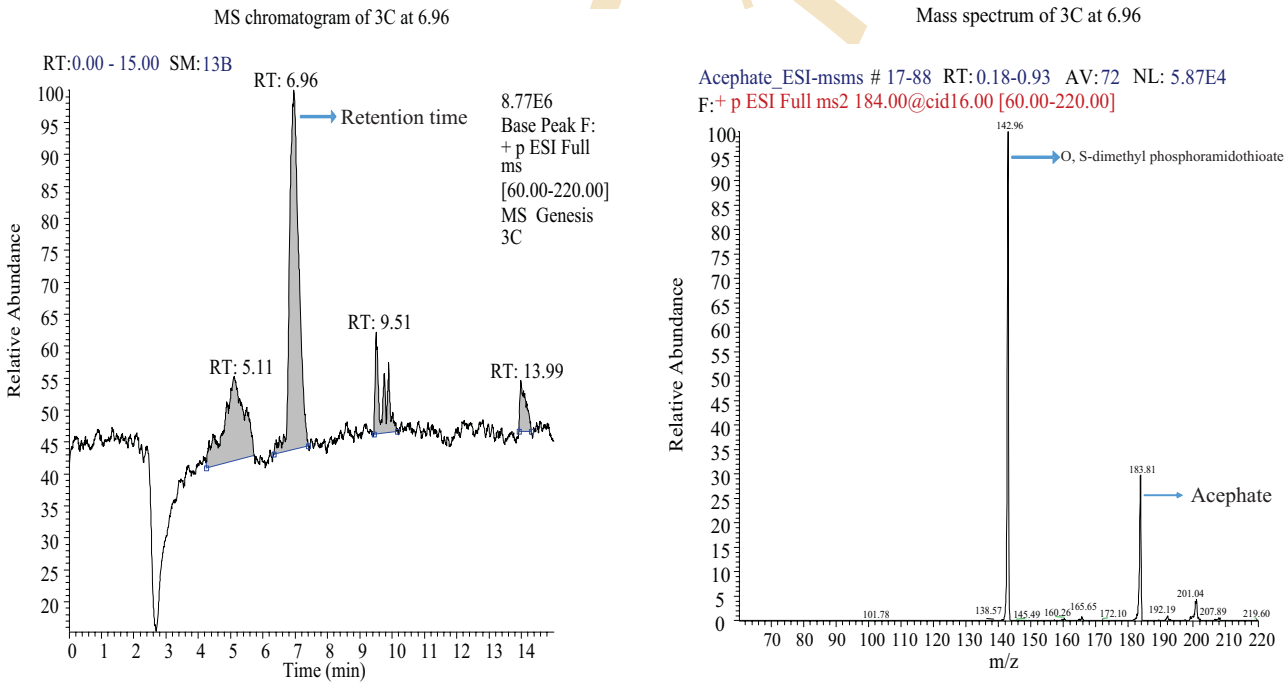

Fig. 5 : Chromatogram and mass spectra showing degradation of acephate into O,S-dimethyl phosphoramidothioate by *B. cereus* (PX-B.C.Or)

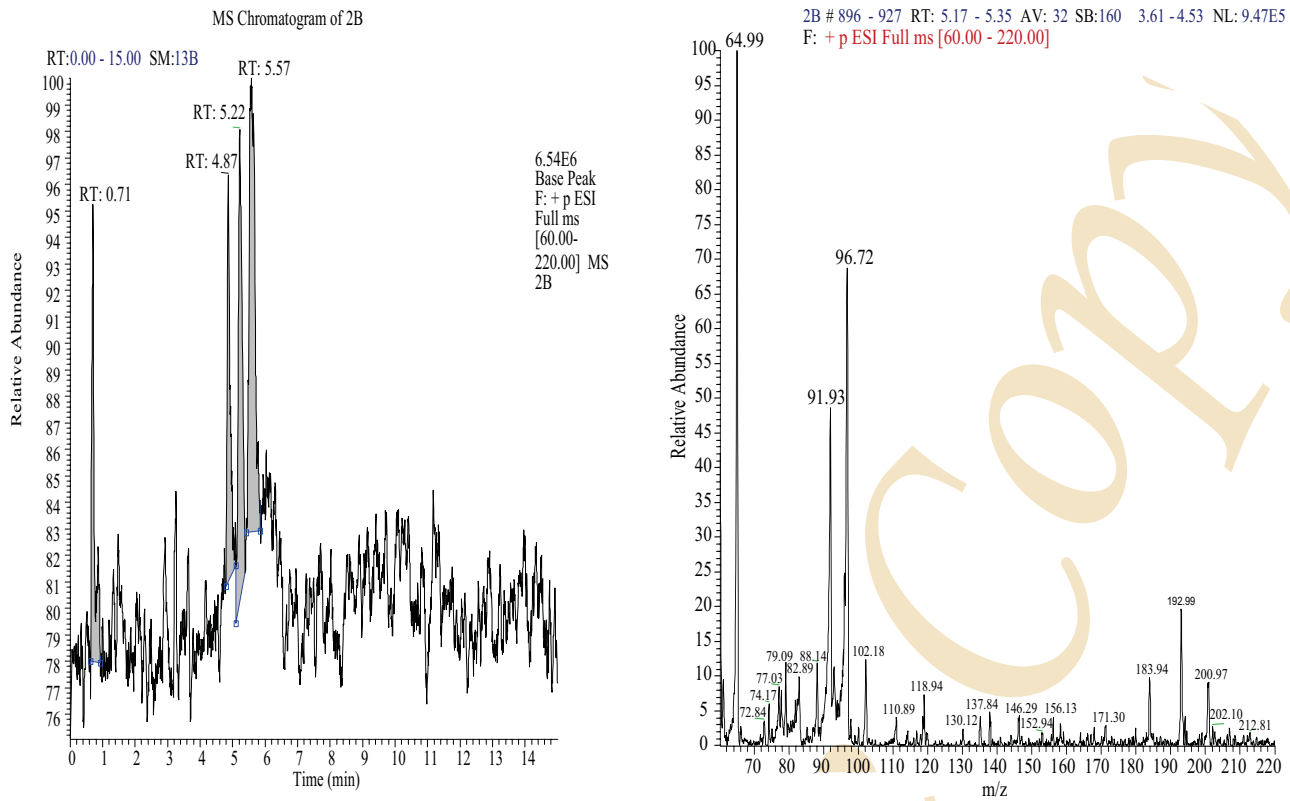

Fig. 6 : Chromatogram and mass spectra for acephate degradation by *P. agglomerans*

Rhodococcus strain isolated from gut of earthworm.

All the three bacterial isolates utilized acephate as sole carbon source, whereas *P. agglomerans* utilized sulphur also as source. In an earlier report, acephate was reported to serve as sole carbon source for growth of *Pseudomonas sp.* Ind01 isolated from soil (Aleem *et al*., 2012). As a potential source of sulphur and nitrogen, acephate was reported in many soil microorganisms like *Penicillium* (Zhao *et al*., 2010). *Hyphomicrobium* and *Luteibacter* have been reported to utilize methamidophos, first degradation product of acephate metabolism, as a sole nitrogen or sulphur source (Wang *et al*., 2011). *Enterobacter* strain B-14 was found to utilize organophosphorus insecticides as a source of carbon and phosphorous (Singh *et al*.,2004). *Bacillus cereus* strain DRY135 utilized acrylamide as a nitrogen source and showed highest growth with 90% degradation completed at 1000 mg l^{-1} of acrylamide after 10 days of incubation. Furthermore, acrylic acid was detected in the media during degradation (Shukor *et al*., 2009).

Bacteria isolated from the gut of *P. xylostella* could utilizing acephate for their metabolism and growth. LC-MS analysis of the extracts from growth study confirmed their acephate-degrading capabilities. As well, bacteria from different genera in the gut of diamondback moth possess numerous enzyme systems capable of degrading acephate. Nevertheless, toxicity of these degraded products to diamondback moth remains to be ascertained. Further, investigation are needed to elucidate the degradation pathway and enzymes involved in the insect gut system, which differs from that of other bacterial niches of soil and aquatic environments.

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