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RESEARCH ARTICLE

INSECTICIDE DEGRADATION BY GUT BACTERIA IN *COTESIA VESTALIS* HALIDAY, A POTENTIAL PARASITOID OF DIAMOND BACK MOTH, *PLUTELLA XYLOSTELLA* (LINNAEUS)

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ABSTRACT

Population of *Cotesia vestalis*, an endolarval parasitoid of Diamondback moth *Plutella xylostella* an important pest of crucifer crops was collected from different geographical locations in India. The gut bacteria (endosymbionts) of *C.vestalis* were isolated and identified. Predominant among them, *Enterobacter cancerogenus* was evaluated for its role in pesticide degradation of the widely used organophosphorus insecticide, Acephate. Growth of the bacterium on minimal salt media utilizing the pesticide as carbon source and reduced peaks of the degradative product in Liquid Chromotagraphy Mass Spectrometry (LCMS) analysis confirmed the role of the bacterium in the degradation of the pesticide. The association of the symbiont contributed to pesticide resistance in the parasitoid. The effective utilization of the symbiont in pest management programme is discussed**.**

INTRODUCTION

Development of insecticide resistance in insect pests has been a concern over the years. The changing cropping patterns, injudicious use of pesticides and pesticide treadmill have contributed to the development of resistance. The diamondback moth (DBM) *Plutella xylostella* Linnaeus is a major pest of crucifer crops world over that had developed resistance to every class of insecticide (Chawla and Kalra, 1976, Saxena *et al*., 1989 Reghupathy, 1996, Shelton *et al*., 2000, Srafaz and Keddie, 2005, Dhumale *et al.,* 2009,). *Cotesia vestalis (plutellae*) Haliday a solitary endolarval parasitoid is widely utilised for the biological control of the pest (Talekar and Shelton, 1993., Liu *et al.,* 2000., Zhi-Hua Shi, 2002., Nofemela and Kfir, 2005). Parasitism of DBM larvae to the tune of 30-50% (Walde *et al.,* 2001), 36.8% (Hemachandra and Singh, 2007), 34% (Mitchell *et al.*, 1997). 53-56% (Seenivasagan *et al.,* 2010), 90-95% under pesticide free conditions (Hasseb *et al.,* 2004) have been documented. Host resistance to insecticides favour development of resistance in the parasitoids also (Iqbal and Wright, 1996. Liu *et al.,* 2001, Liu and Liu, 2001). High levels of resistance to insecticides in the field were reported in certain natural enemies (Rathman *et al.,* 1990, Baker and Weaver 1993, Tabashnik and Johnson, 1999).

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The phenomenon of resistance development in the parasitoids is biologically mediated (Brownlie and Johnson, 2009), since the insect system is a large source of microbial diversity and parasitoids have physiological mechanisms to colonise these and regulate them for their benefit. These gut microbes (symbionts) have most intimate association and inflict metabolic, physiological and reproductive alterations. They play role in nutrition and development (digestive efficiency, supply amino acids and vitamins, degradation of lipids) (Breznak, 1982, Campbell, 1990, Dillon and Dillon, 2004, Rajagopal, 2008) reproduction (cytoplasmic incompatibility, feminisation, parthenogenesis and male killing) (Werren *et.al.,* 2008).

Immunity (resistance to insecticides defense against pathogens) and thermal tolerance (Qi Su *et al.,* 2013). The Symbiont mediated protection in insects has been studied in several insect pests (Scott *et al.,* 2002, Moran 2006, Hiane 2008, Brownlie and Johnson, 2009,. Kikuchia *et al.,* 2012, QiSu *et al*., 2013). The use of naturally or artificially selected insecticide resistant strains of natural enemies has been advocated to enhance the compatibility of biological and chemical methods**.** Here, we report the role of gut bacterium in the parasitoid *Cotesia vestalis,* in degrading insecticide.

MATERIALS AND METHODS

Collection of *C.vestalis*

Parasitized larvae of DBM (20-50 numbers) were collected on cabbage /cauliflower crops from different geographical areas of India *viz.,* Varanasi (Uttar Pradesh), Tirupati (Andhra Pradesh), Hyderabad (Andhra Pradesh), Coimbatore (Tamil Nadu), Pune (Maharashtra), Hoskote (Karnataka), Anand (Gujarat), Bhubaneshwar (Odisha), Jorhat (Assam) and Delhi. where brought to NBAII laboratory and observed for its emergence. The emerged parasitoids were identified as *Cotesia vestalis*.

Mass rearing *C. vestalis*

The parasitoid was reared on a stock culture of Diamondback moth (DBM), *Plutella xylostella* maintained on mustard seedlings raised in ice cream cups (55.2x68.9x45.1mm) containing vermiculite. Mustard seeds were sprinkled as a layer and allowed to germinate by maintaining proper moisture. Seedlings thus raised in cups were placed in acrylic cages (24x24x24") and DBM moths were released in the cages for oviposition.

After 24-48 hours, the cups are removed and kept in trays for egg hatch and larval development. Fresh seedlings were again provided for the moths to oviposit. The mustard seedlings containing 2nd instar larvae of *P.xylostella* are placed in oviposition cages (24x24x24") and were exposed to adults of the parasitoid for 24 hours and kept back in trays to permit the development of the parasitoid. The parasitoids were again provided with fresh batch of 2nd instar larvae and the process continued. The egg + larvae of the parasitoid complete development in 7-8 days which includes 24 hours of egg stage. The cocoons of the parasitoid are collected from the seedlings and placed in containers. The emerged adults were utilised for isolation of gut bacteria

Molecular characterisation- Isolation of gut bacteria strain from *C. vestalis*

The population of the parasitoid obtained from different locations were assayed for the gut bacteria. The head, wings and thorax part of adult *C. vestalis* was dissected out carefully and it was surface sterilized with 75% ethanol for 30 seconds and transferred to in distilled water for 10 min. and rinsed with 0.01% sodium hypochlorite. The specimens were homogenized in 100 µl sterile distilled water in 1.5ml centrifuge tubes. The homogenates were plated in Luria agar media. The colonies were selected based on colony characters (involving colony size, shape, colour, and margin) and morphology of isolate based on Gram's staining technique. The isolates were sub-cultured by streaking in LB agar media (Table 1).

Table 1. Composition of LB-agar media

Ingredient	Quantity
Tryptophane	10g
Yeast extract	5g
Nacl	10g
Agar	15g
Water	1000ml

The colonies obtained were identified according to Bergey's Manual of Systematic Bacteriology (Keddie *et al.,* 1989). The shape and morphology of bacterial cells were determined by light microscopy. A single colony was streaked on to LB slant to obtain the pure culture. The pure culture was then inoculated to LB broth and incubated at 30˚C for 48h for its maximum growth. Plated bacterial cultures were subjected for Gram's staining technique.

Identification of Bacterial Isolate

Bacterial genomic DNA was isolated using Qiagen kit. 16S rRNA gene was amplified using 16S universal primers i.e. 16S F 5'-ACTCCTACGGGAGGCAGCAG-3'and 16S R 5'-ATTACCGCGGCTGCTGG-3'. PCR protocol consisted of an initial denaturation at 95˚C for `1min followed by 30 cycles of 95˚C (1 min), Annealing 55˚C (1min) and 72˚C (2min) and a final extension step of 72˚C for 2min. The PCR product was verified by running samples on a 1.5% agarose gel. The identified bacterium that occurred predominantly in the various populations of the parasitoid was evaluated for pesticide degradation.

Insecticide degradation by the abundant bacteria isolated from the parasitoid

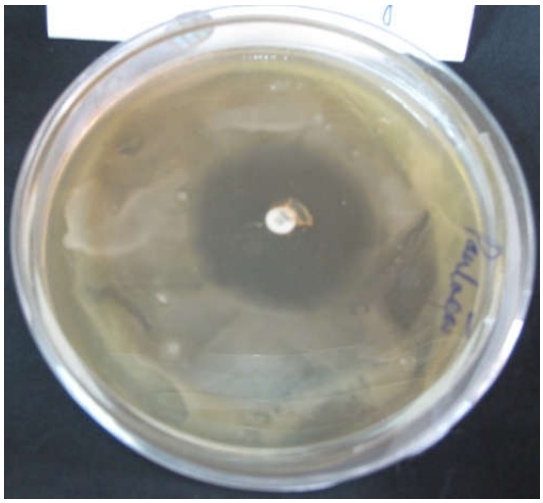
Bacteria were isolated from the various populations of the parasitoid. Among the bacteria, was more predominant. Hence, the bacterium was evaluated for its role in degradation of the widely used organophosphorus pesticide, Acephate 75% SP (O,S-Dimethyl acetylphosphoramidothioate). Technical grade Acephate (LUCID-75) (99%) purity obtained from Cheminova Insecticides Limited, was used for the study. The insecticide was used in different concentrations of 100ppm, 200ppm and 300ppm.

Spectrophotometric method for checking the growth of the bacteria in minimal salt media and LCMS studies to determine pesticide degradation

Minimal salt media (MSM) are those that contain the minimum nutrients possible for colony growth, Minimal medium typically contains a carbon source for bacterial growth, which may be a sugar and various salts that provide essential nutrients to allow the bacteria synthesize protein and nucleic acids. The MSM has the following composition in (g/L): KH2PO4, 4.8; K2HPO4, 1.2; NH4NO3, 1.0; MgSO4.7H2O. 0.2; Ca (NO3)2.4H2O, 0.04; and Fe (SO4)3, 0.001 with pH 7.0 (Rasul *et al.,* 1988). Luria-Bertani (LB) broth (Sambrook *et al.,* 1989) and mineral agar were used according to manufacturer's instructions (HI-MEDIA). MSM-agar medium was prepared and poured in to a Petri-plate in a laminar air flow chamber along with the different concentration of insecticide (Table 2) and mixed properly and allowed for solidification. The control plates were prepared without the insecticide (Acephate). Once the media was solidified a depression was made at the centre of the plate. Bacterial suspension of 2 ml was taken and poured in the depression in the centre the plate. The plates were incubated at 30˚C for 2 days and the bacteria that formed clear zone around the colonies were selectively isolated and used for the further study (Plates 1 & 2).

Table 2. Composition of minimal media, insecticide and bacterial culture considered for degradation study

MSM Media	Acephate (99%)	Bacterial Culture
Control (50ml)	-	-
50ml	-	2ml
Control (50ml)	100ppm	-
50ml	100ppm	2ml
Control (50ml)	200ppm	-
50ml	200ppm	2ml
Control (50ml)	300ppm	-
50ml	300ppm	2ml



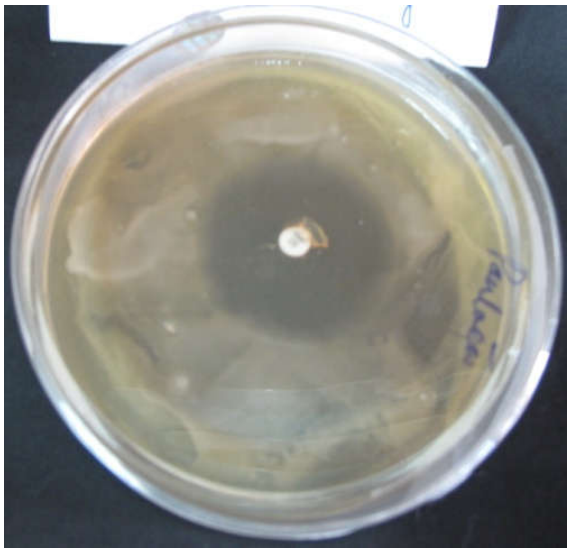


Plate 1 & 2.Clear zone test for *Enteraobacater cancerogenus* on minimal salt media for degradation of insecticide Acephate

Technical grade Acephate (99%) purity was used for the study. Based on clear zone test, the ability of the bacteria, *Enterobacter cancerogenus* to degrade the insecticide was assessed Previously autoclaved series of 250ml conical flasks containing 50ml each of MSM medium was taken and 1ml of isolates and 5ml each of different concentrations of insecticides were added, and the control was maintained.

The flasks were incubated on a shaker at 200rpm at 30˚C for 7 days. Controls for each bacterium were maintained without insecticide treatment and positive control of MSM with insecticide alone was also maintained.

The pH of all media was adjusted to 7.0. Growth was monitored each day spectrophotometrically with the optical density at 660nm. After 7days incubation the cell mass in MSM with and without insecticide was separated by centrifugation at 8000xg for 10min and the supernatant of 50ml was collected separately in the fresh tubes. The supernatant was further analyzed in LC-MS to detect the concentration of insecticides degraded and to elucidate the final degradative product.

RESULTS

Culturable bacteria *Pseudomonas putida, Enterobacter cancerogenus, Bacillus cereus, Pantoea agglomerans* were identified in the populations of the parasitoid collected from different locations, based on the characterisation of 16Sr RNA gene. The Genbank accession numbers were obtained for the bacteria (KC589741, KC13936, KC582828 and KC512244, respectively), *Enterobacter cancerogenus* was predominant and occurred in greater proportion than others. Hence, the bacterium was evaluated for its role in pesticide degradation. The degradation of the pesticide aeephate by the bacterium *Enterobacter cancerogenus* was detected both by spectrophotometric method by checking the growth of the bacteria in minimal salt media and LCMS analysis to elucidate the final degradative product.

The bacterium at 200 ppm showed perfect growth curve at the initial time (0 hr) the log phase started and after 24h the exponential growth was observed, which indicated accelerated growth and after 48h stationary phase initiated and continued till 72h, followed by a gradual decrease (Fig 1).

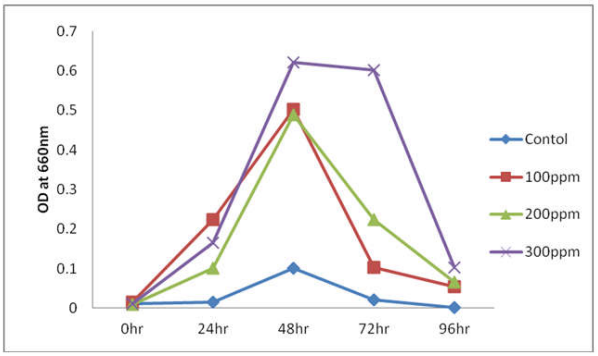


Fig 1. Growth curve for *Enterobacter cancerogenus* in MSM containing different concentrations of Acephate)

Pesticide degradation by bacterium *Enterobacter cancerogenus* was confirmed based on growth curve in minimal media with acephate. Growth curve followed a sigmoid pattern with 200 and 300 ppm of insecticide concentrations indicating utilization of pesticide as source of carbon. No inhibition of growth was observed till 48 hours suggesting the duration required for acclimatization to synthesize the degradative enzymes. The growth curve in the other concentrations did not follow the sigmoid pattern. The sample with perfect growth curve was analysed by LCMS. Further, degradation of acephate by the bacterium was elucidated by Liquid Chromatography - Mass Spectrometry (LCMS). LC-MS works on soft ionization technique and is useful for detection of non volatile compounds, vitamins, amino acids, protein and peptides.

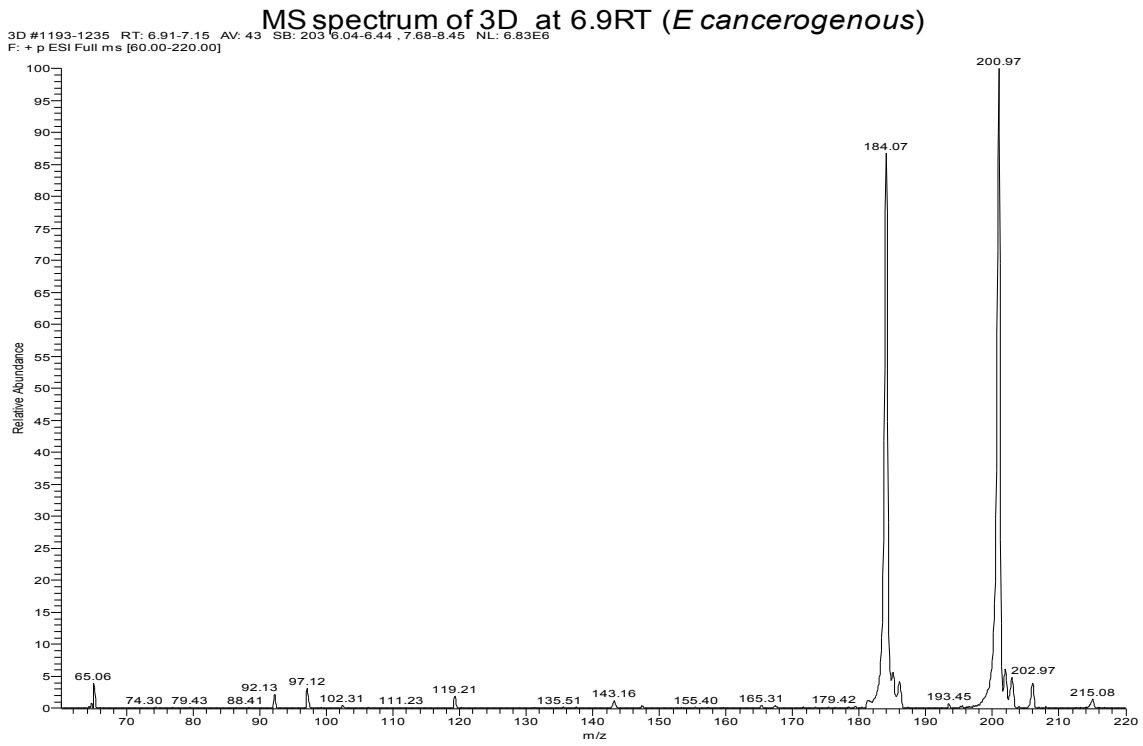


Fig. 2. Mass spectrum of 3D sample (MM+ Acephate 300ppm+*E. cancerogenous*)

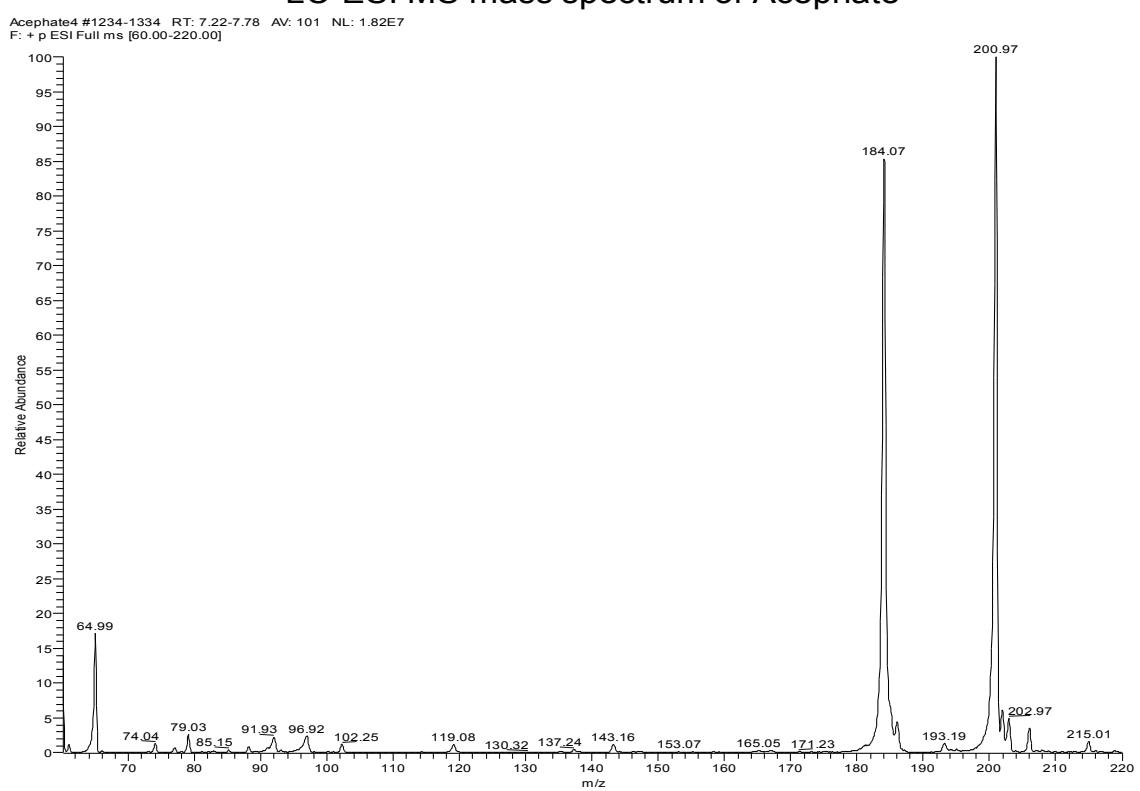


Fig. 3. Mass spectrum of standard acephate

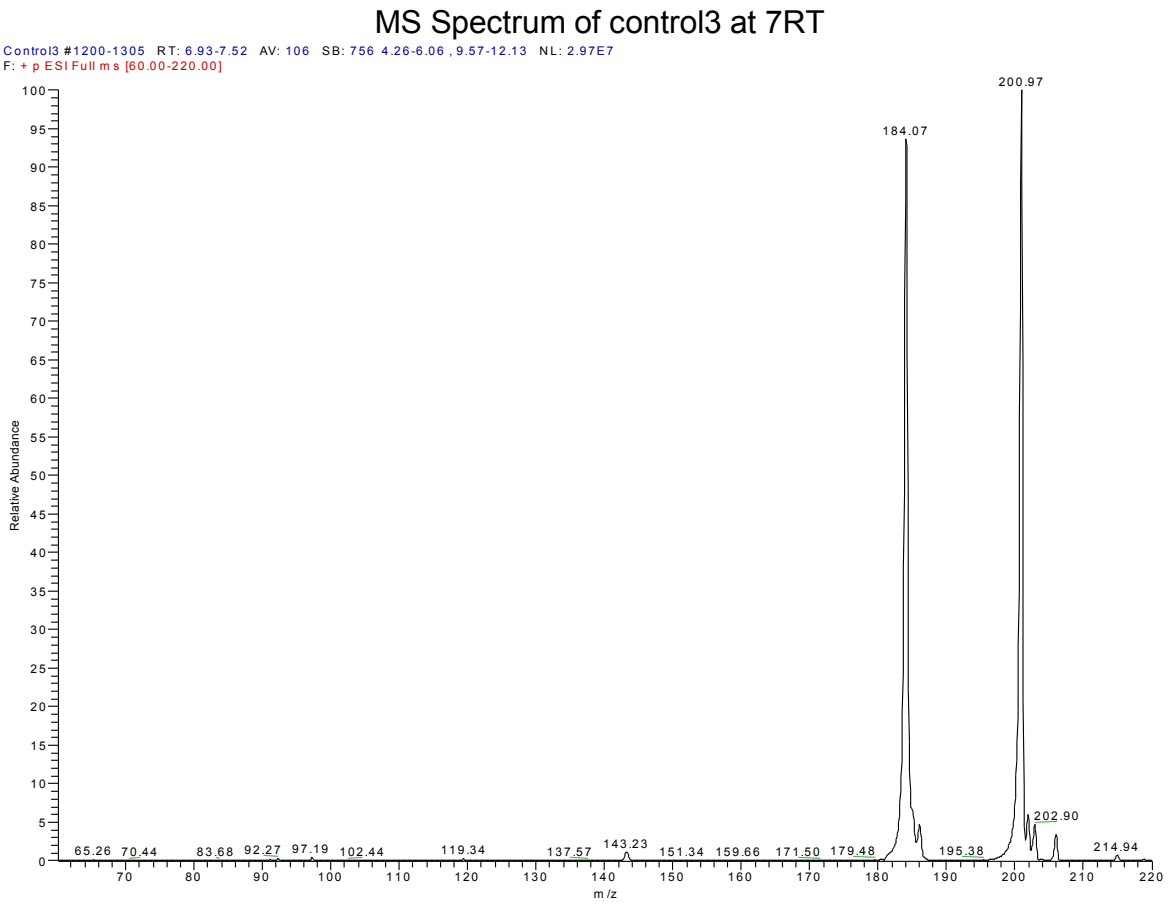


Fig. 4. Mass spectrum of Control 3 (MM+ Acephate 300ppm)

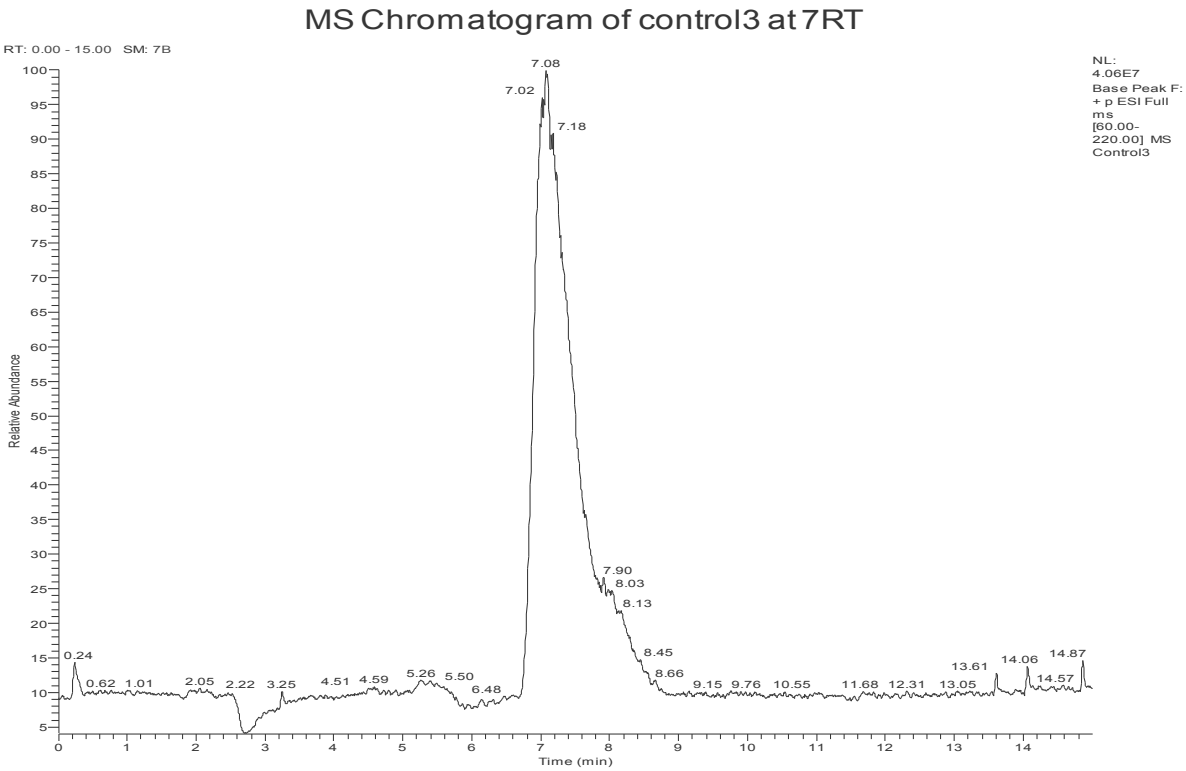


Fig. 5. Chromatogram of Control 3 (MM+ Acephate 300ppm)

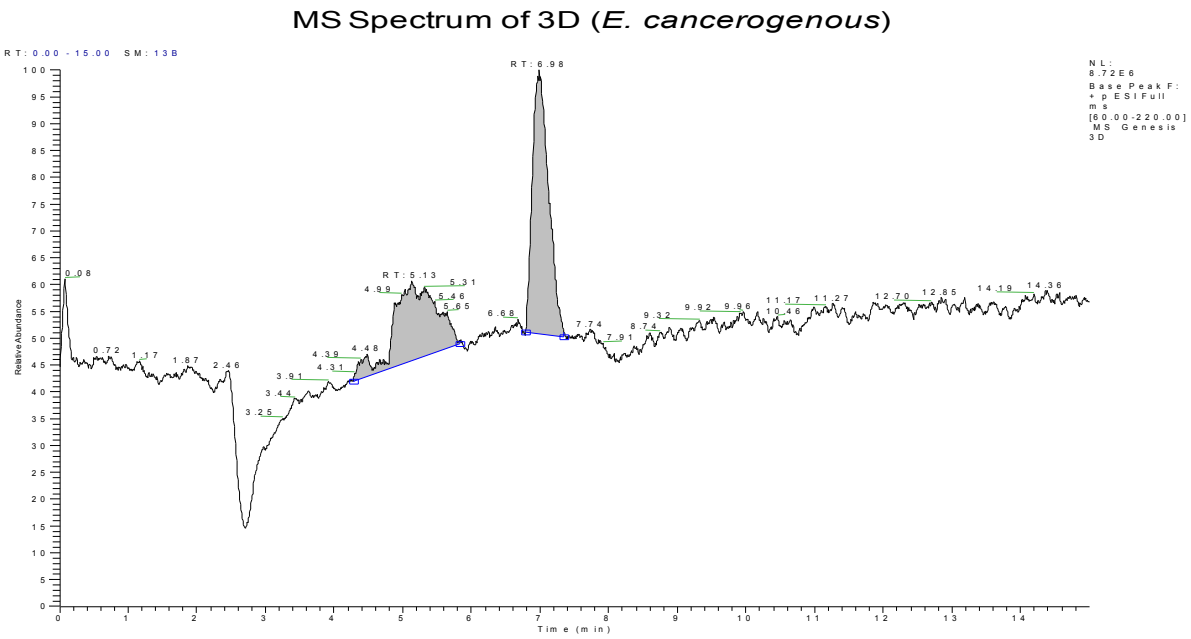


Fig. 6. chromatogram of 3D sample (MM+ Acephate 300ppm+*E. cancerogenous*)

In the present studies, the technique was used to detect the degradation of insecticide by gut micro flora of the parasitoid. The LCMS analysis indicated ability of *E. cancerogenous* to degrade Acephate (183.16 g/mol) in to des-O-methyl acephate (143.2 g/mol) based on the spectrum formed (Fig. 2). The area of the spectrum decreased which indicated degradation of the compound. The mass spectrum of the control sample had a molecular weight of 183 which matched with the standard acephate (Fig 3 & 4). The chromatogram revealed reduced peak in comparison to control (Fig 5 and 6) which indicated degradation. Peak area of 3-control was 1480294729 arbitrary units and the 3D test sample was 69908437.9 arbitrary units. The reduced peak area in the test sample indicated that there could a role of the bacteria in reduction of the insecticide load

DISCUSSION

Our studies have established degradation of organophosphorus insecticide Acephate, by the bacterium *Enteroabacter cancerogenus* in the parasitoid *Cotesia vestalis*, contributing to its survival. Degradation of organophosphorus insecticdes by microorganisms was earlier reported by several researchers, Walker, 2000., Mishra *et.al.*, 2002 and Surekharani *et.al.*, 2008). A similar strategy of growth of the bacterium *Enterobacteria* sp. in the presence of organophosphorus insecticides, phosolone (Lee *et.al.*, 1992), Methomyl (Mohammed, 2009), dichlorvos (Agarry *et.al.,* 2013), was reported previously. The bacteria *Providencia staurtii* (Racke *et al*., 1990) and *Flavobacterium balustinum* (Byoumi *et al.*,2009) utilized chlorpyriphos as source of carbon for growth. The presence of oxidized compounds and absence of phosphorus indicated degradation of monocrotophos in minimal salt by *Klebeiella sp, Pseudomonas flourescens and Bacillus subtilis* (Kaneka *et.al*., 2004, Kavikarunya and Reetha, 2012) for growth utilizing carbon from the pesticide. Our results corroborate with the reports of these workers.

Retention time of acephate was 7.2RT but there other peaks were also noted at different retention time as well which had very low peaks. Agilera and Fernandez, (1998) suggested formation of detoxified compounds due to replacement of sulphur by oxygen during the oxidation processes of degradation. Such a phenomenon could be involved in the degradation of acephate in the present studies contributing to resistance in the parasitoid. Organophosphorus insecticides are widely used for pest management in various crops. Insecticide resistance in endoparasitoid is an acquisition from the host. Symbiont mediated insecticide resistance was earlier reported in the bean bug *Riptortus pedestris* and allied stink bugs that harbour mutualistic gut bacteria of the genus *Burkhoideria* that degraded the insecticide fenitrothion (Kikuchia *et al*., 2012).

Detoxification by symbionts as a mechanism of resistance depends on the ability of the microbes to degrade the compound and rapidly evolve. Symbiont primed parasitoid (paratransgenesis) could be on the anvil through genetic drive (Zablou *et al*., 2004). The co-evolution however, depends on the mode of transmission of these microbes and spread of resistance phenotype (Broderick *et al.,* 2004, Moran, 2007, Werren, 2012). Dunning *et al.* (2007) suggested that co-evolution provides for lateral gene transfer between the host and symbiont and opined that such a process when continued in predators and parasitoids would improve their sustenance in frequently pesticide sprayed areas and contribute to enhanced biological control. Therefore, exploiting their relationship would be fruitful in up regulating the fitness attributes of the parasitoid for effective biocontrol strategies in pest management, as insecticide compatible natural enemies. The breadth of mechanism by which symbionts provide protection is still largely unknown. In several systems, protection results from the production of toxins and antibiotics that target and reduce the effect of predators and pathogens.

The nature of toxins and antibiotics utilized in symbiont mediated protection, might provide novel resources of compounds for control of pests (Brownlie & Karyn, 2009). Thus, utilisation of xenobiotic compounds by microorganisms is a crucial phenomenon in fully understanding the dynamics of symbionts.

Conclusion

Endosymbiotic gut bacteria were isolated from different geographical populations of *C.vetalis,* the larval parasitoid of the diamondback moth, *P.xylostella.* Endosymbionts have been reported to play role in regulation of physiological processes of the insects. Our studies have conclusively established the role of *Enterobacter cancergenus* , an endosymbiotic bacteria in the degradation of organosphosphorus insecticide, which are more widely used against the pest. The gut bacterium aided insecticidal degradation tends to contribute to the fitness and sustenance of the parasitoid under stressed conditions enabling effective biocontrol of the pest.

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