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Acaricidal and antibacterial activities of *Xenorhabdus stockiae* on different culture conditions

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Abstract

Xenorhabdus stockiae is an obligate symbiont bacterium of the entomopathogenic nematode, *Steinernema siamkayai* Stock, Somsook and Reid, and it has been previously shown to have acaricidal and antibacterial activities. In this study, different media were evaluated for enhancing the acaricidal and antibacterial activities of *X. stockiae*. Luria Bertani broth (LB), tryptone soyptone broth (TSB) and modified yeast extract broth (YSG) were used for growing *X. stockiae* for 24, 48 and 72 h. The maximum acaricidal activities against *Luciaphorus perniciosus* Rack, mushroom mite, were found after application with cell-free supernatant of *X. stockiae* grown on TSB medium for 48 h (88.50±1.00%) and 72 h (86.00±3.26%) followed by cell suspension grown on TSB for 48 h (85.00±2.00%). The surviving female mites were found to produce significantly less number of offspring, accounting for 113.50±10.15, 120.50±5.92 and 127.00±7.87 offspring/gravid female respectively, when compared to the control treatment (distilled water) that resulted in 312.75±11.02 offspring/gravid female. The culture supernatant of *X. stockiae* grown using TSB medium for 48 h was shown to have maximum antibacterial activities, particularly against *Staphylococcus epidermidis* and *Klebsiella pneumoniae.* This study suggested that TSB medium was found to be the optimum medium for cultivation of *X. stockiae* to enhance its acaricidal and antibacterial activities.

Keywords: *Xenorhabdus stockiae, Luciaphorus perniciosus,* mushroom mite, acaricidal activity, antibacterial activity

1. Introduction

Xenorhabdus spp. are a gram-negative bacteria, which belong to the family Enterobacteriaceae and are symbiotically associated with the entomopathogenic nematodes in the genus *Steinernema* (Steinernematidae) (1). When this entomopathogenic nematode infests insects, it releases *Xenorhabdus* sp. into the insect's haemocoel which results in bacterial septicemia and death within 48 h (2, 3). This bacterium has been found to produce a variety of metabolites that can help the nematode to destroy the insect's immune system. The metabolites of *Xenorhabdus* spp. include enzymes, such as proteases, lipases, phospholipases that help to generate a food supply during reproduction (4) and also antifungal and antibacterial agents that can preserve the host carcasses, while the bacteria and the nematodes reproduce (2, 5). *Xenorhabdus* spp. have been known to produce xenorhabdin (6), xenocoumacins (7), nematophin (8) and benzylineacetone (9) that have high activities against gram-positive and gram-negative bacteria. The pathogenic potential of this bacteria-nematode complex has been used for controlling a wide range of agricultural pests (10). As *Xenorhabdus* spp. can be grown successfully under laboratory conditions, both of its cell suspension and cell-free supernatant have been used for controlling several insect pests, such as larvae of diamondback moth (*Plutella xylostella* (Linnaeus)), black vine weevil (*Otiorhynchus sulcatus* (Fabricius)), nymphs of desert locust (*Schistocerca gregaria* (Forskal)) (5), beet armyworm (*Spodoptera exigua* (Hűbner)) (8, 11, 12), tobacco hornworms (*Manduca sexta* (Linnaeus)) (13), cricket (*Gryllus firmus* (Scudder)) (14) and forest tent caterpillar moth (*Malacosoma disstria* (Hűbner)) (15). In addition, *Xenorhabdus* bacterium has recently been used as a biological control agent against *L. perniciosus* mite that is endemic in Thailand (16, 17, 18).

Mushroom mite, *L. perniciosus* (Acari: Pygmephoridae), is regarded as a destructive pest in the commercial production of *Lentinus squarrosulus* (Mont.) Singer, *L. polychrous* Lev., *Auricularia auricula* (Bull.) Wettst. and *Flammulina velutipes* Karst (19). This Pygmephorid mite can infest the sawdust spawn that is used for mushroom cultivation and subsequently cause serious damages of the mushroom mycelia and sporophores (20). The chemical insecticides, such as carbamate and organophosphate, and some organic solvents have been used for controlling this mite, but with only little success because this mite tends to have extensive widespread and increasing resistance to such insecticides. Also, due to the concern regarding serious toxicity of the insecticides to a wide spectrum of organisms and environments, there are several attempts to search for the safe biological control measures for this mite (16).

The aim of this study was to examine the effects of different culture media on *X. stockiae* acaricidal activities against adult female *L. perniciosus* mites and antibacterial activities against both gram positive and negative pathogenic bacteria.

2. Materials and Methods

2.1 Bacteria, mushroom and mite cultures

Xenorhabdus stockiae bacterium has been isolated from the symbiotic nematodes (*Steinernema siamkayai*) obtained from the Department of Agricultural, Ministry of Agriculture and Cooperatives using a method described by Kaya and Stock (21). This bacterium was maintained to be in phase I throughout the study. To ensure the phase I, the bacteria were incubated in the dark at 28^{o}C for 24 h on nutrient agar supplemented with 0.004%(w/v) triphenyltetrazolium chloride and 0.0025%(w/v) bromothymol blue (NBTA). The bacterial phase I could be distinguished from phase II by its adsorption of bromothymol blue and forming of blue colonies on NBTA

(22). The seed culture was prepared by inoculating a loopful of phase I colonies from NBTA plate into a 250 ml flask containing 100 ml of Luria Bertani (LB) broth (Sigma-Aldrich, USA) and incubating in incubator shaker (200 rpm) at 28°C for 24 h in the dark (18).

Lentinus squarrosulus, mushroom was obtained from the Mushroom Researchers and Growers Society of Thailand. The mushroom mycelium was cultured on potato dextrose agar (PDA) plate and incubated at 25°C. The mushroom was also grown on the mixture of sawdust and sorghum grain to establish fresh spawn (18).

Luciaphorus perniciosus mites were collected from the infested basidiocarps obtained from the Rapeephan mushroom farm in Khon Kaen province in the northeast of Thailand. The mushroom mites were cultured using *L. perniciosus* spawn in glass bottles and maintained at 28°C for facilitating their reproduction. These in-house bred mites in the glass bottles were used for all of the experiments.

2.2 Culture media for *X. stockiae* cultivation

The media, including Luria Bertani broth (LB) (tryptone, 10.0 g/l; yeast extract, 5.0 g/l; NaCl, 10.0 g/l), tryptone soyptone broth (TSB) (tryptone, 17.0 g/l; soyptone, 3.0 g/l; glucose, 2.5 g/l; NaCl, 5.0 g/l) and modified yeast extract broth (YSG) (glycerol 5.0, yeast extract 5.0, 1 M $MgSO_4$ 5.0 ml, $(NH_4)_2SO_4$ 2.0, 1 M KH_2OP_4 5.0 ml, 1 M K_2HOP_4 5.0 ml and 1 M Na_2SO_4 10.0 ml) were used for *X. stockiae* cultivation. The seed culture of *X. stockiae* (10 ml) was transferred to each of these media (100 ml) in 250 ml flasks and incubated in the dark at 28°C on a rotary shaker at 200 rpm for 24, 48 and 72 h. Each aliquot of the resulting culture broths was centrifuged (10,000 rpm, 20 min, 4°C) and filtered using 0.22-µm syringe filter to separate the bacterial cells from the supernatants. The supernatants were store at 4°C until required.

2.3 Measurement of bacterial cell growth

The growth of bacterial cells was measured by optical density of the cultures at 600 nm and their biomass concentration (g dry cell weight/l) was determined using a calibration curve as described by Wang *et al.* (23). The calibration curve was initially plotted using dilutions of cell suspension of the bacteria and known optical density. A fixed volume of each bacterial dilution was centrifuged at 10,000 rpm for 20 min and the cell pellets were dried at 110°C for 24 h weighted, and finally used for converting the optical density value into dry cell weight (g/l).

2.4 Determination of acaricidal activities

The acaricidal activities of *X. stockiae* were evaluated as previously described by Bussaman *et al.* (18). One hundred adult female *L. perniciosus* mites were transferred to a 50-mm Petri dish plate containing mushroom mycelium growing on PDA medium and then sprayed with 500 µl of each bacterial cell suspensions or supernatants. Sterile distilled water (DW) and commercial miticide (0.005% Omite) in the same volume were used as control groups. The experiments were performed in four replicates. All plates were incubated in the growth chamber at 28°C and 80% relative humidity in the dark. The mortality of mites was recorded daily for 5 days after application with bacterial suspensions or supernatants. The survival female mites were used for evaluating the reduction of their progeny (after 5 days). The pregnant female mites were excised by using a needle and examined for the number and the sex ratios of their progeny.

2.5 Measurement of antibacterial activities

The paper disk diffusion plate assay was used for measurement of antibacterial activities of *X. stockiae* supernatants against gram-positive (*Staphylococcus epidermidis, Staphylococcus pyogenes* and *Bacillus subtilis*) and gram-negative (*Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli*) test bacteria. Briefly, 100 µl of test bacteria (containing 10^6 cfu/ml) were spread onto each NA plate and left at room temperature for 2 h. After that, 6-mm paper disks filters (Whatman No.1) treated with 20 µl of *X. stockiae* supernatants were placed on the plates which were

incubated with bacteria and incubated at 30°C for 24 h. Commercial antibiotic (10 mg/ml Streptomycin) in the same volume was used as a control. The levels of antibacterial activities were measured by the size of diameter (mm) of the inhibition zone.

2.6 Statistical analysis

The data of mite mortality, the reduction of mite progeny and the size of inhibition zone were analyzed by one-way ANOVA. Significance differences between the treatments were compared using the LSD test at $p<0.05$.

3. Results and Discussion

3.1 Effects of different media on *X. stockiae* cell growth

The effects of three different media on *X. stockiae* cell growth were shown in Figure 1. The maximum dry cell weight was found when the bacteria was cultured for 48 h on TSB (11.90 g/l), followed by LB (10.10 g/l) and YSG (9.58 g/l). Therefore, TSB medium was found to be the optimum medium for harvesting biomass of *X. stockiae*.

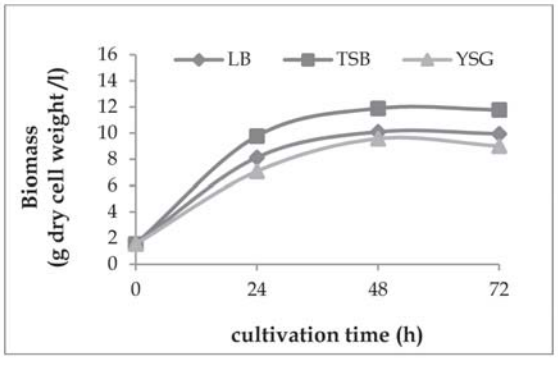

Figure 1. Biomass of *X. stockiae* after cultivating on LB, TSB and YSG for 72 h.

3.2 Acaricidal activities of *X. stockiae* cell suspension and supernatant

The effects of different media on acaricidal activities of *X. stockiae* cell suspensions and supernatants were shown in Table 1. The percentages of mite mortality were measured 5 days after applications with bacterial cell suspensions or supernatants. The highest mortality rates of *L. perniciosus* mites were found after application with *X. stockiae* cell free supernatant grown on TSB medium for 48 h (88.50±1.00%) followed by supernatant grown using TSB for 72 h (86.00±3.26%) and cell suspension grown using TSB 48 h (85.00±2.00%) (Table 1). The differences in cultivation times and source of bacterial culture could play important roles on mortality of mites. TSB medium was found to be the most optimal medium for bacterial growth with high biological activities when compared to LB and YSG medium. The bacterial culture that was grown for 48 h caused the highest mite mortality rates (78.00-88.50%), followed by 72 and 24 h (resulting in 78.00 – 86.00% and 40.00 – 57.00% mortality, respectively) (Table 1). Also, the cell suspensions of *X. stockiae* were found to induce *L. perniciosus* mite mortality rates at levels different from their supernatant counter parts. Indeed cell-free supernatants of *X. stockiae* induced mite mortality at higher rates than their cell suspensions (Table 1). Omite (0.005%) (positive control) caused the highest mite mortality (100%) and no dead mites were observed after application with distilled water (DW) (negative control). In previous report showed that the cell-free supernatants prepared from 48- and 72-h cultures of *Photorhabdus luminescens* ssp. laumondii GPS11 and GPS12 stains showed the highest mortality rates at 94 and 93% for GPS12 and 90 and 88% for GPS11, respectively (17). Furthermore, 48 h bacterial cultures of the *X. nematophila* (X1) and *P. luminescens* (P1), were shown to have strong virulence against the female mites, resulting in 85 and 83% mortality within 3 days after application, respectively (16). Moreover, Bussaman *et al.* (18) reported that 48 h cell-free supernatant of *X. stockiae* culture could result in both the highest mortality rate (89 %) and the lowest reproduction of female mites (41.33 eggs/gravid female).

Table 1. Mortality and fecundity of *L. perniciosus* mites after applications with cell suspensions and supernatants of *X. stockiae* grown on different media (LB, TSB and YSG) for 24, 48 and 72 h.

Media	Cultivation time (h)	Cell suspension			Cell free supernatant		
		Mite mortality (%, mean±SD)	Fecundity (offspring/ gravid female)	Male: female ratio	Mite mortality (%, mean±SD)	Fecundity (offspring/ gravid female)	Male: female ratio
LB	24	43.00±1.15^{g}	244.00±11.28^{d}	1:29.50	48.00±4.32^{g}	189.75±7.80^{d}	1:20.25
	48	81.00±2.00^{cd}	210.50±11.50^{e}	1:13.03	84.50±1.95^{cd}	170.00±8.29^{e}	1:13.88
	72	80.00±1.63^{de}	256.00±4.69^{c}	1:11.80	81.00±2.58^{de}	230.50±2.38^{c}	1:16.40
TSB	24	46.50±1.00^{f}	142.50±6.85^{f}	1:17.14	57.00±1.15^{f}	127.25±6.60^{f}	1:16.18
	48	85.00±2.00^{b}	127.00±7.87^{g}	1:10.21	88.50±1.00^{b}	113.50±10.15^{g}	1:7.50
	72	83.00±2.58^{bc}	131.75±7.41^{fg}	1:10.87	86.00±3.26^{bc}	120.50±5.92^{f}	1:9.32
YSG	24	40.50±1.91^{h}	234.00±7.53^{d}	1:27.36	50.00±3.27^{g}	261.50±14.64^{b}	1:29.76
	48	78.00±1.63^{e}	258.50±6.14^{c}	1:11.92	81.00±2.58^{de}	239.50±13.23^{c}	1:16.74
	72	78.00±2.58^{e}	287.00±9.20^{b}	1:16.94	79.00±5.03^{e}	254.00±12.52^{b}	1:14.87
Control	DW	0.00±0.00^{i}	312.75±11.02^{a}	1:31.08	0.00±0.00^{h}	312.75±11.02^{a}	1:31.08
	Omite	100±0.00^{a}	0.00±0.00^{h}	0:00.00	100±0.00^{a}	0.00±0.00^{h}	0:0.00

Data followed by the different letters in the same row were significantly different (p<0.05) as compared by LSD
DW = distilled water; Omite = commercial acaricide (0.005%)

The reproduction of *L. perniciosus* mites was found to decline after application with *X. stockiae* cell suspensions or supernatants (Table 1). Both cell suspensions and cell-free supernatants of *X. stockiae* grown on TSB medium could significantly reduce mite fecundity when compared to the other treatments. After treatment with cell-free supernatant of *X. stockiae* grown on TSB medium for 48 h, the survival female mites were found to produce significantly less number of the offspring, accounting for 113.50 offspring/gravid female, whereas the females in control treatment (DW) produced 312.75 offspring/gravid female. Also, the offspring of surviving female mites of the same experiment was found to have the lowest ratio of male: female at 1:7.50, while the control treatment (DW) resulted in a ratio of 1:31.08 (Table 1). This study also showed that *X. stockiae* affected the number of progeny and the ratio between female and male offspring in similar fashion to the previous studies (16, 18).

3.3 Antibacterial activities of *X. stockiae* supernatants

The results of antibacterial activities of *X. stockiae* supernatants grown on different media were shown in Table 2. The supernatants of *X. stockiae* grown in all types of media for 24 h were found to have no activities against *S. pyogenes, P. aeruginosa* and *E. coli*. However, the supernatants of *X. stockiae* that were cultured for 48 h were shown to have maximum antibacterial activities against all pathogenic bacteria. Moreover, *S. epidermidis* was shown to be much more sensitive to *X. stockiae* supernatants than other pathogenic bacteria. In our study TSB was found to be the best medium for antibacterial activity as compared to LB and YSG medium. In a previous study, TSB medium was found to be the optimum medium for harvesting biomass and increasing antibiotic activities of *X. nematophilia* TB (24). Fugani *et al.* (25) determined the antibiotic activity of *X. nematophilla, X. szentirmaii* and *X. budapestensis* on mastitis bacteria and showed

that *S. aureus* had the highest sensitivity, *K. pneumonia* was the last susceptible. Furthermore, *Xenorhabdus* spp. have been known to produce broad-spectral antibiotics including xenorhabdin (6) and xenocoumacin (7) which were highly active against gram-positive bacteria, *Streptococcus*, *Staphylococcus* and *Bacillus* species. Moreover, benzylidenacetone is also active against gram-negative bacteria, *Pseudomonas syringae, Pectobacterium carotovorum, Agrobacterium vitis and Ralstonia solanacearum* (9). Thaler *et al.* (4) reported that *Xenorhabdus* spp. might have entomopathogenic properties by producing a variety of metabolites, including the enzymes such as proteases, lipases, phospholipases to generate a food supply during reproduction. In addition, Akhurst and Dunphy (2) and Mahar *et al.* (5) suggested that this bacterial could produce the antifungal and antibacterial agents to prevent the contamination of host carcass due to other microorganisms.

In this study, TSB medium containing carbon source (glucose) and nitrogen source (tryptone and soyptone) was found to result in maximum cell growth and secondary metabolites production. Similar results were obtained from the study of Wang *et al* (23) which reported that glucose and tryptone were optimum carbon and nitrogen sources for cell growth and antibiotic production by *Xenorhabdus nematophila* YL001. However, Fang *et al* (24) working with *Xenorhabdus nematophila* TB, also found that glucose and peptone had the strongest effect on antibiotic activity. In addition, to the best of our knowledge, this study is the first to describe the influence of carbon and nitrogen sources on the acaricidal activities of *X. stockiae* and there are limited information regarding this notion. It was noticed that *Xenorhabdus* had antibacterial activities increased with acaricidal properties. Further experiments therefore remain to be determined to explore regarding the correlation between *Xenorhabdus*'s antibacterial and acaricidal activities and factors affecting them.

Table 2. Antibacterial activities of *X. stockiae* supernatants grown on different media (LB, TSB and YSG) for 24, 48 and 72 h.

Media	Cultivation time (h)	Diameter of inhibition zone (mm, mean±SD)					
		S. epidermidis	*S. pyogenes*	*B. subtilis*	*P. aeruginosa*	*K. pneumoniae*	*E. coli*
LB	24	7.75 ± 0.96^{eA}	-	6.25 ± 0.50^{dB}	-	7.00 ± 1.14^{bcAB}	-
	48	9.00 ± 1.15^{bcdA}	6.75 ± 0.96^{bB}	7.00 ± 0.82^{cdB}	7.25 ± 0.50^{bB}	7.75 ± 0.50^{bcB}	7.25 ± 0.50^{bB}
	72	9.00 ± 1.15^{bcdA}	6.75 ± 0.50^{bB}	7.00 ± 0.00^{cdB}	7.25 ± 1.50^{bB}	7.75 ± 0.50^{bcAB}	7.25 ± 0.96^{bB}
TSB	24	9.25 ± 0.50^{bcdA}	-	7.00 ± 0.82^{cdB}	-	6.75 ± 0.96^{bcBC}	-
	48	10.00 ± 0.00^{bA}	7.00 ± 0.00^{bB}	8.00 ± 0.82^{bB}	8.00 ± 0.00^{bB}	8.00 ± 1.15^{bB}	8.00 ± 0.00^{bB}
	72	9.50 ± 0.58^{bcA}	6.75 ± 0.50^{bC}	7.75 ± 0.50^{bcB}	7.75 ± 0.50^{bB}	7.75 ± 0.50^{bcB}	7.75 ± 0.50^{bB}
YSG	24	8.25 ± 0.50^{deA}	-	6.75 ± 0.50^{dB}	-	6.75 ± 0.50^{bcB}	-
	48	9.00 ± 0.82^{bcdA}	6.75 ± 0.96^{bC}	7.00 ± 1.15^{cdBC}	7.00 ± 0.82^{bBC}	7.00 ± 0.00^{bcBC}	7.50 ± 1.00^{bB}
	72	8.75 ± 0.96^{cdeA}	6.75 ± 0.50^{bB}	7.00 ± 0.00^{cdB}	7.00 ± 1.15^{bB}	7.00 ± 1.15^{bcB}	7.25 ± 0.50^{bBC}
Streptomycin		18.50 ± 0.58^{aA}	15.00 ± 0.82^{aB}	13.00 ± 0.82^{aC}	11.00 ± 0.82^{aD}	14.00 ± 0.82^{aBC}	13.00 ± 0.82^{aC}

Data followed by the different lower case letters in the same column were significantly different ($p<0.05$) as compared by LSD
Data followed by the different upper case letters in the same row were significantly different ($p<0.05$) as compared by LSD
- no zone of inhibition

4. Conclusion

In conclusion, the results in this study demonstrated that TSB medium could improve the acaricidal and antibacterial activities of *X. stockiae.* Morover, 48 h could be the most suitable time for cultivation of *X. stockiae* to obtain the metabolites with high biological activities. Further studies are remained to determine the optimum carbon and nitrogen sources, for cultivation of *X. stockiae,* particularly in large scale settings.

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