INTERNATIONAL JOURNAL OF CURRENT RESEARCH IN BIOLOGY AND MEDICINE
ISSN: 2455-944X
www.darshanpublishers.com
Volume 1, Issue 3 - June - 2016

Original Research Article

SOI: http://s-o-i.org/1.15/ijcrbm-2016-1-3-3

Lipase activity and protein profiling of *Helicoverpa armigera* (cotton boll worm) and *Lucinodes orbonalis* (shoot borer) infected by *Steinernema* sp. and its symbiont *Xenorhabdus* sp. isolated from Western Ghats of Munnar.

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Abstract

The enzyme analyzed in the *Helicoverpa armigera* and *Lucinodes orbonalis* infected with *Xenorhabdus* sp. of Munnar. After 24 hours a clean zone was observed. The lipase has insecticidal toxic activity which had degraded the lipid content of the pest *Helicoverpa armigera* and *Lucinodes orbonalis.* The protein profiling was done in *Steinernema* and *Xenorhabdus* sp. Infected *H.armigera* and *L.orbonalis*. The control had 32.0 to 41.0 kDa and infected showed 31.7 to 45.0 kDa in *Xenorhabdus* sp. and *Steinernema* sp. infected it was 20.0kDa to 43.0 kDa in *H.armigera* and *L.orbonalis.* The control showed 32kDa whereas the infected showed 31 kDa to 98 kDa in *Xenorhabdus* sp and the *Steinernema* infected showed 31.7 kDa. The protein sub unit is high molecular weight compare protein which has death of *H.armigera* and *L.Orbonalis*.

Keywords: *Helicoverpa armigera, Lucinodes orbonalis, Xenorhabdus, Steinernema.*

Introduction

Nematodes are simple roundworms. Colorless, unsegmented and lacking appendages, nematode may be free living, predaceous or parasitic. Many of the parasitic species cause important diseases of plants, animals and humans. Other species are beneficial in attacking insect pests, mostly sterilizing or otherwise debilitating hosts. A very few cause insect death but these species tend to be difficult or expensive to mass produce, have narrow host specificity against pests of minor economic importance, possess modest virulence or are otherwise poorly suited to exploit for the pest purposes. The only insect parasitic nematodes possessing an optimal balance of biological control attributes are Entomopathogenic or insecticidal nematodes in the genera *Steinernema* and *Heterorhbditis*. These multi-cellular metazoans occupy a bio control middle ground between microbial pathogens and predators/parasitoids and are invariably lumped with pathogens, or because of their symbiotic relationship with bacteria.

Entomopathogenic nematodes are extraordinary lethal to many important insect pests, yet are safe for plants and animals. This high degree of safety means that unlike chemicals, or even Bacillus thuringiensis, nematode applications do not require masks or other safety equipment and re-entry time, residues, groundwater contamination, chemical trespass and pollinators are not issues. Most biological require days or weeks to kill, yet nematodes, working with their symbiotic bacteria, can kill insects within 24-48 hours. Dozens of different insect pests are susceptible to infection, yet no adverse effects have been shown against beneficial insects or other non-targets in field

studies (Georgis *et al.*, 1991; Akhurst and Smith, 2002).

Naturally occurring entomopathogenic nematodes and their symbiotic bacteria are important biotic factor in suppression of insect pest populations in soil and cryptic habitats. The virulent species of these nematodes are commercially produced as biological control agents all over the world encompassing North America, Europe, Asia and Australia in glasshouse crops, orchards, ornamentals, turf, lawn, and forestry. India has a great potential to exploit these beneficial nematodes for the suppression of insect pests. Recent emphasis on mass production and formulation technologies of these nematodes in India stresses a need to implement safer and effective pest control methods. This article provides an overview of recent development on formulation and commercialization of entomopathogenic nematodes, and evaluates their potential exploitation in India (Divya and Sanker, 2009).

Materials and Methods

Rearing *Galleria mellonella*

The rearing of *Galleria mellonella* in laboratory is important for multiplication of entomopathogenic nematodes, mandatory for biological control studies. The larvae of greater wax moth *Galleria mellonella* (Lepidoptera: Galleridae) were used for baiting the nematodes. The larvae were maintained in a large container at room temperature on an artificial diet. Aeration was provided by making small holes on the lid. Once in a week larvae were transferred in to a fresh diet. Some of the larvae were kept as such in a container for pupation. During adult stage, they were placed on a container having wax coated butter paper, in which the female laid eggs. The adults do not feed, their duty is to mate, lay eggs and die. The eggs were removed or taken out from the butter paper carefully using sterile blade. Then washed with sterile distilled water and 0.01% formalin and then transferred to fresh diet. The eggs hatch within 3-4 days.

Collection of soil samples

A total of thirteen soil samples were collected from different locations around Ooty, Munnar, Palladam, Madathukulam, Anaikatti, Erode, Sivagiri, Sethumadai, Mettukalam, Karur, Aliyar, Korakadu and Karukkumpalayam. From all location 2500 gram of soil samples were collected at a depth of 15 - 20cm from the surface of soil and transferred into clean polythene bags and were brought to the laboratory and soil samples were maintained at 25 - 32^{0}C for future study.

Isolation of Entomopathogenic Nematode (EPN's)

Entomopathogenic nematodes were recovered from soil sample using the insects baiting methods as described by Bedding and Akhurst, (1975). Insects baits (last instar larvae) of *Galleria mellonella* were placed in 100ml plastic containers which contained 50 grams of collected soil moistured with water. Each collected soil from different areas were kept in separate containers. These containers were covered and holes were made for respiration throughout the baiting period. Larvae were checked for infection every day and the dead ones were removed and live larvae were placed in the containers. The dead larvae were isolated and thoroughly rinsed in 0.01% formalin and placed in White's trap (Kaya and Stock, 1997) until the emergence of third-stage infective juveniles of nematodes in another two to three days. The emerging nematodes were pooled from each sample and stored in culture flask (T-flask). These nematodes were used to infect fresh larvae of *Galleria mellonella* for mass propagation of nematodes for identification and establishment of culture. The culture flasks with nematodes were maintained at 25^{0}C

Isolation of Bacterial Symbiont

Nematode acts as a vector by carrying the bacterium and infects the larvae. They enter through the natural opening like mouth, anus and spiracles. It multiplies within the larvae by using body parts of the larvae as nutrient source. The symbiotic bacteria in the nematodes also multiply along with nematodes. They lead to the death of the larvae by septicemia. The dead larvae were after 24 hours sterilized. The larvae harbour the symbiotic bacteria, so to isolate the bacteria a loopful of haemolymph were streaked on nutrient agar plate containing 0.004% 2,3,5 Triphenyl tetrazolium chloride and 0.025% Bromothymol blue (NBTA). The plates were incubated at 28^{0}C for 24 hours. The two forms phase I and II were differentiated based on the colour of the culture. Only primary form bacteria were used in the study. From the plate, single colony is isolated by inoculation loop and is left into Liquid broth (LB) for multiplication by shaking the conical flask frequently by keeping it at 37ºC or in a shaker. The conical flask is left overnight at 37ºCfor culturing bacteria and is used for further studies.

Enzyme estimation

Lipase

The media was prepared by adding 3g of agar, 2-3 drops of methyl red (pH indicator), 2% of Tween 28 in 100ml of distilled water and kept for sterilization. It was then poured into petri plates and allowed to solidify. After solidification, one plate was streaked with test organism by continuous streaking and the control plate was maintained without the test sample. Plates were then incubated at 37ºCfor 24hours.

Protein profiling using SDS-PAGE gel Electrophoresis

To profile protein present in the sample from infected and uninfected *Helicoverpa armigera* and *Leucinodes orbonalis* larvae, SDS-PAGE were carried out in the method of Laemelli, (1970).

Assemble the clean glass with spacers and seal the bottom. Prepare 12% separating gel with the following components: 3ml acrylamide solution, 1.85ml Tris free base (pH8.8), 2.55ml distilled water, 100µL of 10% SDS, 100µl of 10% APS and 10µL of TEMED for minigel. Add APS and TEMED finally and pour into the glass plates leaving 1/3 rd space for stacking gel. Overlay the separating gel with few drops of n - butanol or ethanol to ensure uniform and quick polymerization (optional). Prepare 4% stacking gel with the following components: 1ml of acrylamide, 1ml of Tris free base (pH6.8), 1ml of distilled water, 100µL of 10% APS and 15µL of TEMED for mini gel. Remove the alcohol layer and pour the stacking gel and place the comb immediately. Allow for polymerization (approx. 10-15 min). Prepare the samples by mixing 50µL of the sample with 10µL of the sample buffer and heat for four min in boiling temperature and cool in ice. Remove the comb from the polymerized gel and fix the gel in the electrophoresis unit with electrode buffer in both upper and lower tank.

.Load the sample in the well carefully using micropipette with protein molecular weight marker in one well. Run the gel at constant voltage of 50 V until the dye front enters separating gel and then run at 100 V until the dye front reaches bottom. Turn off the power supply, remove the gel plates and transfer the gel to staining solution after washing with distilled water once. Incubate the gel in staining solution under shaking for 4 hours to overnight (in dark). Destain the gel by incubating in destining solution (in dark). Visualize the blue protein bands under white/ fluorescent light source.

Results

Enzyme estimation

The enzymes lipase analyzed. After 24 hours, a clear zone was observed around the streaked test organism. This indicated the presence of lipase activity in the sample.

Protein profiling by SDS-PAGE

The crude sample were electrophoresed by SDS-PAGE. The protein markers were also co-electrophoresised with the samples so as to determine the molecular weight of *Steinernema* sp. and *Xenorhabdus* sp. the standard protein markers are 97.4 kDa phosphorylase b, 66 kDa Bovine serum albumin, 43 kDa Ovalbumin, 29 kDa carbonic anhydrase, 20.1 kDa soyabean trypsin inhibitor and 14.3 kDa.

Multi-banding pattern were observed in both infected with *Steinernema* sp. and *Xenorhabdus* sp. and control *H. armigera* and *L. orbonalis*. The patterns of *Helicoverpa armigera* was of range 20.0 kDa to 43.0 kDa in *Xenorhabdus* sp. and 31.75 kDa and 45 kDa in *Steinernema* sp. infected respectively, whereas the control showed a range of 32.0 kDa to 41.0 kDa. In *Leucinodes orbonalis*, the value was in between 31 kDa to 98 kDa in *Xenorhabdus* sp. infected and it was 31.7 kDa in *Steinernema* sp. infected bands. The control showed 32 kDa.

In the present study, the protein sub-unit may be high molecular weight complex proteins which would have caused death *to Helicoverpa armigera and Leucinodes orbonalis* due to its insecticidal toxicity of protein. The larval proteins were degraded by the *Xenorhabdus* sp.

Discussion

In the present study, there were secretions of Protease and Lipase by the *Xenorhabdus* sp. as earlier reported by Gregory, (2010). *Xenorhabdus* sp. is associated with its symbiont *Steinernema* sp. and it secretes lipase and is left in the haemolymph of the pests *H. armigera* and *L. orbonalis*. So the lipase has insecticidal toxic activity which would have degraded the lipid content of the pest. It has also supressed the immunity of the pests *H. armigera* and *L. orbonalis*.

So the host defence mechanism was inhibited by the *Xenorhabdus* sp.

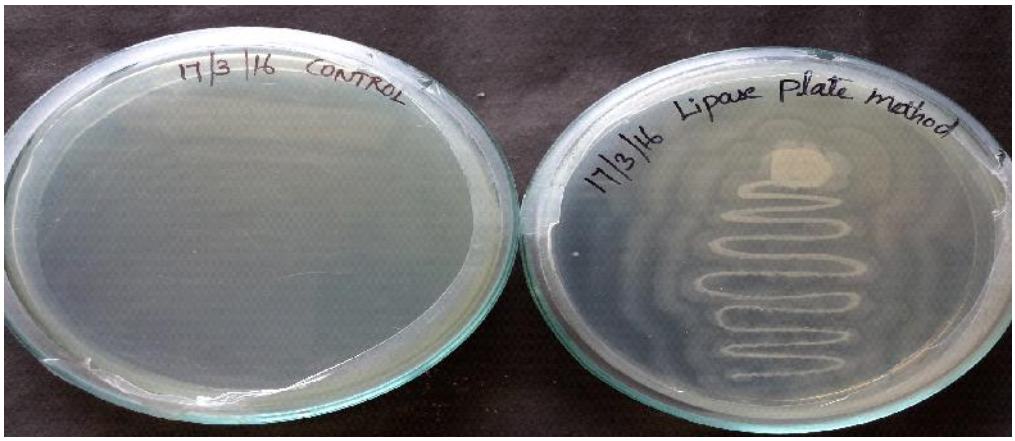
X. nematophila produces at least three distinct haemolysins, two lipases, and two proteases. *X. nematophila* produces a plethora of putative virulence factors, including toxins, lipases, haemolysins, and proteases, which may contribute to disease and modulation of host immunity. *X. nematophila* possesses numerous predicted virulence factors that may contribute to its ability to kill diverse insect hosts, but little is known about the regulation of these factors or their precise involvement in the infection process.

After the release of entomopathogenic bacteria into the insect haemocoel by the EPN's, the bacteria appear to rapidly overcome the insect defence mechanisms and kill the insect. During the growth within the insects, the bacterium is thought to release a variety of compounds including lipases, protease, antibiotics and lipopolysaccharides. Previous studies on the extracellular protease have been equivocal as to their role in insect toxicity (Magda *et al.*, 2007). Bowen *et al*., (2000) characterized extracellular proteases, which are suggested to participate in the activation of toxic complexes. *Xenorhabdus nematophila* also express insecticidal proteins and secrete proteases which are active against insect immune factors.

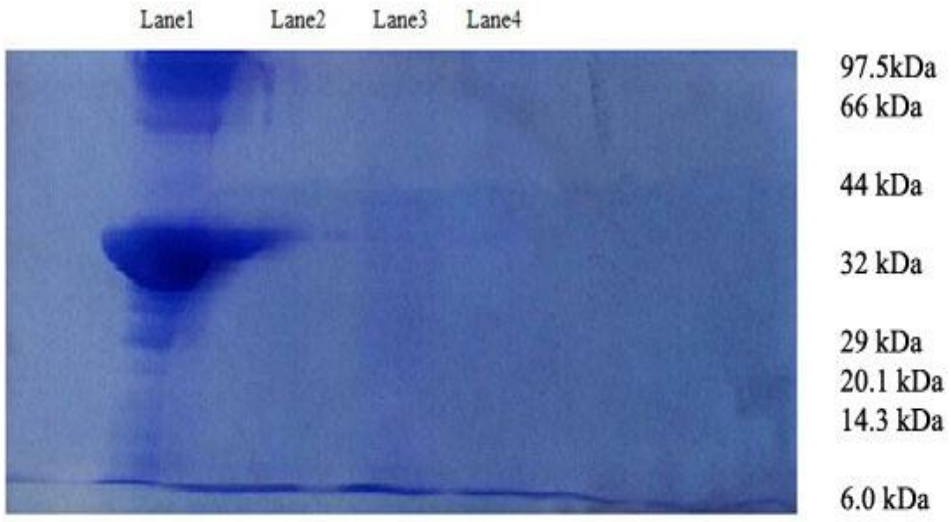
Enzymatic activity of *Xenorhabdus* sp. showing clear zone

A. Lipase activity

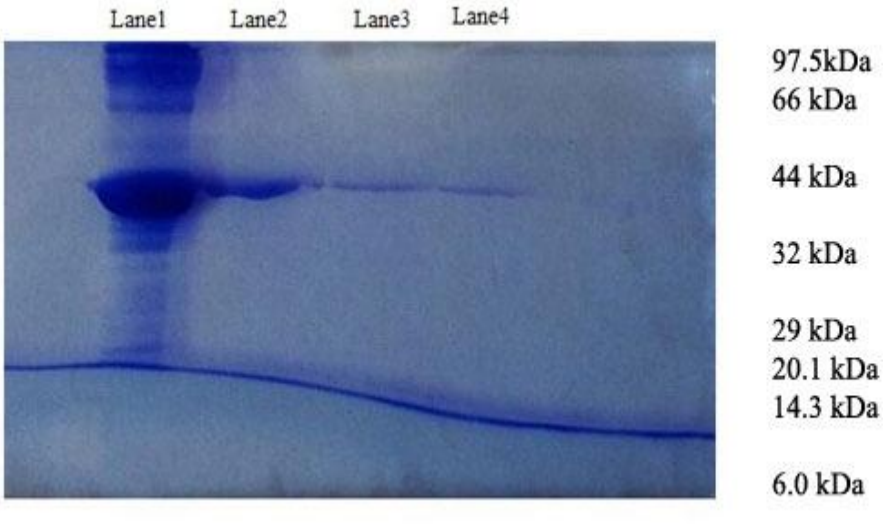
Control **Infected**



Protein profiling by SDS-PAGE of *Helicoverpa armigera*



Lane 1- Marker (BSA)
Lane 2- *Helicoverpa armigera* control
Lane 3- *Helicoverpa armigera* infected with *Xenorhabdus* sp.
Lane 4- *Helicoverpa armigera* infected with *Steinernema* sp.



Lane 1- Marker (BSA)
Lane 2- *Leucinodes orbonalis* control
Lane 3- *Leucinodes orbonalis* infected with *Xenorhabdus* sp.
Lane 4- *Leucinodes orbonalis* infected with *Xenorhabdus* sp.

The protein profiling of *H. armigera* and *L. orbonalis* infected with symbiont *Steinernema- Xenorhabdus* and the bacteria *Xenorhabdus* sp. showed multi-banding patterns with large and small oligomeric proteins in *Helicoverpa armigera* of range 20.0 kDa, 31.80 kDa and 43.0 kDa in *Xenorhabdus* sp. and 31.75 kDa and 45 kDa in *Steinernema* sp. infected lanes respectively, whereas the control showed a low range of proteins with 32.0 kDa to 41.0 kDa. In *Leucinodes orbonalis*, the value was in between 31 kDa to 98 kDa in *Xenorhabdus* sp. infected and it was low oligomeric protein with 31.7 kDa in *Steinernema* sp. infected. The control showed single band of 32 kDa.

So the control in both *H. armigera and L. orbonalis* showed 32 kDa whereas the other low and high protein bands may be due to toxicity of the symbionts in the infected pests. Trypsin-like activity in *H. armigera* larval gut contents is largely associated with a polypeptide fraction of M_r approx. 24,000 as determined by SDS-PAGE.

Morgan *et al*., (2001) described a DNA region of the SDS analysis of the proteins showed that there were a number of proteins in the higher molecular weight range. Similar to proteins found in larvicidal, large polypeptide complexes isolated from the culture supernatant. *X. nematophilus* encoding an insecticidal protein together with other attributes of pathogenicity, including a chitinase gene, organised as a pathogenicity island, suggesting a multicomponent nature of the *Xenorhabdus* toxins. It also contains multiple polypeptides with larvicidal activity, which may be true in this study also as both the pests *H. armigera* and *L. orbonalis* death occurred rapidly due to toxicity of *Xenorhabdus* sp.

Isolated crude protein was estimated (0.03µg/mg) according to Bradford (1976) method and further analyzed using SDS-PAGE with standard molecular weight marker from MBI fermenter (116, 62.2, 45, 35, 25, 18.4 and 14,4kDa). The protein showed the molecular weight of Cry1Ac (~130kDa). The partially purified protease with a dosage of 50mg/ml concentration on *G. mellonella* has showed death of pest in 48 hours. Partially purified toxic protein when sprayed with 1ml/1litre on *A. viarum* termite the kill was observed in 12 hours. In the mosquito larvae of *Culex* when sprayed with toxic protein of *Xenorhabdus* sp. it was killed in 24 hours (Sujatha *et al*., 2016).

So from the present research, it can be concluded that the *Xenorhabdus* sp. showed insecticidal toxicity for both the pests *Helicoverpa armigera* and *Leucinodes orbonalis. Xenorhabdus* sp. has invaded the pest immune system and killed the pest within 11 to 13 hours in the present study. As we are in verge of global warming, the use of chemical pesticide can be minimised as it spoils the soil and changes the pH, it is right time to go for biopesticide as it is

environmentally safe and does not affect non-target organisms. So this *Xenorhabdus* sp. is an effective biopesticide which can be mass produced easily in low cost and so it can be recommended to farmers as efficient biocontrol agent to major pests of economically important crops of our country.

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How to cite this article:

P. Chitra, A. Deepa and K. Sujatha. (2016). Lipase activity and protein profiling of *Helicoverpa armigera* (cotton boll worm) and *Lucinodes orbonalis* (shoot borer) infected by *Steinernema* sp. and its symbiont *Xenorhabdus* sp. isolated from Western Ghats of Munnar. Int. J. Curr. Res. Biol. Med. 1(3): 23-28