BIOCIDAL CAPABILITIES OF *XENORHABDUS* SP. ISOLATED FROM EGYPT

BY

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ABSTRACT

*In this work, **Xenorhabdus** sp. was isolated from a local nematode strain **Steinernema** sp. and a foreign well-identified strain, i.e., **Steinernema riborvae** kindly supplied by Florida State University, USA. The identity of the isolated bacteria was authenticated referring to their cultural, morphological and biochemical traits as well as to their entomopathogenicity against **Galleria mellonella**. The 16S rRNA technique was adopted for conclusive identification of both strains. The antagonistic properties of the isolated **Xenorhabdus** spp. isolates were studied in submerged batch fermentations after 120 hrs using the Disc Diffusion Technique. Both **Xenorhabdus** spp. strains showed various antimicrobial activities against wide spectra of Gram +ve, Gram –ve bacteria as well as yeasts. Oral administration of cell-free culture supernatants of **Xenorhabdus** spp. isolates exhibited high levels of toxicity against **Agrotis ipsilon** and **Spodoptera littoralis**.*

INTRODUCTION

Xenorhabdus sp. is a Gram negative bacterium that has a symbiotic relationship with entomopathogenic soil nematodes of the family *Steinernema* (Forst *et al.*, 1997). *Xenorhabdus* produces active insecticidal protein toxins which are orally toxic to several insect species (Morgan *et al.*, 2001). The nematodes enter the openings of insect body, such as the mouth, spiracle, or anus. After invasion of the insect host, nematodes regurgitate bacteria, which are housed in a vesicle of their intestine, directly into the hemocoel (Ciche and Ensign, 2003). Bacteria produce a range of proteins and metabolites that kill insect host (Bowen *et al.*, 1998). Both nematodes and bacteria replicate in the insect cadaver (Ffrench-Constant *et al.*, 2003). *Xenorhabdus* bacteria can be isolated from infective juvenile nematodes or from infected insect cadaver. They can be *in vitro* cultured as free living organisms on artificial media without a host nematode or insect (Forst *et al.*, 1997.). Pest control efficacy of entomopathogenic nematodes had no much success in field applications because nematodes are not very stable in many environmental conditions in addition to difficulties associated with nematode invasion into insect body. Oral toxicity of *Xenorhabdus nematophilus* living cells and their protein secretions was previously described against neonatal larvae of *Helicoverpa armigera* (Khandelwal and Banerjee-Bhatnagar, 2003). Ffrench-Constant *et al.* (2007) suggested a possible application of *Xenorhabdus* as microbial biopesticides in agriculture. This work was

planned to isolate *Xenorhabdus* from their nematode symbionts and study their cultural, morphological, biochemical and molecular characteristics in addition to examine their oral toxicity against two insect genotypes, as well as their antimicrobial activities against some microbial candidates.

MATERIALS AND METHODS

Nematodes

Two Strains of *Steinernema riborvae* (Sr), *Steinernema* sp (S2) were used in the present study (Table, 1). Nematode strains were cultured in last instar *Galleria mellonella* (L) (Lepidoptera: pyralidae) obtained from Laboratory of Insect Physiology, Plant Protection Research Institute, Agriculture Research Centre (ARC), Egypt.

Microorganisms

Escherichia coli, *Micrococcus* spp., *Candida albicans*, *Staphylococcus aureus*, *Actinomyces* spp., *Bacillus cereus*, *Salmonila typhimurium*, *Saccharomyces cerevisae* from the culture collection of the Department of Microbiology, Faculty of Agriculture, Cairo University were used as test microorganisms to study the antimicrobial activities of the isolated strains of *Xenorhabdus* sp and *Photorhabdus* sp.

Cultural Media

Slants of Lauria Bertani (LB) Agar were used for repeated sub-culturing and maintenance of *Xenorhabdus* spp. and test bacterial strains, while Nutrient Glucose Agar slants were used with actinomycetes and yeast strains. Submerged fermentations for production of *Xenorhabdus* sp. toxins were performed using four fermentation media (Table,

Isolation and characterization of *Xenorhabdus*

Infective juveniles of *Steinernema* were surface-sterilized for 3 hours in 0.1 % merthiolate solution (w/v) containing streptomycin (5,000 units.ml^{-1}) followed by three rinses in sterile Ringers' solution. Fifty juveniles were applied onto a moist Whattman No.1 filter paper (5.5 cm diameter) placed in a 15-mm diameter Petri dish. *Xenorhabdus* sp was isolated from the haemolymph of *G. mellonella* infected with the prepared juveniles. Dead *G. mellonella* larvae were surface-sterilized in 70% alcohol for 10 min, flamed and allowed to dry in a laminar airflow cabinet for 2 min. Larvae were opened where a drop of the oozing haemolymph was streaked onto nutrient agar plates containing 25 mg l^{-1} Bromothymol blue (BTB), 4 ml.l^{-1} of 1% 2,3,5-Triphenyltetrazolium Chloride filtrate. Plates were incubated in the dark for 24 h at 28 °C. A single bacterial colony was repeatedly streaked onto nutrient agar BTB plates until uniform colony size and morphology were obtained. Purity of the isolated bacterial strains was routinely checked using Gram staining and streaking on nutrient agar-BTB plates.

Phenotypic characterization, i.e., cultural, morphological as well as biochemical traits of isolates was examined according to Yamanaka *et al.*, (1992).

Table (1): Sources of *Xenorhabdus* sp. strains and their symbiotic nematodes

Bacterial species	Bacterial strains	Nematode species and strains	Source
Xenorhabdus sp.	xSr	*Steinernema riborvae* (Sr)	University of Florida, USA (H. E. Cabanillas, G. O. Poinar, J. R. Raulston 1994).
Xenorhabdus sp.	xS2	*Steinernema* sp. (S2)	Local nematode isolate from Ismailia soil, Egypt.

Table (2): Components (g/l) of the cultural media used in submerged fermentation batches for toxin production by *Xenorhabdus* sp.

Ingredient	Fermentation Media (g/l)			
	LB	S	YSG	CM
Glucose		10		10
$MgSO_4.7H_2O$			0.6	0.5
Yeast Extract	5		15	4
Peptone		5		2
Hydrolyzed Casein				2
Molasses		10		
K_2HPO4			0.87	
KH_2PO4			0.7	
NaCl	5			
$(NH4)_2SO_4$			2	
Na_2SO_4			0.71	
Tryptone	10			
Glycerol			0	

LB : Luria – Bertani broth YSG: Sundar and Chag (1993).

CM: Hotta *et al* (1985) S: Shaker (1979)

Molecular characterization of bacterial isolates:

Preparations of total genomic DNA were obtained by growing each *Xenorhabdus* strain on LB agar plates for 2 days at 28 °C. Bacterial cells were scraped off in TE8 buffer (50 mM Tris-HCl, 20 mM EDTA, and 50 mM NaCl [pH 8]) and pelleted in a micro-centrifuge tube for 2 min. The cell pellets were washed twice with TE8 buffer and finally re-suspended in 0.5 ml of TE8. The bacterial suspension was lysed by passing the microtubes through liquid nitrogen and boiling water three times for 1 min

each time. DNA samples were further purified by one phenol and one chloroform extraction and then by ethanol precipitation. Each DNA sample was diluted 50- to 100-fold in pure water to be used as a template for the PCR. (Li *et al.*, 2003).

Two oligonucleotide sequences i.e $5^{\prime}$-GGA GAG TTA GAT CTT GGC TC-$3^{\prime}$ (sense) and $5^{\prime}$-AAG GAG GTG ATC CAG CCG CA-$3^{\prime}$ (antisense) were used as primers (Weisburg *et al.,* 1991). Amplification was performed according to the protocol of (Saiki *et al.*, 1988). The reaction products were separated in a 2% agarose gel containing 0.5 mg of ethidium bromide per ml and were visualized under UV light with an Imager (software version 2.03; Appligene Inc.).

Antagonistic properties of *Xenorhabdus* sp. strains:

Submerged fermentation batch cultures were conducted in 250 ml Erlenmeyer flasks each co) (model: SI-100, Bioblock Scientific. France)ntaining 50 ml aliquots of one of the four cultural media i.e. LB broth, YSG (Sundar and Chag, 1993), (Shaker, 1979) and CM medium (Hotta *et al.*, 1985) inoculated with 5ml of 24 hr-old culture of the examined *Xenorhabdus* sp. strain. Inoculated flasks were incubated on a rotary shaker (200 rpm at 28 °C for 5 day. At 24-hr intervals, 5 ml aliquots of the culture were centrifuged at 4100 rpm for 20 min at 10°C under aseptic conditions. Sterilized Whattman No.1 filter paper discs (6 mm diameter) were soaked in 10 µl of the cell-free supernatant of the examined bacterial candidate and placed onto Petri dishes containing 10 ml of the examined Agar medium inoculated with either *E. coli*, *Micrococcus* sp., *Candida albicans*, *Staphylococcus aureus*, *Actinomyces* spp., *Bacillus cereus, Salmonila typhimurium* or *Saccharomyces cerevisae.* Triplicate plates were prepared for each examined cell-free extract and test microorganism. Plates were incubated at 30 °C for 48hrs, after which the plates were inspected for the development of growth-inhibition zones. The diameter of inhibition zones were the measured in centimeter.

Entomopathogenic capabilities of *Xenorhabdus* sp strains:

Pathogenicity of the isolates was promptly authenticated by injecting 0.1 ml cell suspension containing 10^{8} cells. ml^{-1} of the examined strain into the body of *G. mellonella* larvae and streaking the haemolymph of the dead larvae on nutrient Agar BTB plates. A single colony was inoculated into 500 ml of nutrient broth in an Erlenmeyer flask and incubated on a shaking incubator at 150 rpm for 24hr at 28°C. Population density was standardized at 10^{8} cells.ml^{-1} by measuring the optical density of the cell suspension at 600 nm (Elawad, 1998). Cell-free suspensions were obtained by centrifugation of the suspensions at 4100 rpm for 20 min at 10°C. The supernatant was drawn off, replaced with sterile distilled water and standardized at 600 nm to contain 10^{8} cells.ml^{-1}. Tween 80 (3% v/v), as an emulsifier, was added to the cell suspensions to release toxic metabolites from the bacterial cells then the cell suspensions were filtered using sterile Whattman 25mm GD/X membrane (pore size of 0.2 μm). The cell-free toxin solutions were tested on agar plates before application against *G. mellonella* larvae.

Statistical Analysis

All experiments were replicated three times. Data presented in percentage values in the present study were normalized using arcsine transformation. The significance of the main effects was determined by analysis of variance (ANOVA). The significance of various treatments was evaluated by Duncan's multiple range test ($P<0.05$). All

analyses were made using a software package "Costat", a product of Cohort Software Inc., Berkeley, California.

RESULTS

Isolation and Identification of *Xenorhabdus* spp.:

Two isolates of *Xenorhabdus* spp., i.e., xSr and xS2 were isolated from the haemolymph of *G. mellonella* infected with a well identified strain of nematode *Steinernema riborvae* (Sr) isolated from Florida soil, USA and a local strain of nematode *Steinernema* sp isolated from Ismailia soil, respectively. Both are morphologically typical Gram negative non spore-forming non acid fast motile short rods characterized by the formation of polymorphous light brown colonies. The identity of the two *Xenorhabdus* spp strains was first authenticated referring to their colony morphology, physiological and biochemical characteristics appear in Table (3).

Table (3): Biochemical characters of *Xenorhabdus* spp.

Test	*Xenorhabdus* spp. (xSr)	*Xenorhabdus* spp (xS2)
Pigment light brown	+	+
Catalase	-	-
Oxidase	-	-
Lactose	±	±
Arginine dihydrolase	-	-
Citrate utilization	-	±
Hydrogen sulfide	-	±
Urea hydrolysis	-	-
Phenilalanine	-	-
Motility	+	+
Glycerol	-	-
Glucose	±	±
Maltose	+	±
Raffinose	±	±
Salicin	-	-
Esculin hydrolysis	-	-

Molecular characterization of bacterial strains

Conclusive molecular identification was further carried out using the 16S rRNA amplification. DNA of the two strains was amplified with universal ribosomal primers. All produced a single band of about 1,550 bp. A size corresponding to the expected sizes of the published 16S rDNA sequences of *Xenorhabdus* (between 1,536 and 1,539 bp) (Fig. 1).

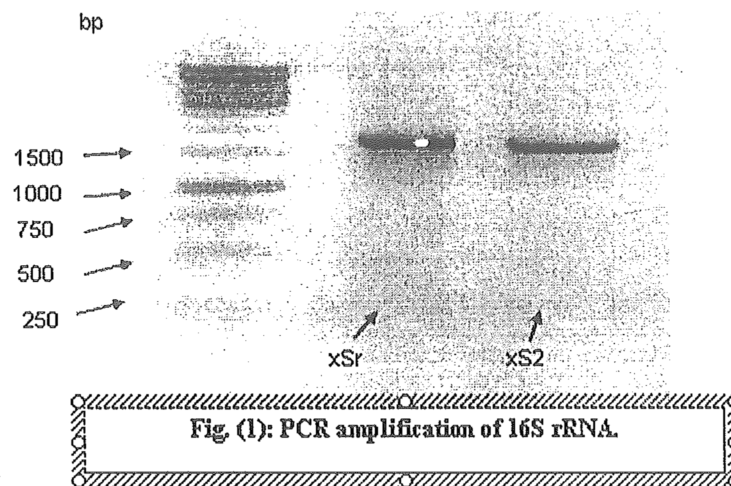

Fig. (1): PCR amplification of 16S rRNA.

Antagonistic properties of *Xenorhabdus* spp. isolates

Production of antimicrobial substances by 120-h-old *Xenorhabdus* sp. culture strains xSr and xS2 grown in different cultural medium was examined using the disc diffusion method (Fig. 2). The bacterial isolates showed low *in vitro* antimicrobial activities when grown in the fermentation broth S medium. However, the other cultural media YSG, CM and LB media provoked high antibiotic capabilities of these strains. The two *Xenorhabdus* sp. strains showed wide-spectrum antimicrobial activities against the examined Gram-negative, Gram-positive bacteria, *Actinomycetes* as well as yeasts. Their utmost antagonism was recorded when *Actinomycetes* sp. was the test organism giving rise to an inhibition zone diameter of as high as 1.4 cm. Yeasts as well as *Staphylococcus aureus*, as test organisms, appeared less affected by the local *Xenorhabdus* sp. strain xSr. the measured diameters of growth inhibition zones did not exceed 3 cm. These test organisms were more sensitive to the antagonistic effect of the strain xSr giving larger growth inhibition zones reaching up to 1.4 cm diameter. The examined *Xenorhabdus* spp. isolates xSr and xS2 grown in YSG. medium suppressed growth of the other Gram –ve test organism, e. g., *S. typhimurium* which indicated by the development of growth inhibition zones measuring 8 and 9 mm in diameter, respectively.

Oral toxicity of bacterial cell-Free supernatant *A. ipsilon* and *S. littoralis*:

Mortality of *A. ipsilon* and *S. littoralis* caused by the examined bacterial cell–free supernatants varied significantly (P=0.005) at 24, 48 and 72 hrs after treatment compared with control (Figs. 3 and 4). Insect mortality percentage increased with time in all treatments and became steady after 72 hrs. Application of cell-free culture supernatant of the bacterial isolate xSr caused 80% morality in *A. ipsilon* after 72 hrs. Cell-free culture supernatant of the strain Sr resulted in a maximum mortality of 93.3±0.02% in *S. littoralis*, while a minimum mortality of 13.3 ± 0.02% was regarded with the bacterial isolate xS2 after 72hrs. No mortality was regarded with two control insects fed with the standard plain cultural medium supernatant. However, the lethal susceptibility was different among both insect species. Statistical analysis revealed high significant differences between all cell-free supernatant and days (df =5 , F=16.6 ,P=0.000), (df=2 , F=14.4 ,P=0.0000) of *A .ipsilon* and (df=5 ,F=44.01 ,p=0.0000),

(df=2 ,F=22.6 ,P=0.0000) of *S .littoralis,* respectively. On the other hand, no significant differences were recorded for all interactions between treatments (df=10, F=1.95, P=0.079) in case of *A. ipsilon* although the high significant differences (df=10,F=6.8,P=0.0000) recorded with *S. littoralis*.

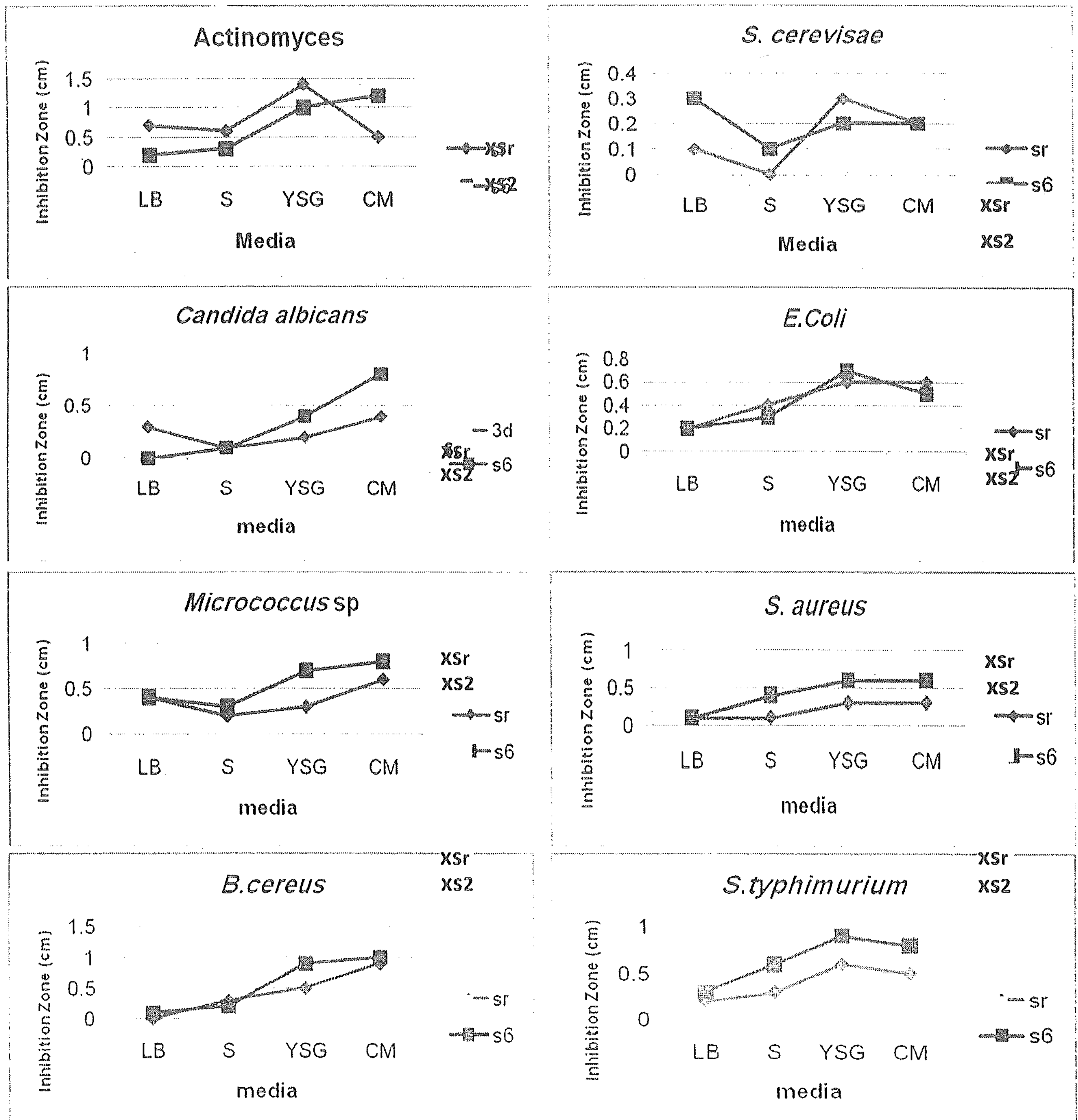

Fig. (2): Antibiotic activities of *Xenorhabdus* sp. (xSr) and *Xenorhabdus* sp. (xS2) using four different fermentation media and eight test microorganisms.

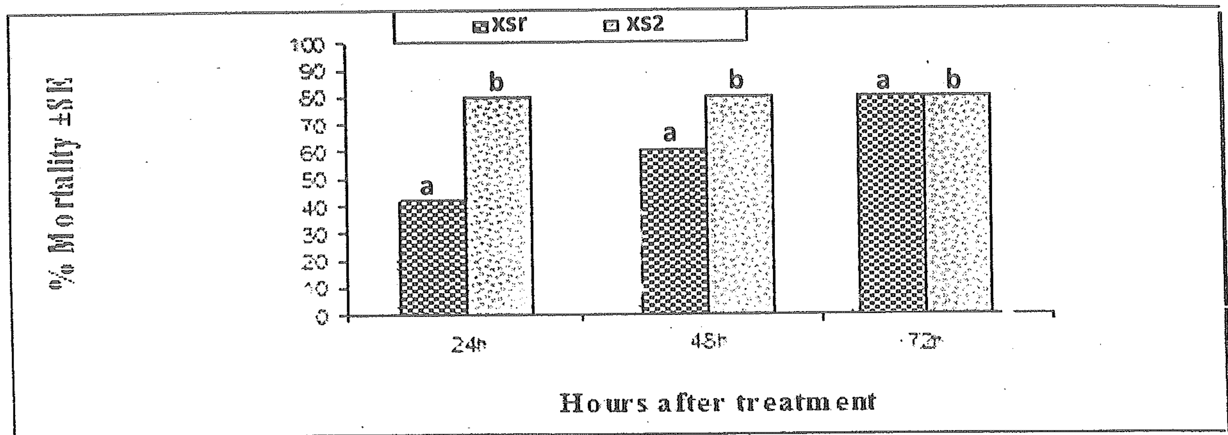

Fig. (3): Moralitiy of *Agrotis ipsilon* after 24, 48 and 72 h of treatment with *Xenorhabdus* cell-free supernatant. Bars (mean ±SE) in the same and between time intervals with the same letter(s) are not significantly different (P<0.005).

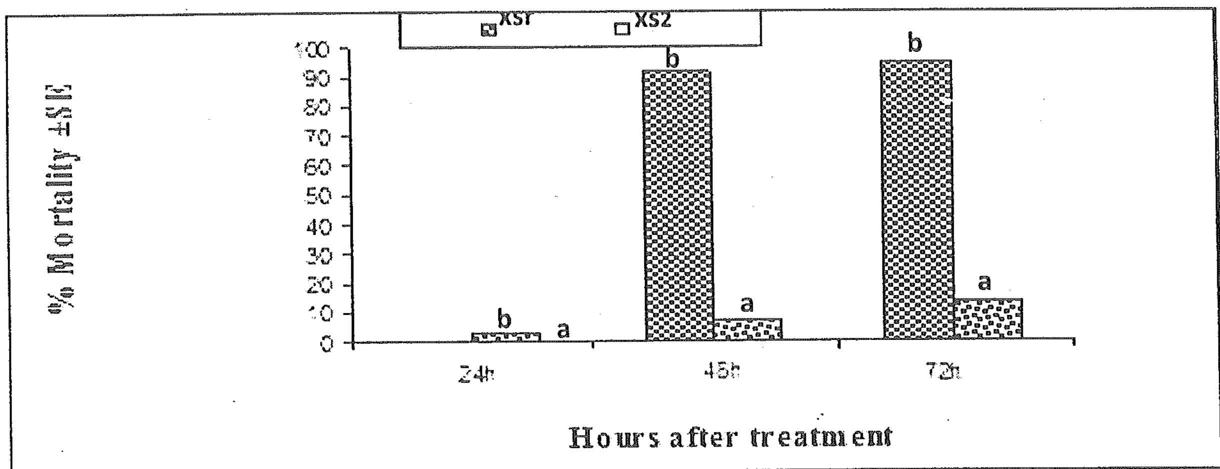

Fig.(4): Mortalitiy of *Spodoptera littoralis* after 24, 48 and 72 h of treatment with *Xenorhabdus* cell-free supernatant at 1X10^8 .Bars (mean ±SE) in the same and between time intervals with the same letter(s) are not significantly different (P<0.005).

DISCUSSION

According to Bergey's manual of systematic bacteriology the difficulties associated with identifying *Xenorhabdus* members were attributed to the biochemically-inactive nature of *Xenorhabdus* candidates (e.g., many of the traits used to distinguish the genera that form the *Enterobacteriaceae* are negative for the *Xenorhabdus/Photorhabdus* group) in addition to the high level of similarities in cultural and morphological characteristics with other members of *Enterobacteriaceae* (Yamanaka *et al.*, 1992). Herein, both bacterial isolates isolated in the present work were tentatively related to *Xenorhabdus* Sp. relying on their cultural, morphological and biochemical traits. However, their potent entomopathogenicity against *G. mellonella* besides the 16S rDNA analysis conclusively fit in well with the criteria

stated by (Yamanaka *et al.*, 1992) for phenotypical identification and Steven and Kenneth (1996) for molecular characterization of *Xenorhabdus* sp.

A possible exploitation of the Disc Diffusion Technique for *in vitro* assaying the antagonistic potency of cell-free cultures of *Xenorhabdus* was most recently approved by András *et al.*, (2010) and Yonghong *et al.*, (2011). Adopting such technique both examined *Xenorhabdus* Sp. strains exhibited wide spectrum antibiotic capabilities against Gram-ve, Gram +ve bacteria and yeasts. In parallel with these results, previous reports pointed out to the wide spectra antimicrobial activities of cell free liquid culture against wide range of test organisms particularly those not genetically related to the family *Enterobacteriaceae* (András *et al.*, 2010). This was attributed to the fact that *Xenorhabdus* species can resist the response of insect larvae to immunity and can rapidly colonies the larvae, producing compounds with antibacterial and antifungal activity (Issacson and Webster, 2002). According to Dongjin *et al.*, (2004), *Xenorhabdus nematophila* shows antibacterial activity in cultures of *Agrobacterium vitis*, *Pectobacterium carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *tabaci* and *Ralstonia solanacearum*. With few exceptions, phase one *Xenorhabdus* produced antibiotics capable of inhibiting the growth of many bacteria and fungi (Nealson. *et al.*, 1990).

Results recorded in this study demonstrate some differences in the pathogenicity between the two bacterial strains against the *A. ipsilon* and *S. littoralis*. Pathogenicity bioassays of six cell-free supernatants indicate high rate of mortality caused by both xSr bacterial isolates, and xS2 with highest mortality at a concentration of $1X10^8$ cells/ml. These bacteria produce insecticidal factors which are critical for their pathogenic activities on insects (Ffrench-Constant *et al.*, 2007). Both nematodes and bacteria replicate in the insect cadaver (Ffrench-Constant *et al.*, 2003). The bacteria secrete entomopathogenic factors directly into the growth medium. Interestingly, these bacteria or their toxic factors are insecticidal when they are ingested through the mouth and when they are injected into the hemolymph (Ffrench- Constant *et al.*, 2003). There have been several reports of oral toxicity of symbiotic bacteria and their toxins. The live cells and secreted proteins of *Xenorhabdus nematophilus* were orally toxic to neonatal larvae of *Helicoverpa armigera* (Khandelwal and Banerjee-Bhatnagar, 2003). Toxins from entomopathogenic bacteria have a very wide spectrum of activity against soil organisms including plant parasitic nematodes (Samaliev *et al.*, 2000) and soil fungi (Chen *et al.*, 1994). Patents for the use of toxins from *X. nematophila* against various insect pests have been issued i.e., for the control of fire ants (Dudney, 1997), for *Pieris brassicae* (Jarrett *et al.*, 1997) and for several insect species by various species of *Xenorhabdus* (Ensign *et al.*, 2002). Brown *et al.*, (2004) showed that *X. nematophila* had an active secretion system and others (Ffrench-Constant *et al.*, 2000; Duchaud *et al.*, 2003) have shown that genome of *P. luminescens* contains many proteins predicted to be involved in secretion systems. The Tc toxins in *P. luminescens* have a high level of redundancy, where knockout of any one of the *tc* gene products causes a reduction but not a loss of toxicity (Bowen *et al.*, 1998; Duchaud *et al.*, 2003). Brown *et al.*, (2006) reported Txp40, a ubiquitous insecticidal toxin protein from *Xenrhabdus* bacteria, which occurs widely and highly conserved toxin in these bacteria. Txp 40 is important for the broad insecticidal activity and is a significant component of the extensive array of toxins that the bacteria and nematodes use to destroy their insect hosts. These toxins are proteins, excreted by the bacteria. (Lonne *et al.*, 2005) proved that the thrips were killed after

sucking from leaves covered with the toxins. Toxins have a negative effect on thrips fecundity. Mahar *et al.*, (2008) said that direct application of either cell solutions or cell free-filtrates from symbiotic bacterium *Xenorhabdus nematophila* can control insects on leaves and in soils. Larvae of the beet armyworm (*Spodoptera exigua*), diamondback moth (*Plutella xylostella*), black vine weevil (*Otiorhynchus sulcatus*) and nymphs of desert locust (*Schistocerca gregaria*) were killed by both cell treatments and cell-free filtrates, which were equally effective (Mahar *et al.*, 2008)Therefore, the toxin present in both treatments was responsible for the lethal effects. Cells of *X. nematophila* recovered from the haemocoele of all treated insects, indicating that cells were able to move from the external environment and enter insects in the absence of the nematode vector.

Brown *et al.*, (2004) described that there is still some debate on the species classification of *Xenorhabdus*, but because the invertebrates evolved before vertebrates, it is possible that theses insect pathogens are the ancestors of the animal pathogen. It is also clear that *Xenorhabdus* may be potentially rich resource for the discovery of additional virulence mechanisms and proteins with other novel bioactivities. *Steinernema carpocapsae* carries the bacterium *X. nematophila* into insects were exempted from normal pesticide legislation (Kaya and Gaugler, 1993). Ansari *et al.* (2003) showed that *P. luminescens* and *X. bovienii* were more virulent to *G. mellonela* and *Hoplia phylanthus* than were other bacterial species. Bussaman *et al.*, (2009) proved that the pathogenicity of six the entomopathgenic bacteria *Photorhabdus* and *Xenorhabdus* resulting in 83 to 85% of mushroom mite *Luciaphorus pernicious* within 3 days after application. (Bussaman *et al.*, 2006) reported that bacterial cultures of *L. pernicious* subsp. *laumondii* strain GPs11 were more virulent against *L. pernicious* female mites a *X. nematophila* strains Thai and All, isolated from *Steinernema thailandens* and *S. carpocapsae*, respectively.

The toxins produced by entomopathogenic bacteria are essentially insecticides and probably require full acute toxicity studies before they can be used commercially (Akhurst and Smith, 2002). Therefore, treatments of insect pests with *X. nematophila* bacterial cells or cell-free filtrates require further studies before being used in the field.

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استخدام *Xenorhabdus* Sp عزلة مصرية كمبيد حيوي

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في هذه الدراسة تم استخدام عزلتين إحداهما معزولة من النيماتودا المحلية *Steinernema* sp. والاخرى من سلالة *S. riborvae* المعزولة والمعرفة والتى تم الحصول عليها من جامعة فلوريدا بالولايات المتحدة الامريكية ، تم تعريف العزلتين اعتمادا على خواصهما المورفولوجية, المزرعية و البيوكيميائية الى جانب التاثير الممرض على *G. mellonella* كذلك تم استخدام تقنية 16S rRNA لتاكيد تعريف العزلتين . تم دراسة النشاط المضاد للميكروبات للعزلتين بتنميتهما على بيئات تخمرمختلفة واستخدمت تقنية Disc Diffusion Technique لدراسة هذا التاثير على مجال واسع من الميكروبات السالبة والموجبة لجرام الى جانب الخمائر . وقد اظهرت العزلتين تأثير سمي على دودة ورق القطن والدودة القارضة وكانت نسبة الموت عالية خلال 72 بعد المعاملة .