CHAPTER – 4

Results and Discussion
4.0. RESULTS AND DISCUSSION

The experimental results have been presented in Tables 3-9; Plates 15-30 and Figures 3-16.

Out of 86 soil samples, only two samples, one each collected from the rhizosphere of sapota (Achras zapota) in Agriculture Research Farm, Shillong district of Meghalaya and from the rhizosphere of banyan tree (Argeria speciosa) in Rampanpudam, Kerala revealed the presence of entomopathogenic nematodes. These two strains/isolates were found to have very high insect biocontrol potential since they could induce insect mortality within 24–48 hrs and there infective juveniles emerged en masse from the cadaver with in 3 to 4 days after the inoculation, at 25-30°C. Infective juveniles of Meghalaya isolate were characterized by having 430-630 µm long body, excretory pore located 32-40 µm from anterior end and 44-58 µm long tail. Based on its biology, morphological features of infective juveniles and complete taxonomic studies, it was described and published as new species of Steinernema, namely Steinernema meghalayensis (Ganguly et al., 2011). Based on biology and morphological features of infective juveniles of Kerala strain/isolate, it was designated as a new isolate of Steinernema, namely IARI-EPN-SGkr1.

4.1 Bacteria associated with Nematodes

The isolations of symbiotic bacteria from the IJs of S. meghalayensis (Ganguly et al., 2011) and the Steinernema sp. IARI-EPN-SGkr1 on NBTA indicator plates, revealed the presence of blue colonies of Xenorhabdus. Besides, some other bacteria were also observed in some plates. The 16s rDNA gene of these bacteria were then sequenced and the strains showing novelty were further characterised as per the methods described earlier.
4.2 Symbiotic bacteria, *Xenorhabdus* species

All the characterisation experiments were conducted on three strains of each type of bacteria isolated from the nematode IJs. The symbiotic bacteria isolated from IJs of *S. meghalayensis* and the Steinernema sp. IARI-EPN-SGkr1 were designated as strains IARI-Xeno-SKmg and IARI-Xeno-SKkr respectively. Symbiotic bacteria used in the present study were *Xenorhabdus indica* from Steinernema thermophilum already reported from India and these two new isolates IARI-Xeno-SKmg and IARI-Xeno-SKkr.

4.3 Cultural characteristics

Different cultural test were carried out for *Xenorhabdus* strains/isolates of symbiotic bacteria. On LB agar, 3 days old colonies were found circular to irregular, with smooth to irregular edges, convex, raised, shiny and opaque. On NBTA, colonies were blue while yellow on NA media, pinkish red on Mackonkey agar and brown on Triple sugar iron agar medium. After several transfers, colony variants were observed, which do not accumulate the respective dye on NBTA medium (Plates- 15, 16, 17, 18 & 23).

Cells of the bacteria were gram negative, non spore forming, motile, straight rods having 1-2 inclusion bodies. Different size of the strains/isolates was found ie. *Xenorhabdus* isolate IARI-Xeno-SKmg: 3.0-6.5 X 1.0-2.0 µm and IARI-Xeno-SKkr: 2.5-10 X 1.5- 2.0-µm respectively (Plates- 19, 20, 21 & 22).

4.4 Biochemical characterization

*Xenorhabdus* isolates IARI-Xeno-SKmg and IARI-Xeno-SKkr were tested for their positive and negative response to different biochemical tests on the basis of which they were compared to different representative genera in Bergey’s manual of determinative bacteriology. Both the strains were found positive for casein hydrolysis by forming a clear cut zone for IARI-Xeno-SKmg (25mm) and IARI-Xeno-SKkr(28mm) (Plate- 25); Gelatin hydrolysis test as
liquefication of the gelatin was observed; Lipase Activity by forming clear cut zone of lipid hydrolysis for IARI-Xeno-SKmg (13 mm) and IARI-Xeno-SKkr (19 mm) (Plates- 26): Starch hydrolysis as zone of clearing around growth was observed (Plates-28); lecithinase activity by forming clear zone of hydrolysis for IARI-Xeno-SKmg (15 mm) and IARI-Xeno-SKkr (7 mm) (Plates-27) and for hemolysin activity as clear zone of hemolysis of blood around the growth was observed (Plates-24). Both the strains were found catalase negative.

For Urease activity isolate IARI-Xeno-SKmg was positive while IARI-Xeno-SKkr isolate was found to be weak. IARI-Xeno-SKmg was found negative for Cytochrome oxidase activity while IARI-Xeno-SKkr isolate was found positive.

4.5 Substrate utilization tests

Both the new isolates/ species IARI-Xeno-SKmg and IARI-Xeno-SKkr along with Xenorhabdus indica were subjected to both the substrate utilization kits. Data from Enterobacteriaceae Identification kit KB003 Hi25TM tests strips and BIOLOG GN plates are summarized in Tables-4 and 5, respectively. According to the Enterobacteriaceae Identification kit KB003 Hi25TM tests strips, the majority of tests including Deamination, H$_2$S production, Esculin hydrolysis, Lactose, Celllobiose, Melibiose, Saccharose, Raffinose, Trehalose, Xylose, Adonitol, Rhamnose, Voges Proskauer’s, Methyl red and Indole were negative for both the isolates along with X. indica. Tests shown to be positive for all strains are indicated in the legend of Tables-4. On BIOLOG GN2 Plates majority of the results obtained with panels including N-Acetyl-D-Galactosamine, L-Arabinose, D-Arabitol, D-Celllobiose, i-Erythritol, L-Fucose, D-Galactose, Gentiobiose, α-Cyclodextrin, Dextrin, α-D-Lactose, Glycogen, Tween 40, Tween 80, D-Mannitol, D-Melibiose, β-Methyl- D Glucoside, D-Psicose, D-Raffinose, L-Rhamnose, D-Sorbitol, Sucrose, Turanose, Xylitol, Succinic Acid Mono-Methyl- Ester, Acetic Acid, D-Serine, γ-Amino Butyric Acid, Citric Acid, Formic Acid, D-Galactonic Acid Lactone, D-Galacturonic Acid, D-Glucosaminic Acid, D-Glucuronic Acid, α-Hydroxybutyric Acid, β-
Hydroxybutyric Acid, γ-Hydroxybutyric Acid, Itaconic Acid, α-Keto Butyric Acid, α-Keto Glutaric Acid, α-Keto Valeric Acid, Malonic Acid, Propionic Acid, Quinic Acid, D-Saccharic Acid, Sebacic Acid, Succinamic Acid, Glucuronamide, L-Alaninamide, Hydroxy-L-Proline, L-Leucine, L-Ornithine, L-Phenylalanine, L-Threonine, Urocanic Acid, Thymidine, Phenylethylamine, Putrescine, 2-Aminoethanol and 2,3-Butanediol were found negative for all the three species. Tests shown to be positive for all strains are indicated in the legend of Tables-5.

Some tests in the BIOLOG panels were identical to both the new species, however IARI-Xeno-SKmg and IARI-Xeno-SKkr were distinct from X. indica in response to Adonitol, m-Inositol, Maltose, D-Trehalose, Cis-Aconitic Acid, Hydroxy Phenylacetic Acid, L-Alanylglucose, Uridine and a-D-Glucose-1-Phosphate utilization; on KB003 Hi25TM tests strips they vary in response Citrate utilization, Esculin hydrolysis, Adonitol where in X. indica was positive for esculin hydrolysis, adonitol utilization while negative for citrate utilization.

Both the new isolates were also compared for their distinctiveness from each other on the basis of substrate utilization tests. IARI-Xeno-SKmg was different from IARI-Xeno-SKmkr in response to Tween 80, N-Acetyl-D-Glucosamine, D-Gluconic Acid, D,L-Lactic Acid, Succinic Acid, Bromosuccinic Acid, D-Alanine, L-Alanine L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, Glycyl-LAspartic Acid, Glycyl-LGlutamic Acid, L-Proline, L-Pyroglutamic Acid and D,L-Carnitine on BIOLOG GN2 Plates; and in response to Arabinose, Urease, ONPG (ortho-Nitrophenyl-β-galactoside) and Oxidase on KB003 Hi25TM tests strips. These result was also compared with the data (not Shown) observed for other Xenorhabdus species (Lengyel et al., 2005, Somvanshi et al., 2005 and Tailliez et al., 2006) and it is in agreement with the earlier results for strains of the genera Xenorhabdus.
4.6 Antimicrobial result

As reported for most of the described Xenorhabdus species, both the isolates IARI-Xeno-SKmg and IARI-Xeno-SKkr exhibited strong antimicrobial activity against both the test organisms, forming an inhibition zone of around 1.2 and 1.4 cm against Micrococcus luteus and around 1.0 and 1.2 cm against Bacillus cereus respectively.

4.7 Antibiotic test result

Both the test cultures/isolates of Xenorhabdus ie. IARI-Xeno-SKmg and IARI-Xeno-SKkr were found susceptible to Chloramphenicol (24 & 30mm), Tetracycline (16 & 20 mm), Gentamycin (25 & 22mm), Kanamycin (24mm for both) and Streptomycin (28mm for both) with having clear cut zone of inhibition respectively however both the isolates were resistant to Ampicillin and Vancomycin as per the results obtained according to National committee for clinical laboratory standards (NCCLS) (Plates- 29 & 30).

4.8 Results of gnotobiological tests

The infective juveniles from which they had been isolated recover, grow and propagate on them in TSY agar plates, producing fertile progeny through generations. The nematodes, of course, grow poorly on each other's bacterial symbionts.

4.9 Electrophoresis and isozyme visualization

Isozymic patterns of esterase(EST), Superoxide dismutase (SOD) and Malate dehydrogenase (MDH) showed polymorphism, which could differentiate both the new native species/isolates along with X. indica and other Xenorhabdus native isolate IARI-Xeno-as1 (Table-6).

EST patterns (Figure-4) showed good polymorphism among Xenorhabdus species. All four species/isolates exhibited species-specific bands. X. indica possessed four bands at Rf 0.46, 0.56, 0.71 and 0.75. IARI-Xeno-SKmg exhibited five distinct bands at Rf 0.22, 0.29, 0.42, 0.59 and 0.74.
IARI-Xeno-SKkr possessed four species specific bands at Rf 0.22, 0.41, 0.58 and 0.74 and IARI-Xeno-as1 showed only two species specific bands of Rf 0.68 and 0.75.

Isozyme profiles of SOD for all the four species/isolates showed species-specific phenotypes but first three bands in each of the three species were at almost the same level (Rf=0.11, 0.25 and 0.36 respectively) except the IARI-Xeno-as1 isolate which showed four bands at Rf 0.44, 0.58, 0.76 and 0.87. Species-specific phenotypes represented by X.indica with rest six bands (Rf 0.49, 0.59, 0.66, 0.73, 0.86 and 0.94), isolate IARI-Xeno-SKmg with rest four bands (Rf 0.50, 0.65, 0.71 and 0.90) and IARI-Xeno-SKkr with rest four bands (Rf 0.45, 0.53, 0.65, and 0.84), respectively (Figure-5).

Malate dehydrogenase profiles of isolate IARI-Xeno-SKmg and IARI-Xeno-SKkr showed identical phenotypes with one non-specific band at Rf 0.71 while X. indica and IARI-Xeno-as1 revealed species-specific phenotype with four band in each at Rf 0.42, 0.59, 0.68, 0.91 and at Rf 0.19, 0.42, 0.59 and 0.70 respectively (Figure-6).

4.10 Hierarchical Cluster Analysis based on isozymic profiles

The dendrogram obtained from hierarchical cluster analysis based on esterase, SOD and MDH isozymes (Figure-7) placed X. indica and isolate IARI-Xeno-as1, in one cluster showing 44% similarity with other isolates placed in another cluster. In cluster II, IARI-Xeno-SKmg and IARI-Xeno-SKkr showed 69% similarity with each other. This dendrogram based on band sharing amongst the isolates showed the relationship between the native isolates of the Xenorhabdus (Kumar et al., 2009). This should not be viewed as absolute measures of the evolutionary relationships with in the Xenorhabdus as it contains only four species / isolates and addition of other species may significantly alter the topography (Dubey et al., 2009). It includes data only for three isozymes (i.e. esterase, SOD and MDH), and subsequent addition of more isozymes may alter the topography.
Isozymic profile has demonstrated the utility of Esterase, Superoxide dismutase and Malate dehydrogenase isozymes of *Xenorhabdus* for the differentiation of some of the species of *Xenorhabdus*. Further studies on isozymic profiles of several populations of *Xenorhabdus* and *Photorhabdus* comprising different species and strains, will yield useful information for the identification of species in these genus.

Compared to DNA based technologies, this technique is simpler, less time-consuming and less costly and therefore can be widely applied for identifying the samples obtained from large surveys. Keeping in view the high biocontrol potential of *Xenorhabdus* along with its nematode symbiont, *Steinernema* species, strains are continuously being isolated from different parts of the world and several strains are already in queue waiting for identification. Combination of Esterase, Superoxide dismutase and Malate dehydrogenase isozymic profiles from *Xenorhabdus*, supplemented with morphological and cultural details, can be useful for preliminary screening and differentiation of *Xenorhabdus* and *Photorhabdus* species.

### 4.11 Molecular Characterization

The Polymerase chain reactions successfully amplified the ITS region of ribosomal DNA, and the PCR-amplified products obtained from both the isolates/ species of *Xenorhabdus* IARI-Xeno-SKmg and IARI-Xeno-SKkr were of the same size (1530 bp) as evident from the position of single intense band of DNA *(Figure-3)*. This was in accordance with the earlier reports on size of ITS region of rDNA of other *Xenorhabdus* species (Rainey *et al*., 2000 & Somvanshi *et al*., 2006). The purified PCR- amplified ITS region of the rDNA was sequenced and the length of ITS region (flanked by the above primers) was found to be of 1461 bp for IARI-Xeno-SKmg and 1454 bp for and IARI-Xeno-SKkr respectively . The base sequences (1-1461 bp) of IARI-Xeno-SKmg and (1-1454 bp) of IARI-Xeno-SKkr were deposited in GenBank, NCBI under...
accession no. JN177510.1 as Xenorhabdus sp. SKmg (Figure-8) and accession no. JN177511.1 as Xenorhabdus sp. SKkr (Figure-9) respectively.

4.11.1 Multiple alignments

The base sequences (1-1461 bp) of IARI-Xeno-SKmg and (1-1454 bp) of IARI-Xeno-SKkr were then subjected to BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Xenorhabdus isolate IARI-Xeno-SKmg, showed maximum identity with Xenorhabdus kozodoii (97%) followed by X. stockiae (95%) and X. hominickii (95%) and IARI-Xeno-SKkr showed maximum identity with X. indica (97%), X. szenirmaii (95%) and X. miraniensis (95%). The sequence alignments of IARI-Xeno-SKmg and IARI-Xeno-SKkr along with their four closely related species were done using the default parameters of the software BioEdit (Figures- 10 & 11). The alignments were used to compare the similarities and differences in nucleotides of IARI-Xeno-SKmg and IARI-Xeno-SKkr with other species, which revealed remarkable differences as diagnostic characters in sequence length and base composition. Classified length of the amplified region and the nucleotide composition along with diagnostic characters for IARI-Xeno-SKmg and IARI-Xeno-SKkr and their 4 closely related species is summarized in the Tables 7 & 8.

4.11.2 Phylogenetic tree construction

In continuation with there molecular differentiation, it was also undertaken to know there phylogenetic relationships with other species of the genus on the basis of sequences of ITS region of rDNA. For the Xenorhabdus species IARI-Xeno-SKmg the tree exhibited trichotomy (Figure-12), which could be correlated with the morphological features of these species. The species were grouped in to 4 main clusters: (i) X. pionarii groups with X. bovioni and no other species; (ii) X. stockiae group, wherein X. hominickii was grouped alongwith three other species. (iii) X. kozodoii + X. graffiniae group: which
contain X. ehlersii, X. beddingii along with X. budapestensis. IARI-Xeno-SKmg was located along with other species in the X. kozodoii group and (iv) X. indica group: IJs of nematode symbiont of these species clustered in this group are characterized with/ without having paired horn-like structure in the lip region of infective juveniles (Nguyen & Adams, 2003). This was the biggest cluster with maximum number of the species in it along with X. nematophila. It has been demonstrated that phylogenetic relationships based on molecular characteristics of Xenorhabdus species are in conformity with their morphological features of their nematode counter part. Within X. kozodoii group, IARI-Xeno-SKmg was most closely related to X. kozodoii, and the clade was well supported by bootstrap analysis. IARI-Xeno-SKmg isolate with its distinctive position in the phylogenetic tree separated it from other species.

For the Xenorhabdus species/isolate IARI-Xeno-SKkr the tree exhibited trichotomy (Figure-13), which could be correlated with the morphological features of these species. The species were grouped in to 3 main clusters: (i) X. pionarii + X. indica group with X. bovionii and other species. IARI-Xeno-SKkr was located along with other species in the X. indica group. IJs of nematode symbiont of these species counter-part clustered in this group are characterized with having paired horn-like structure in the lip region of infective juveniles (Nguyen & Adams, 2003). This group is subdivided in to X. nematophila, X. miraniensis and X. romanii groups along with other species in it. This was the biggest cluster with maximum number of the species in it; (ii) X. stockiae + X. hominickii group, wherein X. japnica was grouped alongwith five other species. (iii) X. kozodoii group: which is only species in the group alone. Within X. pionarii group, IARI-Xeno-SKkr was most closely related to X. indica, and the clade was well supported by bootstrap analysis. IARI-Xeno-SKkr isolate with its distinctive position in the phylogenetic tree separated it from other species.
4.11.3 Restriction Fragment Length Polymorphism profiles

The RFLP profiles obtained from the restriction enzymes AluI, Rsal, HinfI, Hhal, HaeIII and MspI for both species / isolates are given in Figures-14 & 15. The RFLP profiles obtained for both isolates could be grouped into 9 phenotypes I, II, III, IV, V, VI, VII, VIII & IX with 4, 4, 3, 3, 3, 4, 4, 5 & 4 restriction fragments, respectively (Table-9). Restriction enzymes AluI, HaeIII and MspI showed species specific banding pattern for both the species however enzymes Rsal, HinfI, Hhal shared similar banding profile with III, IV and V phenotypes respectively for both IARI-Xeno-SKmg; IARI-Xeno-SKkr isolates with three bands in each. The RFLPs obtained from AluI showed two phenotypes I-4 for IARI-Xeno-SKmg (with 4 bands of 200, 350, 550 and 750 base pairs) and II-4 for IARI-Xeno-SKkr (with 4 bands of 200, 250, 350, 600 bps). RFLPs were unique with species specific banding patterns from HaeIII with two phenotypes VI-4 (for IARI-Xeno-SKmg with 100, 200, 300, 1000 bps) and VII-4 (for IARI-Xeno-SKkr with 150, 200, 300, 350 bps). Phenotypes VIII-5 (for IARI-Xeno-SKmg with 350, 500, 650, 800, 1000 bps) and IX-4 (for IARI-Xeno-SKkr with 300, 500, 700, 800 bps) were exhibited by MspI which also showed species specific polymorphism. Both the isolates are indicated to be distinctive based on RFLP profiles, which is indicative of nematode-bacterium specificity. It is indicated that both the isolates represent distinct species of Xenorhabdus as these have been isolated from different Steinernema isolates extracted from diverse geographical regions. These findings are in conformity with the protocols suggested by (Hominick et al., 1997), wherein the utility of restriction enzymes in the taxonomy of entomopathogenic nematodes, have been emphasized. In the present investigation, the restriction enzymes AluI, HaeIII and MspI were found to be useful diagnostic markers for differentiating native isolates of Xenorhabdus.

The sequence variation in ITS region as evident from the differences in RFLPs, is a useful molecular tool which is integrated with morphological,
cultural and biochemical data for differentiating the EPB species and deriving their phylogenetic relationships.

4.11.4 Hierarchical Cluster Analysis

The dendrogram was obtained from hierarchical cluster analysis based on RFLP profile of ribosomal DNA (Figure-16) by using restriction enzymes Alul, Rsal, HinfI, Hhal, HaeIII, MspI. All the 9 native isolates (data not shown here for other seven isolates) of Xenorhabdus (IARI-Xeno-SKmg; IARI-Xeno-SK kr; IARI-Xeno-as1; IARI-Xeno-gj; IARI-Xeno- dl1; IARI-Xeno –chh1; IARI-Xeno –jk; IARI-Xeno –ouat and IARI-Xeno–champ) along with X. indica exhibited atleast 51% similarity in their RFLP patterns. The phylogenetic tree placed these species/isolates in 2 clusters. Cluster I was further subdivided into cluster-Ia comprised of 6 native isolates (IARI-Xeno-as1; IARI-Xeno-dl1; IARI-Xeno–chh1; IARI-Xeno–jk; IARI-Xeno–ouat and IARI-Xeno–champ) along with X. indica showing 75–92% similarity with each other. IARI-Xeno-gj formed a separate clade (cluster-Ib) having 58% similarity with cluster-Ia. Cluster II comprised of both the new isolates (IARI-Xeno-SKmg; IARI-Xeno-SKkr showing 80% similarity with each other and 52% with cluster-I.

This study has shown that the ITS region is an ideal candidate for molecular taxonomy. However, it should not be viewed as absolute measures of the evolutionary relationships within the Xenorhabdus as it is based on only six restriction enzyme, use of more restriction enzymes specific for differentiating Enterobacteriaceae family may significantly alter the topography and it includes data only for a subset of ITS region (ie. The restriction enzyme recognition sites), and subsequent nucleotide sequence data of the entire spacer may also alter the topography.

Conclusively, RFLPs of ITS region of rDNA using the restriction enzyme Alul, Rsal, HinfI, Hhal, HaeIII and MspI differentiated the native isolates from one another, which demonstrated the robustness of this highly conserved
region of rDNA as diagnostic tool. The restriction enzymes EcoRI has been used as a diagnostic marker for differentiating the species of another enterobacter of the genus Providencia but its utility for Xenorhabdus has been demonstrated for the first time (Somvanshi et al., 2006a). In this study it is extended to 6 Restriction enzymes and AluI, HaeIII and MspI proved to be the distinct diagnostic marker for differentiating the Xenorhabdus species. It also indicated that each of the native isolate used in the study might represent a distinct species since these have been isolated from diverse geographical regions.

Based on morphological, cultural, biochemical, molecular characterization and the distinct phylogenetic position of strains/isolates IARI-Xeno-SKmg and IARI-Xeno-SKkr among the other known Xenorhabdus species as well as differentiating metabolic properties, both the isolates represented a new species which are being described as Xenorhabdus meghalayensis sp. nov. and Xenorhabdus keralensis sp. nov. respectively.

**DESCRIPTION**

*Xenorhabdus meghalayensis* sp.nov.,

meghalensis, megha'. lensis. L. fem. Adj. meghalaya, pertaining to Meghalaya, a north-eastern state of India, the origin of the novel bacterial and nematode species.

Meghalaya (Latitude of 25°30`N; Longitude of 91°00`E; Altitude 1965 m above sea level) covers the Eastern Himalayan agro climatic zone having annual rainfall 1150 cm and an average temperature from 4-28°C is one of the North eastern states of India. It is bound on the north and east by Assam, and on the south and west by Bangladesh. Meghalaya, literally meaning the abode of clouds, is essentially a hilly state. Meghalaya is basically an agrarian state, in which about 80 per cent of the population depends primarily on agriculture for their livelihood. The State has a vast potential for developing horticulture due to agro-climatic variations, which offer much
scope for cultivation of temperate, sub-tropical and tropical fruits and vegetables.

A new species of entomopathogenic bacterium population of *Xenorhabdus* isolated from Shillong district of Meghalaya, one of the seven states in north-eastern hilly regions of India, could not be assigned to any of the known species based on the morphological biochemical, ecological features as well as molecular characters, and hence described as a new species namely *Xenorhabdus meghalyensis* sp.n.

Cells Gram negative, oxidase and catalase negative, glucose-fermenting straight rods of 3.0-6.5 x 1.0- 2.0 μm in size (Plate-19). Aged rods show pronounced 1 to 2 inclusion bodies (Plate-20). After 3 days on nutrient agar individual colonies are circular to irregular, 2.7 to 3.2 mm in diameter, with smooth to irregular edges, convex, raised, shiny and opaque with a brownish pigmented centre (Plate-15). On NBTA, the colonies are blue (Plate-17), while red on MacConkey agar. Even after several transfers, no change to colony variants (phase II) was observed, which do not accumulate the respective dyes. On Trypticase Soy Agar, aged colonies grow with the formation of concentric rings and characteristic brown colour. Maximum growth temperature (Tmax): 40° C in Trypticase Soy Broth, 35° C in Nutrient Broth. Strains caused complete haemolysis on Columbia-blood agar plates (Plate -24). It is different from other known species of the genus in their ability to cause distinct starch hydrolysis, being positive for Tween 80, Arabinose and for N-Acetyl-D-Glucosamine and D-Gluconic Acid utilization (Tables 4 & 5). Strains of this species are variable for utilization of trehalose. On Biolog substrate panels, negative for utilization of D,L-Lactic Acid and Glycyl-L-Aspartic Acid (all the other members of *Xenorhabdus* are reported positive for these substrates). Isolated from the crushed infective juveniles (IJs) of *Steinernema meghalayensis* collected from Shillong district of Meghalaya, one of the seven states in north-eastern hilly regions of India.
**Type strain:** IARI-Xeno-SKmg (deposited at EPN Genomics Laboratory, IARI, New Delhi, also submitted ribosomal DNA sequence to NCBI Genbank, USA).

**DESCRIPTION**

*Xenorhabdus keralensis* sp. nov.,

*keralensis*, kerala’. ensis. L. fem. Adj. kerala, pertaining to Kerala, a southern state of India, the origin of the novel bacterial and nematode species.

Although Kerala lies closer to the equator than most Indian states, it is blessed with pleasant and equable climate throughout the year. This is because of the land’s nearness to the sea and the presence of the natural fort like Western Ghats on the east that protect the state from dry and hot winds blowing from the north. Kerala receives copious rain (average 3000 mm) each year. The temperature normally ranges from 28º C to 32º C (82º F to 90º F) on the plains but drops to about 20º C (68º F) in the highlands. The Highlands of Kerala, enjoys a cool and invigorating climate the year-round.

Owing to its diversity in geographical features, the climatic condition in Kerala is diverse. It can be divided into 4 seasons - Winter, Summer, South-West Monsoon and North-East Monsoon.

Cells Gram negative, oxidase and catalase negative, glucose-fermenting straight rods of 2.5-10.0 x 1.5-2.0 µm in size (Plate -21). Aged rods show pronounced 1 to 2 inclusion bodies (Plate -22). After 3 days on nutrient agar individual colonies are circular to irregular, 2.0 to 3.0 mm in diameter, with smooth to irregular edges, convex, raised, shiny and opaque with a brownish pigmented centre. On NBTA, the colonies are blue (Plate-18), while red on MacConkey agar. Even after several transfers, no change to colony variants (phase II) was observed, which do not accumulate the respective dyes. On Trypticase Soy Agar, aged colonies grow with the formation of concentric rings and characteristic brown colour. Maximum growth temperature (Tmax): 42º C in Trypticase Soy Broth, 37º C in Nutrient Broth. Strains caused complete haemolysis on Columbia-blood agar plates (Plate-
It is different from other known species of the genus in their ability to cause weak starch hydrolysis, ability to lecithinase activity (70mm zone), being positive for L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, Glycyl-L-Glutamic Acid, D,L-Carnitine utilization on BIOLOG plates.

Isolated from the crushed infective juveniles (IJs) of *Steinernema isolate IARI-EPN-SGkr* collected from the soil of Kerala, located in the southern region of India.

**Type strain:** IARI-Xeno-SKkr (deposited at EPN Genomics Laboratory, IARI, New Delhi, also submitted ribosomal DNA sequence to NCBI Genbank, USA).

Finding new *Xenorhabdus* species from *S. meghalayensis* and *Steinernema* isolate IARI-EPN-SGkr is in accordance with the previous investigations which have also shown species specific nematode- bacterium relationships in entomopathogenic nematodes (Boemare, 2002, Somvanshi *et al.*, 2005, Tailliez *et al.*, 2006). It is likely that symbiotic bacteria co-evolve with their nematode counterparts; there is a high probability of finding a new *Xenorhabdus* species from a newly described *Steinernema* species (Frost & Nealson, 1996). This is indeed the case for the strains/isolates investigated in this study which have been isolated from the recently described *Steinernema meghalayensis* (Ganguly *et al.* 2011) and *Steinernema* sp. IARI-EPN-SGkr from Indian soil. The present species *X. meghalayensis* sp.nov and *X. keralensis* sp.nov. may be the twenty second and twenty third species of the genus respectively.

The abovementioned morphological, cultural, biochemical and molecular data obtained from various techniques will be useful in re-defining and diagnosing these species along with other new species. Proposal of two bacterial species, is the second record of new species of *Xenorhabdus* from India and first in their respective agro climatic zone. The precise species identification of symbiotic bacteria associated with *Steinernema* nematodes
will form the basis for pursuing future basic and strategic research on these bacteria for better understanding of complex nematode-bacterium relationships, and identifying the biotoxins and insect toxic genes for developing biotechnological management methods against insect pests. Future research on the species identity of Xenorhabdus bacteria along with its nematode symbionts, is urgently needed so that it can suitably be incorporated in integrated pest management schedules for its biopesticidal potential.