

## Extracellular Novel Metalloprotease from *Xenorhabdus indica* and Its Potential as an Insecticidal Agent<sup>S</sup>

Kumar Pranaw<sup>1</sup>, Surender Singh<sup>1</sup>, Debjani Dutta<sup>2</sup>, Nirpendra Singh<sup>3</sup>, Garima Sharma<sup>4</sup>, Sudershan Ganguly<sup>5</sup>, Vinay Kalia<sup>4</sup>, and Lata Nain<sup>1\*</sup>

<sup>1</sup>Division of Microbiology, Indian Agricultural Research Institute, New Delhi-110012, India

<sup>2</sup>Department of Biotechnology, National Institute of Technology, Durgapur-713209, India

<sup>3</sup>Central Instrumentation Facility, Biotech Center, University of Delhi South Campus, New Delhi-110021, India

<sup>4</sup>Division of Entomology, Indian Agricultural Research Institute, New Delhi-110012, India

<sup>5</sup>Division of Nematology, Indian Agricultural Research Institute, New Delhi-110012, India

Received: June 26, 2013  
Revised: August 1, 2013  
Accepted: August 2, 2013

First published online  
August 9, 2013

\*Corresponding author  
Phone: +91-011-25847649;  
Fax: +91-011-25846420;  
E-mail: latarajat@yahoo.co.in

**S**upplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2013 by  
The Korean Society for Microbiology  
and Biotechnology

Proteases produced by *Xenorhabdus* are known to play a significant role in virulence leading to insect mortality. The present study was undertaken to purify and characterize protease from *Xenorhabdus indica*, an endosymbiont of nematode *Steinernema thermophilum*, and to decipher its role in insect mortality and its efficacy to control *Helicoverpa armigera*. A set of 10 strains of *Xenorhabdus* isolated from different regions of India were screened for protease activity on the basis of zone of clearing on gelatin agar plates. One potent strain of *Xenorhabdus indica* was selected for the production of protease, and the highest production (1,552 U/ml) was observed at 15–18 h of incubation at 28°C in soya casein digest broth. The extracellular protease was purified from culture supernatant using ammonium sulfate precipitation and ion-exchange chromatography. The enzyme was further characterized by SDS-PAGE and zymography, which confirmed the purity of the protein and its molecular mass was found to be ~52 kDa. Further MALDI-TOF/TOF analysis and effect of metal chelating agent 1,10-phenanthroline study revealed the nature of the purified protease as a secreted alkaline metalloprotease. The bioefficacy of the purified protease was also tested against cotton bollworm (*Helicoverpa armigera*) and resulted in 67.9 ± 0.64% mortality within one week. This purified protease has the potential to be developed as a natural insecticidal agent against a broad range of agriculturally important insects.

**Keywords:** Entomopathogenic nematode, alkaline metalloprotease, *Xenorhabdus indica*, MALDI-TOF, *Helicoverpa armigera*

### Introduction

*Xenorhabdus*, an endosymbiont of *Steinernema*, has a unique life cycle causing pathogenicity in the insect host [1, 3]. The infective juveniles (IJ's) of Steinernematidae release the bacterium into the nutrient-rich hemolymph within 5 h of invasion [4, 31]. The bacteria proliferate and produce a wide range of toxins, antibiotics, bacteriocins, and hydrolytic exoenzymes, resulting in a bacterial septicemia and death of the host within 24–48 h [12, 18]. The bacteria also provide a suitable nutrient-rich environment for nematode growth

and reproduction. The immature nematode develops in the IJ's that again carry away the bacteria, in search of a new insect host to continue the cycle. A very interesting significant feature of bacterium *Xenorhabdus* is that there are two phenotypic variants, primary (Phase-I) and secondary form (Phase-II). They differ in their antibiotic production profile, outer membrane proteins (fimbriae and flagellae), symbiotic capabilities with nematode partner, and exoenzyme production potential. The complete genomic analysis of *Xenorhabdus* has shown that it has many genes that encode different toxins, proteases, and lipases [9]. The protease enzyme plays

an important role in the pathogenicity of the nematode–bacteria complex. It is a well accepted phenomenon that secreted proteolytic enzymes of *Xenorhabdus* play a significant role in virulence by suppressing the immune response of the insect host and helping in tissue penetration [23]. Unraveling such systems of the pathogen, secreted proteases can provide insight regarding their role in a host's defense mechanism. Surprisingly, despite the importance of proteases in insect pathogenesis, only a few studies have been undertaken to explore the nature of protease and its production under axenic cultivation based upon their substrate specificity [11].

In this paper, we have for the first time reported the isolation and characterization of an alkaline metalloprotease from different isolates of *Xenorhabdus* species. *X. indica* has been found to produce the maximum of secreted alkaline protease. The MALDI-TOF/TOF analysis of the homogeneously purified protease confirmed its identity as secreted alkaline metalloprotease from *Xenorhabdus*. The bioefficacy of the purified protease was evaluated against *H. armigera* (cotton bollworm).

## Materials and Methods

### Bacterial Strain and Growth Condition

The strains of *Xenorhabdus* sp. were isolated from IJ's of different *Steinernema* sp. The primary form was differentiated on the basis of characteristic blue colony on NBTA medium (Peptone 5; Beef extract 3; NaCl 4; Bromothymol blue 0.025; Triphenyl-2,3,4-tetrazolium chloride 0.04 in g/l), unlike the secondary form, which had a chocolate brown color.

### Screening of *Xenorhabdus* Isolates for Protease Enzyme

**Qualitative assay.** Qualitative assay of protease enzyme was carried out on gelatin agar plates (Peptone 5; Beef extract 3; NaCl 4; Gelatin 12 in g/l, pH 7.2.) by spot inoculating 2 µl of cell suspension and incubation at 28°C [21]. After 48 h, the plates were flooded with 5 ml of HgCl<sub>2</sub> solution and the zone of hydrolysis observed. On the basis of the qualitative assay, the most promising *Xenorhabdus* strain was selected for further studies.

### Selection of Medium for Protease Enzyme

For optimum production of protease enzyme, five different media were evaluated: skim milk (1%) in nutrient broth (NB), skim milk (1%) in NB (Half strength), gelatin (1%) in NB, gelatin (1%) in NB (half strength), soya casein digest medium. The media were inoculated with 2% (v/v) cell suspension and incubated at 28°C, 150 rpm for 24 h. Samples were withdrawn at intervals of 3 h upto 24 h for the enzyme assay.

### Enzyme Assay

The culture suspension was centrifuged at 10,000 rpm for 5 min

and culture supernatant was used as the enzyme source. Protease activity was assayed by incubating 250 µl of azocasein (Megazyme, 2% (w/v)) with 150 µl of enzyme solution in a water bath at 30°C for 30 min [28]. After incubation 1.2 ml of 10% trichloroacetic acid was added to stop the reaction and the mixture was allowed to stand for 15 min. Enzyme blanks were prepared by mixing buffer, azocasein, trichloroacetic acid, and enzyme. The content was centrifuged at 10,000 rpm for 5 min to remove any undigested azocasein. The optical density of reaction supernatant was determined by adding 1.4 ml of NaOH (1 N) in supernatant (1.2 ml). One unit of enzyme is defined as the amount of enzyme required to produce an absorbance change of 0.01 in a 1 cm cuvette under the standard assay conditions.

### Purification of Protease Enzyme

Extracellular protease enzyme was extracted by centrifugation of cell suspension grown in soya casein digest medium at 10,000 rpm after 18 h of incubation. Cell-free supernatant was saturated with ammonium sulfate (80%), and precipitate was collected after centrifugation at 10,000 rpm, dissolved in 0.1 M TrisHCl buffer (pH 7.6.), and dialyzed overnight at 4°C against the same buffer. The dialysate was ultrafiltered using a 30 kDa Amicon ultra filtration unit (Millipore) followed by anion-exchange chromatography using a Macro-Prep High Q (Biorad) pre-packed column equilibrated with the same buffer. The protein was eluted with a NaCl gradient (0.5–1.5 M) in the same buffer at a flow rate of 1 ml/min. Fractions having protease activity were pooled and concentrated using a 3 kDa Amicon ultra filtration unit. The purified fraction was stored at –20°C, and its purity verified using SDS-PAGE and zymography [19, 30].

### Characterization of Protease Enzyme

**Determination of optimum pH and temperature.** The optimal pH of partially purified protease enzyme was determined in 0.1 M sodium phosphate buffer (pH 6.2–7.4), 0.1 M TrisHCl buffer (pH 7.8–8.6), and 0.1 M Glycine NaOH buffer (pH 9.0–9.8). The optimal temperature was investigated by exposure of enzyme to temperatures in the range of 26–42°C. After exposing the enzyme to different pH and temperature values for 30 min, the protease activity was estimated as per the standard protocol described earlier.

**Effect of metal ions and inhibitors.** The effect of metal ions were determined in the presence of different metals such as Mn<sup>2+</sup>, Ca<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>3+</sup>, and Mg<sup>2+</sup> at a concentration range 5–25 mM. The effect of inhibitors was studied using EDTA, PMSF, and 1,10-phenanthroline (10–25 mM) on the protease activity.

**Enzyme kinetics.** Kinetic constants such as V<sub>max</sub> and K<sub>m</sub> were identified under steady-state conditions using various concentrations of azocasein (5–30 mg/ml) as substrate [29].

### Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF/TOF)

Purified protein sample was digested with sequencing grade trypsin according to the manufacturer protocol [20]. In brief,

**Table 1.** Qualitative assay of protease in different *Xenorhabdus* isolates on the basis of zone of clearing on gelatin agar plates.

Strain name	Nematode host	NCBI Accession No	Zone of hydrolysis (mm)	Place
<i>X. indica</i>	<i>Steinernema thermophilum</i>	GQ373384	20	Delhi
<i>X. indica</i> strain KB-3	<i>S. thermophilum</i>	GQ923884	26	Kerala
<i>X. meghalayensis</i>	<i>S. meghalayensis</i>	JN177510	15	Meghalaya
<i>Xenorhabdus</i> sp. SGgj	<i>Steinernema</i> sp.	HM749976	17	Gujarat
<i>Xenorhabdus</i> sp. SGor	<i>Steinernema</i> sp.	HM749977	19	Bhubaneswar
<i>Xenorhabdus</i> sp. SGchamp	<i>Steinernema</i> sp.	GU731206	19	Champawat
<i>Xenorhabdus</i> sp. SGkr	<i>Steinernema</i> sp.	GU980747	18	Kerala
<i>Xenorhabdus</i> sp. SGjk	<i>Steinernema</i> sp.	GU731205	18	J&K
<i>Xenorhabdus</i> sp. XB	<i>Steinernema</i> sp.	JN547413	17	Haryana
<i>X. stockiae</i> strain XOK	<i>S. siamkayai</i>	JX221724	19	Tamilnadu

purified protein was denatured with 8 M urea and disulfide bridges were cleaved using 10 mM DTT at 55°C for 30 min. Cysteine was alkylated using 25 mM iodoacetamide (IAA) at room temperature for 30 min in the dark. The denatured and alkylated protein was digested with MS-grade trypsin in the ratio of 50:1 for 3 h. Digested peptides were extracted using 5% ACN and 0.1% TFA in water. Peptides were desalted and concentrated by speed vac before analysis. Digested peptides (1 µg) were injected onto the chromolith Caprod RP-18e (150-0.1 mm) column [20, 34]. The extracted peptides were spotted with  $\alpha$ -cyano-4-hydroxycinnamic acid matrix in the ratio of 1:1 and spot analyzed on an Absciex 4800 Plus TOF/TOF analyzer in reflector positive-ion mode for PMF spectra MS/MS. Data were converted from the 4000 series explorer file in the form of a peak list using the Mascot peak converter. The data were searched using the online Mascot server in the NCBI database for identification. Samples were similarly processed in ESI LC-MS for protein ID and its peaks were also searched using the same data server in the NCBI database for protein ID.

#### Bioassay of Purified Protease

Larvae of *H. armigera* were reared on a chickpea-based semi-synthetic diet in the laboratory as described by Nagarkatti and Prakash [24], modified by Kalia *et al.* [17], at 27 ± 2°C temperature and 60 ± 5% RH. The adult moths were given 10% honey solution fortified with multivitamins throughout their egg-laying period. Five pairs of adults were kept in each jar covered with rough cotton cloth for egg laying. Neonates were used in bioassays to evaluate the toxicity of protease.

The efficacy of the purified protease was assessed on the neonates of *H. armigera* by the diet incorporation method as per Gujar *et al.* [13]. The diet was incorporated with 0.1 ml of solution containing 1, 5, 10, 25, and 100 µg of the purified protease/gm of diet, in three replicates, while equal quantity of 0.1 M TrisHCl buffer (pH 8.2) was used in the control. Total cell protein extract was prepared as per Dulmage *et al.* [10] and was evaluated *per os* at 100 µg/g concentration (on the basis of total protein content) by

the diet incorporation method against neonates of *H. armigera*. Ten neonates were released per replicate on the purified protease incorporated diet in a plastic container of 2.5 cm diameter. Mortality was observed up to day 7 at 24 h intervals. The median lethal concentration (LC<sub>50</sub>) value was calculated using the maximum likelihood programme MLP 3.01 [27].

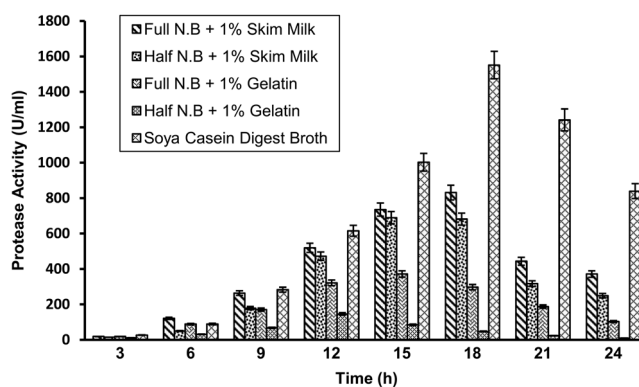
## Results

### Screening of *Xenorhabdus* Isolates for Protease Enzyme

Ten isolates of *Xenorhabdus* were screened for extracellular protease activity on gelatin agar plates. In the qualitative plate assay, all *Xenorhabdus* strains had shown zone of hydrolysis ranges from 16 to 26 mm (Table 1). The maximum zone of clearance was observed in *X. indica* strain KB-3 (Supplementary information 1).

### Production of Protease Enzyme

*X. indica* strain KB-3 was selected for further analysis on the basis of qualitative plate assay. The culture was grown

**Fig. 1.** Proteolytic activity during the growth of *X. indica* strain KB-3 in different growth media.

**Table 2.** Purification of protease from *Xenorhabdus indica* strain KB-3.

	Volume (ml)	Total protein (mg)	Total activity	Specific activity (U/mg of protein)	Recovery	Fold purification
Crude	100	14,385	116,400	8.09	100	0
Ammonium sulfate	20	1,966.24	69,680	35.43	59.86	4.38
Macro-Pep High Q	5	408.725	26,040	63.71	22.37	7.87

One unit of enzyme is defined as the amount of enzyme required to produce an absorbance change of 0.01 in a 1 cm cuvette under the conditions of the assay.

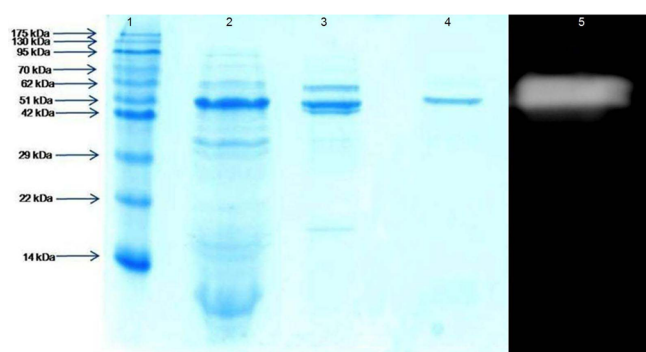
aerobically in all five different sets of media at 28°C, 150 rpm. Among the different growth media, proteolytic activity was maximum (1,552 U/ml) at 18 h in soya protein rich medium (soya casein digest broth). Similarly, milk protein (skim milk supplemented nutrient broth) was able to induce only 832 U/ml of protease activity within 18 h (Fig. 1).

### Purification of Protease Enzyme

The protease enzyme was purified by ammonium sulfate precipitation at 80% saturation. After dialysis, the dialysate was subjected to anion-exchange chromatography using a Macro-Pep High Q column (BioRad). A single peak with protease activity was obtained with a gradient (0.5–1.5 M) of NaCl (Supplementary information 2). The total recovery of protease after purification was 22.3% with ~8-fold purification by this step (Table 2). The purified fraction was used for zymography and SDS-PAGE. A single band of ~52 kDa was detected in the zymograph possessing protease activity (Fig. 2).

### Characterization of Protease Enzyme

The optimal pH of the purified enzyme was 8.2 as determined by hydrolysis of azocasein (Fig. 3A). The optimal temperature for the protease activity was observed to be 34°C at pH 8.2.



**Fig. 2.** SDS-PAGE and zymogram profile of protease enzyme. Lane 1: Marker; lane 2: crude enzyme; lane 3: ammonium sulfate precipitates after ultrafiltration (30kDa); lane 4: purified enzyme; lane 5: zymogram of purified protease.

(Fig. 3B). At 50°C, purified protease lost 90% of its proteolytic activity. The purified enzyme was found to be stable for several days at –20°C. Kinetic analysis of the hydrolysis of azocasein was carried out and  $V_{max}$  and  $K_m$  were found to be 109.89 U/ml/min and 2.626 mM, respectively (Supplementary information 3). The reaction rate was constant beyond a substrate concentration of 20 mg/ml of azocasein.

### Effect of metal ions and inhibitors on protease activity.

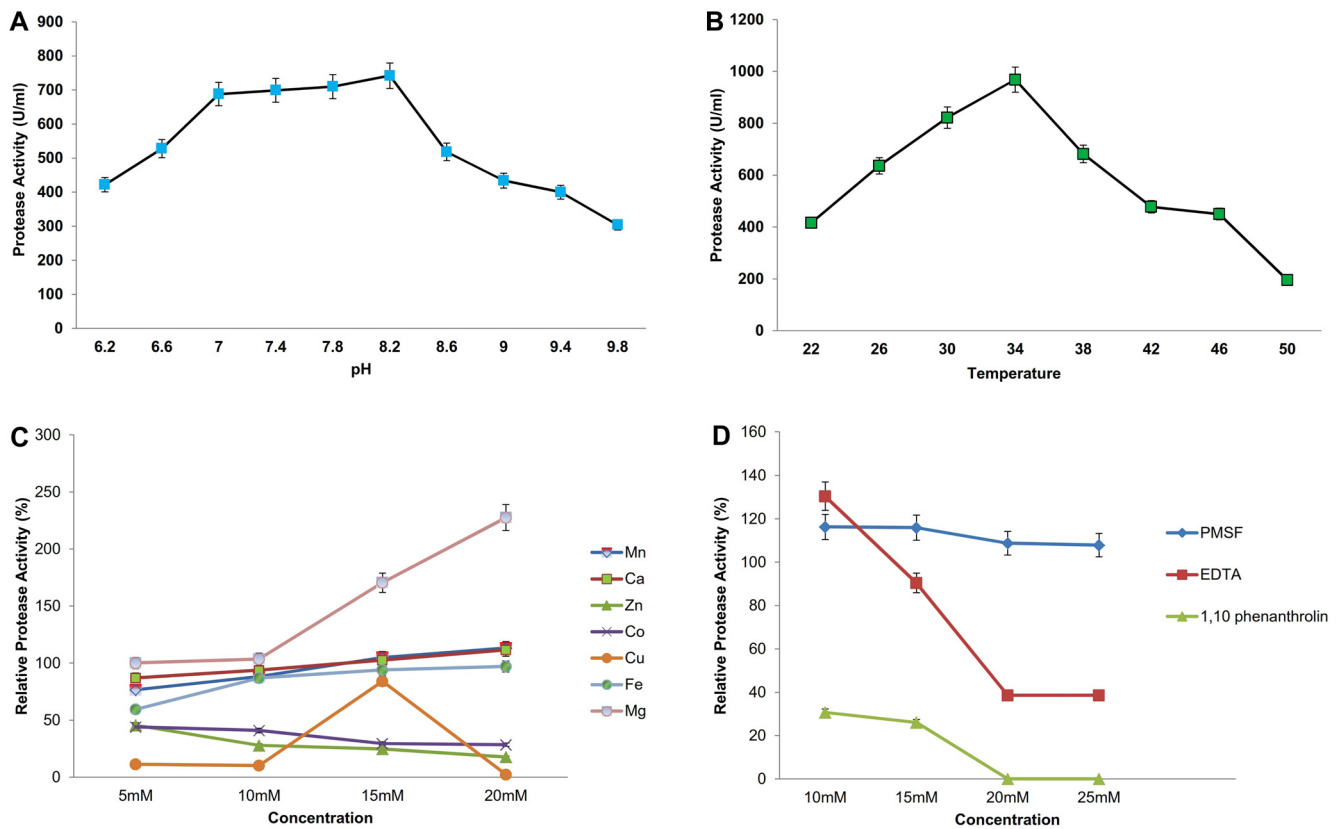
It was found that among metal ions,  $Mg^{+2}$  significantly increased the activity, whereas higher concentrations 25 mM of  $Zn^{+2}$ ,  $Co^{+2}$ ,  $Cu^{+2}$ , and  $Fe^{+2}$  resulted in reduction of protease activity (Fig. 3C). EDTA and 1,10-phenanthroline were found to inhibit the protease activity. The complete inhibition of protease was observed at 20 mM concentration by 1,10-phenanthroline. The same concentration of PMSF did not inhibit the protease activity.

### Mass Spectrometry Analysis

The MALDI-TOF/TOF analysis of digested protein revealed its similarity with secreted alkaline metalloproteinase from *X. nematophila* (Accession No. gi/300724813) in the NCBI database. This protein is not documented in the Swiss Prot database. The reported molecular mass of this protein is 52.5 kDa, and it matched well with our observation from SDS-PAGE data (Fig. 2). We could not detect any other protein in the MALDI-TOF/TOF analysis with significant score (Fig. 4). This further authenticated our anion-exchange purification analysis (Supplementary information 2) about the purity of protein, which was subsequently used for downstream experiments. The protein sequence alignment using ClustalW revealed alkaline metalloprotease from *X. indica* had 99%, 79%, 65%, 65%, and 51% identical amino acid residues with *X. nematophila* (YP\_003714138), *X. bovienii* (YP\_003466436), *Photorhabdus temperata* (AAX99100), *P. luminescens* (AA039316), and *Pseudomonas aeruginosa* (WP\_003150973) respectively (Fig. 5).

### Bioefficacy of Purified Protease

The  $LC_{50}$  of protease was 16.56  $\mu$ g/gm of diet against neonates of *H. armigera* after 7 days. A positive correlation



**Fig. 3.** Proteolytic activity of protease at different (A) pH, (B) temperatures, (C) metal ions, and (D) inhibitors.

( $r = 0.920$ ) was observed between the concentration of protease and insect mortality. The perusal of results showed that purified protease gave higher mortality ( $67.9 \pm 0.64\%$ ) as compared with total protein of *X. indica* ( $33.33 \pm 1.04\%$ ) at  $100 \mu\text{g/gm}$  of diet concentration.

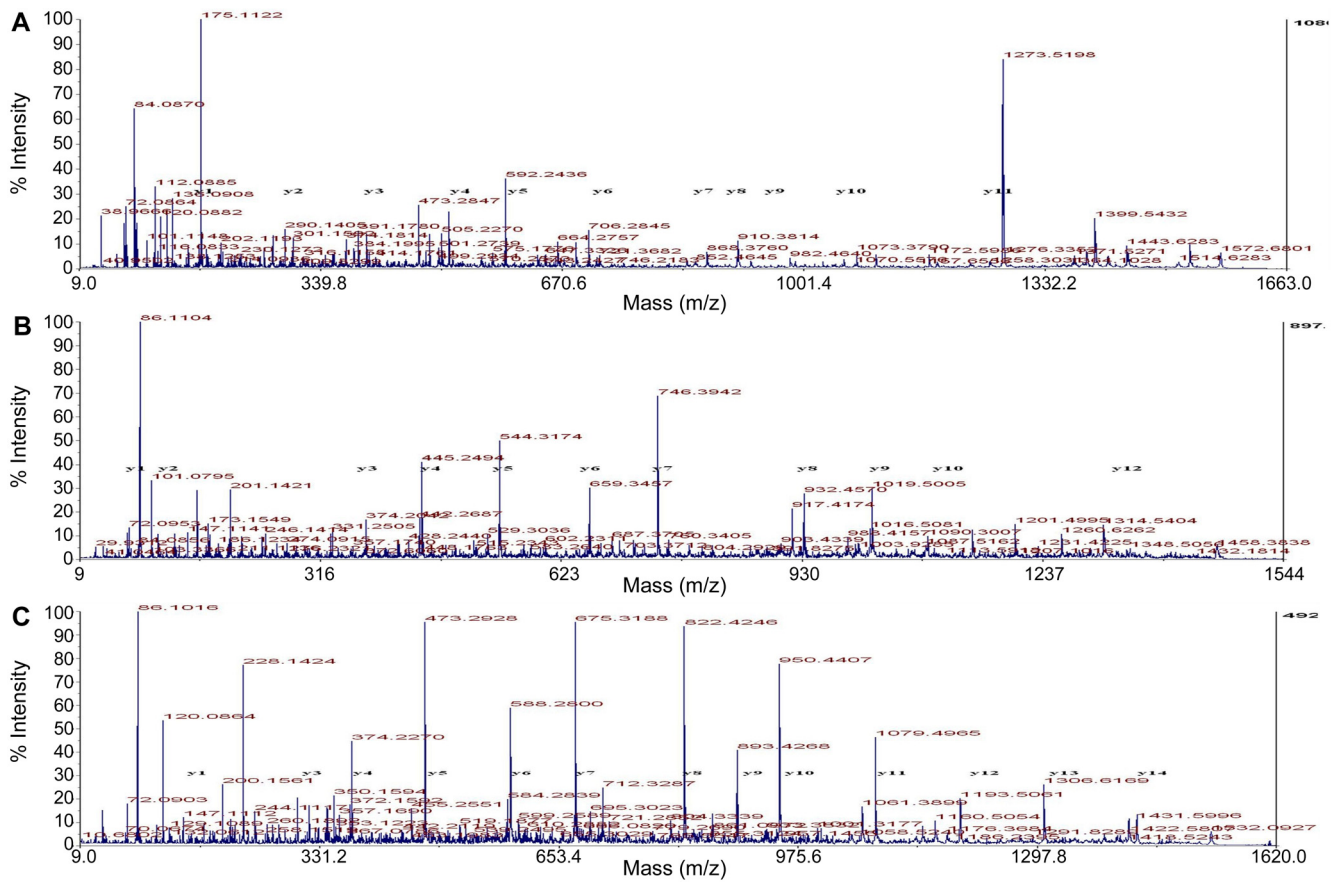
### Discussion

*Xenorhabdus* sp., a member of family Enterobacteriaceae, is mutually associated with the nematode *Steinernema*. The IJ's nematode serves as a vector to carry bacteria into insect larvae. The nematode releases bacterial symbiont *Xenorhabdus* sp. inside the insect and rapidly kills the insect larvae. 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 [5]. *Xenorhabdus* sp. is highly virulent against a wide range of insect larvae and possesses the capacity to produce many toxins and antibiotics [6, 15], 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 [26]. However, data on the bio-efficacy of purified enzymes on insect are lacking and hence this study was undertaken to assess and establish the insecticidal capacity of purified protease from *X. indica*.

The initial screening of *Xenorhabdus* sp. isolated from different locations of India was performed on the basis of gelatin liquefaction and zone of hydrolysis due to protease production, a method previously used by Marokházi *et al.* [21]. The zone of hydrolysis varied from strain to strain and provided a very clear idea about the most promising strain for protease production. *X. indica* was explored for protease production with different substrates; namely, gelatin, skim milk, and casein hydrolysate medium (soya casein digest medium). In our study, the highest protease activity was observed in casein hydrolysate medium.

The enzyme production in *Xenorhabdus* differs qualitatively and quantitatively and depends on the strains, species of bacteria, and their culture conditions. Many environmental factors, including temperature and aeration, affect enzyme production in *Xenorhabdus* sp. [2, 7]. In the earlier studies of enzyme secretion by *Xenorhabdus* strains, protease activity was assayed by nonspecific and relatively insensitive



**Fig. 4.** MALDI TOF-TOF MS/MS spectra of peptides (A) y ion assignment of fragment on the peptide of molecular mass 1,573.74 Da, (B) y ion assignment of fragment of peptide of molecular mass 1,460.78 Da, and (C) y ion assignment of fragment of peptide of molecular mass of 1,533.8 Da.

methods, which were based on the release of amino acids from substrate proteins like gelatin. Now, with the availability of nonselective chromogenic protein substrates like azocoll, azocasein, hide powder azure, etc., the sensitivity of protease assays has increased several folds. Gelatin zymography, which can detect even small amounts of protease, was employed for detection of protease in SDS-PAGE. The crude protease was purified by different techniques like ammonium sulfate precipitation, Amicon ultra filtration, and ion-exchange chromatography techniques. The single band on SDS-PAGE and zymography revealed that the preparation contains a single protease of approximately 52 kDa. The nature of the protease was further confirmed using MALDI-TOF/TOF data, which also strengthened our claim for the purified protease. The molecular mass of this kind of protease ranges from 51–60 kDa in different species and strains of *Xenorhabdus* sp. [5, 23].

**1** MTSKKKYSNV GLSDSHSAQD VNALLTAYVP NSDPNVRVAH EVLADEAPDE  
**51** LVRGHYKWAN KYVNSSGTL E LSYHFLKAS DPLMRIFKVS GFSAFNEEQK  
**101** DAAK**LSLQSW** SDVANIKFTE VSSIYKANIT FGPFDKSVNK DYAFANLPQG  
**151** QKMVYTWYNA KSHTFVDNDI DVNGYIRQTF THEIGHTLGL EHPADYDASD  
**201** EIRPNYINSA EYFEDCRAYT **VMSYFSEKFT** QQDFKYGYSS APLLNDISAI  
**251** QELYGANMET **RKGDVTYGFN** SNTDRDFMTA TDANSKLIFS VWDAGGEDTF  
**301** **DFSGFTQNGR** INLNAGSFS D VGGLKGNVSI ARGVVIENAI GSGDDILVQ  
**351** **NSADNILKGG** VGDDIYGG L GGDHLWGGEG NDFVYLSGK ESLKNNPDWI  
**401** HDFVSGEDKI DLSDFNFGGD GDKIFVDSFS GKAGEVLFTY DEENDVTDLE  
**451** ISLGGDLAGN DFLVKVIGQP LTEADFI V

**Fig. 5.** Identified peptide sequence (bold red) mapped on alkaline metalloproteases of *Xenorhabdus nematophila* (Accession No. YP\_003714138 in NCBI database).

The optimal pH and temperature of the purified protease were found to be 8.2 and 34°C, respectively, unlike other proteases from *Xenorhabdus* sp. [22, 30]. In our study, the metal ions Mn<sup>+2</sup>, Ca<sup>+2</sup> increased the protease activity slightly

whereas the activity was considerably increased by more than 2-fold with  $Mg^{+2}$ . Metal ions act as a salt or ion bridge to maintain the structure conformation of the enzyme or to stabilize the binding of the substrate and enzyme complex in metallic proteases [32]. Protease showed maximum inhibition by 1,10-phenanthroline at 20 mM, which is in agreement with a previous report by Schmidt *et al.* [30]. However, at the same concentration, PMSF did not show significant inhibition, which clearly indicated that protease enzyme produced by *Xenorhabdus* is a metalloprotease. The calculated  $V_{max}$  (109.89 U/ml/min) and  $K_m$  (2.63 mM) for the homogeneously purified protease was comparable to the earlier report of this kind of alkaline metalloprotease [25].

The role of different extracellular metabolites produced by *Xenorhabdus* sp. like toxins and antibiotics in insect nematode interaction has been well documented [8, 33]. Many proteases have also been purified and characterized from these endosymbiotic bacteria, but to the best of our knowledge no report is available on the bioefficacy of these purified proteases against the *H. armigera*. The bioefficacy of purified protease (100 ppm) was tested using neonates of *H. armigera*, which showed 70% mortality after 7 days. The blackened dead larvae probably resulted as a result of an activation of enzyme cascade leading to melanization in *H. armigera* [35]. Proteases are known to damage the peritrophic matrix, which leads to growth retardation of insects and even mortality due to increased permeability of the midgut. In several species of Lepidoptera and Diptera, proteases inhibit inducible antibacterial peptides Cercopin A that specifically inhibit gram-negative bacteria [14]. The inhibition of antibacterial peptides leads to immunosuppression in insects that favor the bacteria to establish itself in the hemolymph, causing septicemia and leading to death of the insect [16]. This is the first report of the identification and characterization of a secreted alkaline metalloprotease from the native species of *X. indica* and its application as a natural biocide against *H. armigera*. MALDI-TOF/TOF analysis confirmed the findings of the biochemical and bioefficacy data about the nature of the protein and revealed that the purified protein is a secreted alkaline metalloprotease of *Xenorhabdus*.

## Acknowledgments

The authors acknowledge World Bank funded National Agriculture Innovation Projects (NAIP-ICAR) Component 4 (IARI-70:13) for sponsoring this research project. The authors also acknowledge the central instrumentation facility

of Delhi University-South Campus for providing the proteomic facility.

## References

- Adams BJ, Fodor A, Koppenhöfer HS, Stackebrandt E, Patricia Stock S, Klein MG. 2006. Biodiversity and systematics of nematode-bacterium entomopathogens. *Biol. Control* **37**: 32-49.
- Akhurst RJ. 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *J. Gen. Microbiol.* **128**: 3061-3065.
- Bode HB. 2009. Entomopathogenic bacteria as a source of secondary metabolites. *Curr. Opin. Chem. Biol.* **13**: 224-230.
- Caldas C, Cherqui A, Pereira A, Simões N. 2002. Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. *Appl. Environ. Microbiol.* **68**: 1297-1304.
- Chaston JM, Suen G, Tucker SL, Andersen AW, Bhasin A, Bode E, *et al.* 2011. The entomopathogenic bacterial endosymbionts *Xenorhabdus* and *Photorhabdus*: convergent lifestyles from divergent genomes. *PLoS ONE* **6**: e27909.
- Chattopadhyay A, Bhatnagar NB, Bhatnagar R. 2004. Bacterial insecticidal toxins. *Crit. Rev. Microbiol.* **30**: 33-54.
- Chen G, Zhang Y, Li J, Dunphy GB, Punja ZK, Webster JM. 1996. Chitinase activity of *Xenorhabdus* and *Photorhabdus* species, bacterial associates of entomopathogenic nematodes. *J. Invertebr. Pathol.* **68**: 101-108.
- Dowling A, Waterfield NR. 2007. Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon* **49**: 436-451.
- Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taourit S, *et al.* 2003. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nature Biotechnol.* **21**: 1307-1313.
- Dulmage HT, Correa JA, Martinez AJ. 1970. Coprecipitation with lactose as a means of recovering the spore-crystal complex of *Bacillus thuringiensis*. *J. Invertebr. Pathol.* **15**: 15-20.
- Felföldi G, Marokházi J, Képiró M, Venekei I. 2009. Identification of natural target proteins indicates functions of a serralyisin-type metalloprotease, PrtA, in anti-immune mechanisms. *Appl. Environ. Microbiol.* **75**: 3120-3126.
- Furgani G, Böszörményi E, Fodor A, Máthé-Fodor A, Forst S, Hogan JS, *et al.* 2008. *Xenorhabdus* antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. *J. Appl. Microbiol.* **104**: 745-758.
- Gujar G, Kumari A, Kalia V, Chandrashekar K. 2000. Spatial and temporal variation in susceptibility of the American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* var. *kurstaki* in India. *Curr. Sci.* **78**: 995-1001.
- Han R, Ehlers RU. 2000. Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema*

- carpocapsae* under axenic *in vivo* conditions. *J. Invertebr. Pathol.* **75**: 55-58.
15. Herbert EE, Goodrich-Blair H. 2007. Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nature Rev. Microbiol.* **5**: 634-646.
  16. James RR, Xu J. 2012. Mechanisms by which pesticides affect insect immunity. *J. Invertebr. Pathol.* **109**: 175-182.
  17. Kalia V, Chaudhari S, Gujar G. 2001. Changes in haemolymph constituents of American bollworm, *Helicoverpa armigera* (Hübner), infected with nucleopolyhedrovirus. *Indian J. Exp. Biol.* **39**: 1123.
  18. Kim D, Forst S. 2005. *Xenorhabdus nematophila*: mutualist and pathogen. *ASM News* **71**: 174-178.
  19. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
  20. Mann M, Hendrickson RC, Pandey A. 2001. Analysis of proteins and proteomes by mass spectrometry. *Annu. Rev. Biochem.* **70**: 437-473.
  21. Marokházi J, Lengyel K, Pekár S, Felföldi G, Patthy A, Gráf L, et al. 2004. Comparison of proteolytic activities produced by entomopathogenic *Photorhabdus* bacteria: strain- and phase-dependent heterogeneity in composition and activity of four enzymes. *Appl. Environ. Microbiol.* **70**: 7311-7320.
  22. Marokházi J, Mihala N, Hudecz F, Fodor A, Gráf L, Venekei I. 2007. Cleavage site analysis of a serralyisin-like protease, PrtA, from an insect pathogen *Photorhabdus luminescens* and development of a highly sensitive and specific substrate. *FEBS J.* **274**: 1946-1956.
  23. Massaoud MK, Marokházi J, Venekei I. 2011. Enzymatic characterization of a serralyisin-like metalloprotease from the entomopathogen bacterium, *Xenorhabdus*. *Biochim. Biophys. Acta* **1814**: 1333-1339.
  24. Nagarkatti S, Prakash S. 1974. Rearing *Heliothis armigera* (Hb.) on artificial diet. *Tech. Bull. Commonw. Inst. Biol. Contr.* **17**: 169-173.
  25. Patil U, Chaudhari A. 2009. Purification and characterization of solvent-tolerant, thermostable, alkaline metalloprotease from alkalophilic *Pseudomonas aeruginosa* MTCC 7926. *J. Chem. Technol. Biotechnol.* **84**: 1255-1262.
  26. Richards GR, Goodrich-Blair H. 2010. Examination of *Xenorhabdus nematophila* lipases in pathogenic and mutualistic host interactions reveals a role for *xlpA* in nematode progeny productions. *Appl. Environ. Microbiol.* **76**: 221-229.
  27. Ross GES. 1977. *Maximum Likelihood Programme. The Numerical Algorithms Gr.* Rothmested Experiment Station, Harpendon, UK.
  28. Sarath G, Dela Motte RS, Wagner FW. 1989. Protease assay methods. In Beynon RJ, Bond JS (eds.). *Proteolytic Enzymes; A Practical Approach.* Oxford University Press, Oxford, England.
  29. Schmidt T, Kopecky K, Neelson K. 1989. Bioluminescence of the insect pathogen *Xenorhabdus luminescens*. *Appl. Environ. Microbiol.* **55**: 2607-2612.
  30. Schmidt TM, Bleakley B, Neelson KH. 1988. Characterization of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. *Appl. Environ. Microbiol.* **54**: 2793-2797.
  31. Simões N, Caldas C, Rosa JS, Bonifassi E, Laumond C. 2000. Pathogenicity caused by high virulent and low virulent strains of *Steinernema carpocapsae* to *Galleria mellonella*. *J. Invertebr. Pathol.* **75**: 47-54.
  32. Tork SE, Shahein YE, El-Hakim AE, Abdel-Aty AM, Aly MM. 2013. Production and characterization of thermostable metallo-keratinase from newly isolated *Bacillus subtilis* NRC 3. *Int. J. Biol. Macromol.* **55**: 169-175.
  33. Wang Y, Fang X, An F, Wang G, Zhang X. 2011. Improvement of antibiotic activity of *Xenorhabdus bovienii* by medium optimization using response surface methodology. *Microb. Cell Fact.* **NOV. 14**: 98.
  34. Wilkinson J. 1986. Fragmentation of polypeptides by enzymic methods, pp. 121-148. In Darbre A (ed.). *Practical Protein Chemistry: A Handbook.* John Wiley and Sons, New York, NY.
  35. Yang J, Zeng H-M, Lin H-F, Yang X-F, Liu Z, Guo L-H, et al. 2012. An insecticidal protein from *Xenorhabdus budapestensis* that results in prophenoloxidase activation in the wax moth, *Galleria mellonella*. *J. Invertebr. Pathol.* **110**: 60-67.