International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 4, Issue 2 - 2017

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.02.015

Effect of temperature and pH on protease activity of *Xenorhabdus* (the symbiont of EPN *Steinernema*) against *Helicoverpa armigera*

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Abstract

Entomopathogenic nematodes (EPNs) are used to control several agriculturally important insect pests of different orders. With the aim of determining virulence of different EPN's species, the present study aimed to investigate the ability of EPNs in control of *Helicoverpa armigera* in laboratory and field conditions. Knowledge about enzyme properties is first and main step in physiological based control methods. Enzymes are affected by changes in pH and Temperature. Entomopathogenic nematodes were recovered fromsoil sample in Vellalore. The protease activity was studied in the *H. armigera* infected with *Steinernema* sp. And non infected.Protease enzyme was studied,since it is known to cause toxicity for the pest. The maximum activity was observed at 55°C and there after it declined in untreated control. In biopesticide treated *H. armigera* the enzyme was active till 50°C and then after it declined. The enzyme activity of *Steinernema* sp. Treated *H. armigera* showed less activity compared to the untreated control. The enzyme was active upto high temperature 65°C in control and 55°C in Vellalore *Steinernema* sp. The activity showed 50% low in infected compared to control. The enzyme was thermostable. The activity of enzyme was observed in the presence of different pH ranging from 4.5 to 9.5 using phosphate buffer and both infected and control the activity was maximum at pH 8.

Keywords: Entomopathogenic nematode, *Steinernema*, *Xenorhabdus*, *Helicoverpa armigera*, Protease.

Introduction

Entomopathogenic nematodes (EPNs) are used to control several agriculturally important insect pests of different orders. Several species of EPNs are used worldwide against a variety of pests. Some important EPN species of *Steinernema* and *Heterorhabditis* are obligate pathogens and are characterized by their association with symbiotic bacteria present in the digestive tract; *Xenorhabdus* in Steinernematids and *Photorhabdus* in Heterorhabditids (Boemare *et al*., 1996). With the aim of determining the virulence of different EPN species, the present study was aimed to investigate the ability of EPNs in control of *Helicoverpa armigera* in laboratory and field conditions.

Recent studies and researches provide useful information about digestive system of insects and tended researchers to find new control methods. In addition, with their safety to environment and bio-control agents also are effective in IPM programs (Lawerence and Koundal, 2002). Knowledge about enzyme properties is first and main step in physiological based control methods. Enzymes are affected by changes in pH and temperature. The most favourable pH value and the temperature where the

enzyme is highly active is known as the optimum pH and optimum temperature respectively. Extreme changes in pH and temperature values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes (Eisenthal and Danson, 2002). In any enzyme studies, it is essential to assay its activity at regular intervals under standard conditions to check that if it remains constant. It is a much better procedure however, to try to find conditions under which the stock enzyme solutions may be stored without appreciable loss of activity over the time involved.

Proteases are very important enzymes in insects, as they hydrolyze the peptide bonds in dietary proteins to liberate the amino acids needed for growth, survival, and reproduction, and because they detoxify protein toxins ingested as a consequence of plant and pathogen feeding (Terra *et al*., 1996). The lepidopteran larvae need a proteolytic enzyme complex including trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases, and carboxypeptidases for protein digestion, and many serine proteases are dominant in the larval gut. Since there is significant variation among the biochemical properties of insect digestive proteases, their characterization is necessary for designing a safe control strategy that utilizes plant-proteinaceous inhibitors (Wilhite *et al*., 2000).

Protease are also a type of exotoxin, which is a virulent factor in bacterial pathogenesis. Bacterial exotoxic protease destroy extra cellular structure of the host there by it kills the pest. Extra cellular protease by entomopathogenic bacteria plays a role in insect in insect toxicity. Their physiological importance is highly conflicting protease might have a role in insect toxicity by analogy with protease produced by other pathogens. This protease suppresses antibacterial peptides involved in the insect's immune response, thereby providing a role for it in the pathogenic bacteria.

Materials and Methods

Isolation of entomopathogenic nematodes (EPN's)

Entomopathogenic nematodes were recovered from soil sample of Vellalore (Coimbatore District) 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 container were covered and holes were mate for respiration throughtout 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 throughly 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 25^0C.

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 with 70% ethanol by immersing the larvae in ethanol for 3 times and air dried. 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 28^0C 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.

Enzyme assay

Casein was used at a concentration of 1% in a 10ml assay reaction mixture containing Phosphate buffer, pH 6 and appropriate amount of enzyme. The reaction mixtures were incubated for 45 min at room temperature terminated by adding 0.5ml of trichloroacetic acid. They were incubated for 15 min at 4^0C, followed in turn by centrifugation at 5,000 rpm for 5 min at room temperature and absorbance was recorded at 440 nm.

Effect of pH on enzyme activity

The effect of pH on the enzyme activity was studied over the range of 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 and

9.5. The buffers of different pH were prepared by using phosphate buffer. After incubation the assay procedure was followed. The activity of enzyme was read at 440nm.

Effect of Temperature on enzyme activity

The effect of temperature on enzyme activity was conducted at varying temperatures ranging from 30^0C, 35^0C, 40^0C, 45^0C, 50^0C, 55^0C, 60^0C, 65^0C, 70^0C, and 75ºC under controlled conditions. The substrate was pre incubated at desired temperature before the addition of enzyme. The activity of enzyme was read at 440 nm.

Effect of temperature on protease activity of *Helicoverpa armigera*

The protease enzyme activity against *H. armigera* was studied at different temperatures ranging from 30^0C to 75^0C, in inoculated and uninoculated larvae. In the bio-pesticide, untreated and treated, protease enzyme activity showed at 30^0C – 36.48, 16.28, 35^0C – 43.36, 25.46, 40^0C – 45.17,30.52, 45^0C – 50.42, 37.93, 50^0C – 51.23, 40.26, 55^0C – 54.10, 39.85, 60^0C – 33.59, 26.19, 65^0C – 30.31, 19.30, 70^0C – 21.60, 16.28 and 75^0C – 19.47, 12.41IU/ml activity respectively. The maximum activity was observed at 55^0C and there after it declined in untreated control. In biopesticide treated *H. armigera* the enzyme was active till 50^0C and then after it declined.

Effect of pH on protease activity of *Helicoverpa armigera*

The activity of the enzyme was observed in the presence of different pH ranging from 4.5 to 9.5 using phosphate buffer. In both untreated and treated protease of *Helicoverpa armigera* showed pH 4.5 – 58.61, 27.25, pH 5 – 72.15, 40.53, pH 5.5 – 100.36, 59.17, pH 6 – 123.82, 65.31, pH 6.5 – 139.54, 65.37, pH 7 – 142.69, 71.82, pH 7.5 – 155.21, 75.18, pH 8 – 161.17, 77.29, pH 8.5 – 95.38, 50.52, pH 9 – 62.14,42.79 and pH9.5 – 50.47, 23.16 protease activity respectively. The *Helicoverpa armigera* showed maximum activity of protease at pH 8.0 and thereafter it declined in untreated and treated showed maximum activity at pH 8.0 and thereafter it decreased and the least activity was observed in pH 9.5. The percentage of activity of the protease enzyme, the treated showed less activity compared to the untreated.

Results

(A) EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY OF *HELICOVERPA ARMIGERA* LARVAE

S.No.	Temperature in °C									
	30°C	35°C	40°C	45 °C	50°C	55 °C	60°C	65 °C	70°C	75 °C
1	0.28	0.39	0.33	0.42	0.48	0.46	0.32	0.28	0.22	0.19
2	0.44	0.35	0.48	0.50	0.43	0.58	0.32	0.34	0.20	0.15
3	0.40	0.48	0.46	0.49	0.58	0.56	0.27	0.26	0.25	0.20
Mean	0.37	0.41	0.42	0.47	0.50	0.53	0.30	0.29	0.22	0.18
SD±	0.08	0.07	0.08	0.04	0.08	0.06	0.03	0.04	0.03	0.03
Enzyme activity (IU/ml)	36.48	43.36	45.17	50.42	51.23	54.10	33.59	30.31	21.60	19.47

(B) EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY OF *HELICOVERPA ARMIGERA* INFECTED WITH *STEINERNEMA* SP. OF VELLALORE

S.No.	Temperature in °C									
	30°C	35°C	40°C	45 °C	50°C	55 °C	60°C	65 °C	70°C	75 °C
1	0.10	0.16	0.33	0.37	0.36	0.47	0.21	0.21	0.18	0.12
2	0.13	0.26	0.21	0.31	0.40	0.39	0.30	0.18	0.07	0.10
3	0.18	0.28	0.34	0.37	0.40	0.35	0.25	0.15	0.20	0.07
Mean	0.14	0.23	0.29	0.35	0.39	0.40	0.25	0.18	0.15	0.10
SD±	0.04	0.06	0.07	0.03	0.02	0.06	0.05	0.03	0.07	0.03
Enzyme activity (IU/ml)	16.28	25.46	30.52	37.93	40.26	39.85	26.19	19.30	16.28	12.41

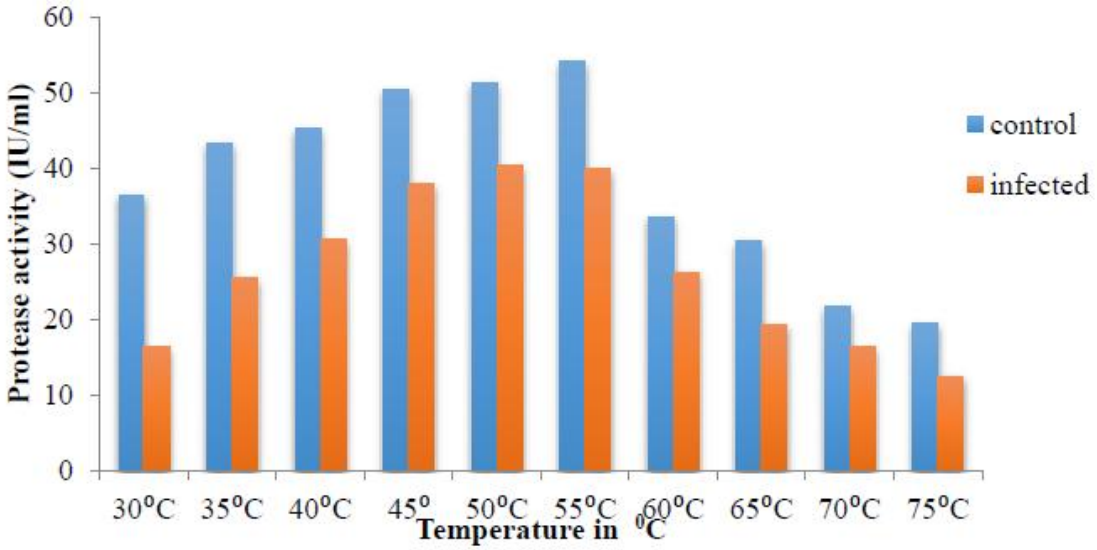
(A) EFFECT OF pH ON PROTEASE ACTIVITY OF *HELICOVERPA ARMIGERA* LARVAE

S.No.	pH										
	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5
1	0.62	0.77	1.05	1.05	1.69	1.53	1.39	1.51	1.02	0.59	0.51
2	0.53	0.65	1.09	1.21	1.29	1.45	1.63	1.67	0.91	0.64	0.47
3	0.60	0.68	0.91	1.33	1.31	1.41	1.58	1.62	0.88	0.56	0.55
Mean	0.58	0.70	1.02	1.20	1.43	1.46	1.53	1.60	0.94	0.60	0.51
SD±	0.05	0.06	0.09	0.14	0.23	0.06	0.13	0.08	0.07	0.04	0.04
Enzyme activity (IU/ml)	58.61	72.15	100.36	123.82	139.54	142.69	155.21	161.17	95.38	62.14	50.47

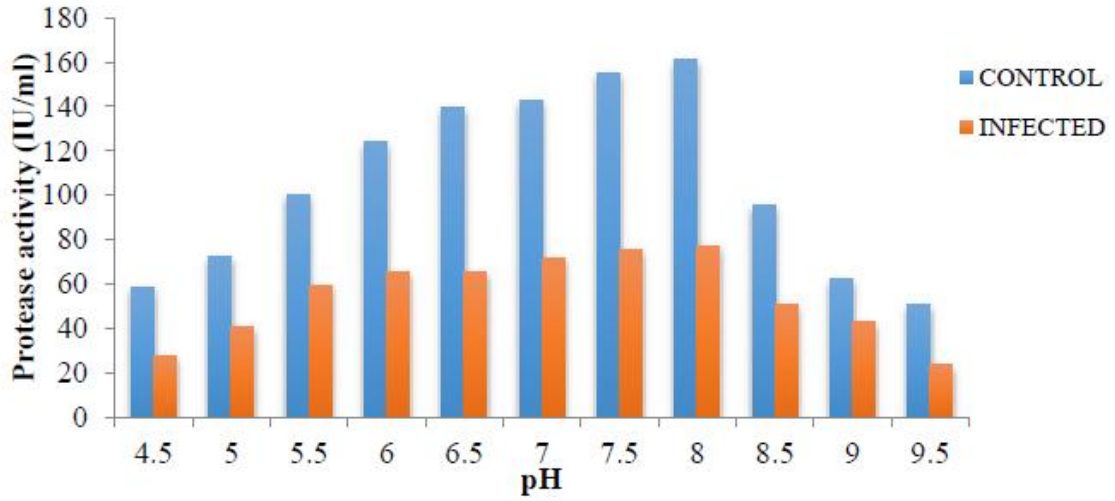
(B) EFFECT OF pH ON PROTEASE ACTIVITY OF *HELICOVERPA ARMIGERA* INFECTED WITH *STEINERNEMA* SP. OF VELLALORE

S.No.	pH										
	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5
1	0.31	0.44	0.54	0.63	0.71	0.74	0.8	0.83	0.48	0.40	0.28
2	0.28	0.31	0.69	0.59	0.68	0.78	0.71	0.68	0.55	0.31	0.18
3	0.20	0.48	0.50	0.69	0.60	0.65	0.68	0.76	0.43	0.48	0.18
Mean	0.26	0.41	0.58	0.64	0.66	0.72	0.73	0.76	0.49	0.40	0.21
SD±	0.06	0.09	0.10	0.05	0.06	0.07	0.06	0.08	0.06	0.09	0.06
Enzyme activity (IU/ml)	27.25	40.53	59.17	65.31	65.37	71.82	75.18	77.29	50.52	42.79	23.16

EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY OF *HELICOVERPA ARMIGERA* LARVAE CONTROL AND INFECTED WITH *STEINERNEMA* SP. OF VELLALORE



EFFECT OF pH ON PROTEASE ACTIVITY OF *HELICOVERPA ARMIGERA* LARVAE CONTROL AND INFECTED WITH *STEINERNEMA* SP. OF VELLALORE



Discussion

The protease activity was studied in the *H.armigera* infected with *Steinernema*sp. and non infected. Bacterial protease is a important extracellular enzyme are mainly involve in providing peptide nutrients for the micro organisms, these protease are main virulence factor produced by the entomopathogenic bacteria play main role in insect toxicity (Magda *et al*., 2007).

After the release of entomopathogenic bacteria into the insect haemocoel by the EPN's the bacteria appear to rapidly overcome the insects defense mechanisms and kill the insect. During growth within the insects, the bacterium is thought to release a variety of compounds including lipases, antibiotics, lipopolysaccharides and protease. Previous studies on the extracellular protease have been equivocal as to their role in insect toxicity. Thus, some authors have directly implicated them as toxic components. While others have suggested that they play a specific role in attacking antibacterial defense system of the insect (Magda *et al*., 2007).

Also as Kumar *et al*.,(2013)found that the protease plays an important role in the pathogenicity of the nematode – bacteria complex. It is a well accepted phenomenon that secreted proteolyitic enzymes of *Xenorhabdus* play a significant role in virulence by suppressing the immune response of the insect host and helping in tissue penetration. Unraveling such system of the pathogen, secreted protease can provide insight regarding their role in a host's defense mechanism. So, this may be true in this study also as the protease activity in the *Steinernema*sp. infected *Helicoverpa armigera* showed 50% low than control protease.

The tripartite relationship of bacteria – nematode and insect has been studied extensively, and the role of *Xenorhabdus* sp. in killing of insects has been documented. *Xenorhabdus* sp. is highly virulent against a wide range of insect larvae and possesses the capacity to produce many toxins and antibiotics, which makes it a very potent insect killer when associated with nematodes. Besides toxins and antibiotics, it also produces many enzymes, including lipase, lecithinase, esterase, and protease, which are also implicated in killing insect larvae as reported by Kumar *et al*., 2013. This may be true in this study also, since the field study and pathogenicity study has revealed that this *Steinernema* – *Xenorhabdus*symbiont was highly virulent against the three lepidopterans and the GAC samples has worked well at field level against *H.armigera, S.litura and L.orbonalis.*

Reasonable protease activity at temperature 45^0C to 60^0C with maximum activity at 55^0C, was observed, rapid decrease was observed at 65^0C onwards, no complete inactivation was noted upto 75^0C. So the enzyme isolated in this study was active at high temperature and the protease is thermostable, so even in adverse temperature or due to global warming the temperature increase can be coped up by the nematodes and its bacterial symbiont to inactivate the pest defense mechanism.

The affect of different pH of the reaction mixture and activity of the crude protease was investigated in pH range from 4.5 to 9.5 at room temperature. It was found that the crude protease had relatively wide pH range of activity between 6 to 8 with maximum enzyme activity at pH 8, in both control and infected *H.armigera*. But the activity at each pH compared to control, infected protease activity was less than 50%. So the protease is alkaliphilic (Abdelnasser *et al*., 2007).

According to Kumar *et al*., (2013) the optimum pH of the purified protease were found to be 8.2. This was on par with our study.

X. nematophila PA II to digest some protein of the insect haemolymph hydrolysis of these proteins may provide nutritional factors to the associated nematode necessary for its complete development and reproduction inside the infected insect cadaver, other possible function of metalloprotease for *X. nematophila* PA II including the inactivation non-specific insects defense mechanisms.

X. nematophilia PA II is an alkaline metalloprotease. Its substrate specificity strengthness the possibility that it is involved in degradation of insect tissues for providing nutrients to the associated nematode, which is unable to grow on insects without a previous bioconversion of the insect cadavers by the symbiotic bacteria. The protease isolated in the present study is alkaline protease since its activity is high at 6 to 8. This was on par with the study of Magda *et al*, 2007. Since they have isolated alkaline protease from *X.nematophila* alkaline. So the protease isolated in this study is alkaliphilic since it has maximum activity at pH 8 in both infected and control. This study was on par pH with study of Abdelnasser *et al*., (2007).

Conclusion

Protease enzyme was studied, since it is known to cause toxicity for the pest. The activity was studied in different temperature and pH. The enzyme was active upto high temperature 65^0C in control and 55^0C in Vellalore *Steinernema* sp. The activity showed 50% low in infected compared to control. The enzyme was thermostable.

The pH was studied from 4.5 to 9.5 showed that both infected and control the activity was maximum at pH 8. So the pH is alkaline and so the protease is alkaliphilic.

So from this study it is clear that the Vellalore isolate of *Steinernema –Xenorhabdus* symbiont has performed well in lab and field studies and caused statistically significant control over the lepidopteran pests especially the devastating *H.armigera* which is polyphagous. These EPNs are environmentally safe as it does not affect the non target organism and is highly potential biopesticide which can be sprayed individually as biocontrol agent or can be incorporated along with other IPM technology for economically important crops in sustainable agriculture.

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DOI:10.22192/ijarbs.2017.04.02.015	

How to cite this article:
M Tamilselvi and K Sujatha. (2017). Effect of temperature and pH on protease activity of *Xenorhabdus* (the symbiont of EPN *Steinernema*) against *Helicoverpa armigera*. Int. J. Adv. Res. Biol. Sci. 4(2): 137-144.
DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.02.015