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(RESEARCH ARTICLE)



Effect of isolated bacteria and their supernatant from American cockroach's ootheca on its hatching as a safety method for control in Jeddah governorate

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Abstract

Periplaneta americana is one of the hygienic pests and is considered a vector of several human diseases. They transfer pathogens biologically by vertical transmission from the mother to embryos through egg cases (ootheca). Forty types of bacteria were isolated from different parts of *P. americana* ootheca to study their effects on ootheca hatching. The objective of this study was to investigate the pathogenicity of isolated *Bacillus subtilis* and their free cells to demonstrate their mechanism of action. Positive effects of bacterial cells and their free-cells on ootheca hatching were observed. Bacterial cells and their free cells were given to the ootheca by sparing method for 1, 2, 3 and 4 times in a month with different concentrations. The results showed that treatments of high concentrations of bacterial cells and their free cells had a significant effect on the mortality of all treated ootheca for four times as compared to the control. Also, high concentrations of each treatment were exhibit high shrinkage, dehydrated and rigid. The present study reveals that the toxicity of bacterial cell of *B. subtilis* has great potential more than free-cells for biological control against *P. americana* ootheca.

Keywords: *P. americana*; Ootheca; Hatching; Isolated bacteria; Hatchability; Toxicity

1. Introduction

American cockroaches (*Periplaneta americana*) (Dyctioptera: Blattidae) are one of the most important insects in medicine [1]. They are among the most abundant and obnoxious insect pests [2]. *P. americana* live in warm environment and in unfavorable area for human [3].

P. americana can transmit many pathogenic microorganisms [4]. They play effective role in diseases transmission either mechanically or biologically [5]. *P. americana* live in sewage, sewer pipe which contain high density of pathogens [6]. *P. americana* habits make them ideal carriers serious pathogenic microorganisms [7]. Cockroaches spread pathogens on their cuticle, as their nymphal cuticle is removed or as they lose body parts [8]. All of these pathogens used as bioterrorism agents attack animal or human populations which they are all harmful to them [9].

Serious problems of increasing costs of using synthetic pesticides have pointed the need for using biological insecticides [10]. Microbial insecticides do not affect humans and other organisms [11]. Bacteria, viruses, fungi, nematodes and protozoa are main groups of entomopathogens which used to control insect pests in the field [12].

Bacteria are easily and cheaply produced [13]. Different types of entomopathogenic bacteria were affected against different groups of insect pests, according to their toxin, such as *Bacillus thuringiensis* var. *kurstaki* which effect on

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caterpillars. *Bacillus thuringiensis* var. *israelensis* was effected against flies and *Bacillus thuringiensis* var. *san diego* eas effected against larvae of beetles [14].

Not much is known about the contagion of *P. americana* ootheca via bacterial cells and free-cells extracted from their egg cases, as biological pesticides, but discovering a new way for controlling *P. americana* ootheca can bring new methods of controlling, since there are no insecticides are effective when applied topically on oothecal phase and it is important to inhibit embryogenesis of *P. americana* to prevent them from hatching.

2. Material and methods

2.1. Insects collecting and rearing

Insects collecting and rearing Lab strain were used in this study. Large population of *P. americana* was collected from sewers from Al- Aziziah District, Jeddah province, kingdom of Saudi Arabia by using glass jars covered with dark cloth according to the method of [15]. Glass gars were placed into sewers. Cockroaches were gathering every two days and transferred in glass containers (30 × 60 × 30 cm). The collected adults and nymphs were separated in another glass containers, nymphs were maintained until they reach adult stage. Containers were coated with petroleum jelly 2 cm from the inside inner top and provided with wetted cotton, dry dog food, cardboard and dry cotton for collecting more of ootheca. The cockroaches were then kept under the laboratory condition of 25 ± 3 °C and 75 ± 5% RH. To get the lab strain of *P. americana*, ootheca were daily collected from glass rearing containers and removed to another one until they reach adult stage.

2.2. Ootheca collecting

After 7 days, ootheca were collected and removed to plastic container (3x5 cm) to make sure that nothing affects them and can be used for the experiments.

2.3. Preparation of nutrient agar medium

To make a nutrient agar medium, 6.8 gram of nutrient agar powder was added to 250 ml of distilled water and autoclaved at 121 °C. After overnight incubation of plates, samples from the liquid media is inoculated into the nutrient agar plates [16].

2.4. Isolation of bacteria from inside and outside of ootheca

Bacteria were isolated from inside and outside the ootheca and cultured on nutrient agar. In the isolation from Outside of ootheca, 100 ootheca were frozen individually at 0 °C for 10 minutes then collected in test tube (15 ml) according to the [17]. 2 ml of normal saline and distilled water separately was added to both tubes and tested samples were shaken for 2 minutes. 100 µl of the wash was cultured by spreading (using spreader) on nutrient agar. In the isolation from Inside of ootheca, 100 ootheca were sterilized, opened with sterilized needles and a drop of the hemolymph was smeared onto nutrient agar plates. The plates were incubated at 30 °C for 48 hours. Bacterial colonies were then sub-cultured until pure colonies were obtained according to the method of [18]. The isolated bacteria then were kept in stock culture for further experiments in the Microbiology Laboratory Department Faculty of Sciences – King Abdul-Aziz University (KAU), Jeddah Province, Saudi Arabia.

2.5. Biochemical reactions of bacterial isolates

Identifications of bacterial isolates were morphological and biochemical tests using Analytical profile index test (API-20E test strip). A bacterial suspension is used to rehydrate each of the wells.

2.6. Preparing of bacterial solution

A single colony from each isolated bacterial was selected and inoculated into 50 ml of nutrient broth and incubate in shaking incubator for 48 hours at 30 °C. The concentration of bacterial cells in nutrient broth were estimated using the number of cells which was adjusted to 4 × 10⁷ cells Ml⁻¹, a concentration that had been showed to be effective against ootheca. To get bacterial free-cells solutions containing toxic metabolites, the suspension was filtrated through a bacterial filter (with pore size of 0.2 µm). Different concentrations of suspension were used by adding distilled water to each one.

2.7. Mode of treatment of ootheca with bacteria and their supernatant

The experimental ootheca were divided into four groups. Ootheca in group 1 were not treated with anything known as positive control. Ootheca in group 2 were treated with distilled water known as negative control. Group 3 were treated with bacterial cells, and group 4 were treated with bacterial free-cells. The experimental design was completely randomized for ootheca, with a total of 120 individuals per experiment (30 ootheca in each group) with three replications. Treated ootheca were cleaned with deionized water. 100 µl of bacterial cells and bacterial free-cells was applied using hand sprayer. Specimen were stored in plastic containers (5 x 3 cm) with moistened cotton at 25 ± 3 °C in the dark, RH \geq 80%. Mortality was checked daily for 30 days.

3. Results

In the present study, *B. subtilis* cells and their free-cells were isolated from different parts of *P. americana* ootheca and tested against their hatching percentage.

3.1. Isolation and identification of bacteria

10 species of Gram-negative and Gram-positive bacteria were isolated from outside of the *P. americana* ootheca (*B. subtilis*, *Micrococcus* spp, *Clostridium* spp, *Enterobacter*, agglomerans, *Aeromonas sobria*, *Vibrio metschnikovii*, *Enterobacter cloacae*, *Xantho maltophilia*, *Proteus vulgaris*, *Rahnella aquatilis*, *Sphomon paueimobilis*, *Pseudomonas* spp). 12 species of Gram-negative and Gram-positive bacteria were isolated from outside of the *P. americana* ootheca (*Enterobacter cloacae*, *Enterobacter amnigenus*, *Chryseomonas luteola*, *Micrococcus* spp, *Proteus pemmri*, *B. subtilis*, *Enterobacter sakazakii*, *Weeksella virosa*, *Pseudomonas* spp, *Aeromonas sobria*)

3.2. Effect of *B. subtilis* on *P. americana* ootheca hatching

Isolated bacteria and their free-cells of *B. subtilis* from *P. americana* ootheca were able to prevent oothecal hatching. We investigated that high shrinkage, dehydrated and rigid were topically highest when the bacterial cells and free-cells reached the stationary phase (4×10^7), respectively. Hatching percentage of ootheca was depending on treatment times by spraying on cuticle. Treatments with cells solution and free-cells for four times in a month had significant effect of all treated ootheca on their hatching as compared to control.

The required values, i.e. IT_{50} and IT_{90} are presented in (Fig. 1). The results clearly showed that bacterial cells of *B. subtilis* were effective insecticide, the time per week that inhibit 50 % of nymphs emergence of the exposed ootheca (2.1 week), whereas the time per week that inhibit 90% of nymphs emergence of the exposed ootheca was 4.6 weeks. Hatchability of *P. americana* ootheca treated with bacterial cells were decreased by increasing the time (weeks) as in Table 1.

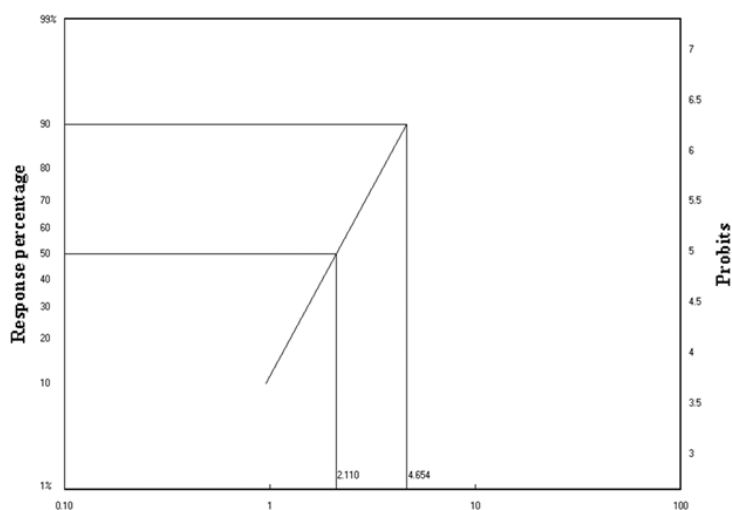


Figure 1 IT_{50} and IT_{90} of *P. americana* ootheca treated with bacteria cells of *B. subtilis*

Table 1 The effect of treatment of *P. americana* ootheca with the *B. subtilis* bacterial cells on hatching

Treated time per month	Ootheca hatchability (%)*		Decrease in hatchability (%)
	Bacterial free-cells treated	Control	
1	86.66	100	13.34
2	60.00	100	40.00
3	24.44	100	75.56
4	0	100	100

* 3 replicates, 10 ootheca of *P. americana* in each.

Bacterial free-cells of *B. subtilis* showed a high level of insecticidal activity. Ootheca of *P. americana* was inhibited when treated with four times in a month. Hatchability was decreasing when free-cells concentration was increased. 25% caused the highest mortality percentage for *P. americana* ootheca (100%) while, 0.1% caused the lowest mortality percentage 95.55% when treated for one time (Table 2). The concentration that inhibit 50 % of nymph's emergence of the exposed ootheca when treated for one time in a month was 7.4 % (Fig. 2).

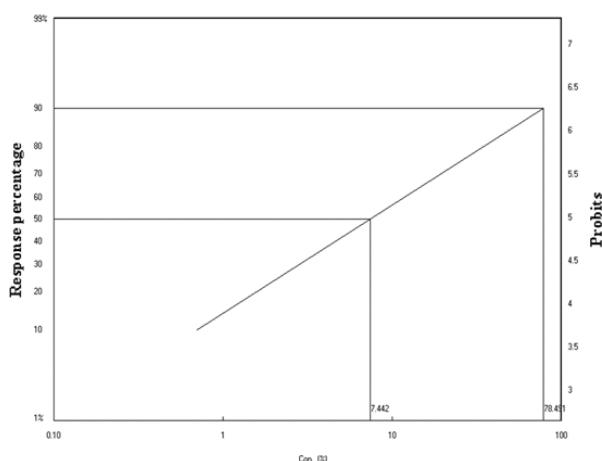


Figure 2 LC₅₀ of *P. americana* ootheca treated with bacteria free-cells of *B. subtilis* for one time in a month

Table 2 The effect of treatment of *P. americana* ootheca with *B. subtilis* bacterial free-cells on hatching when treated with one time in a month

Concentrations (%)	Ootheca hatchability (%)*		Decrease in hatchability (%)
	Bacterial free-cells treated	Control	
0.05	100	100	0
0.1	95.55	100	4.45
0.5	90.88	100	9.12
1	86.44	100	13.56
3	80.44	100	19.56
5	69.11	100	30.89
7	56.44	100	43.56
10	44.22	100	55.78
15	28.44	100	71.56
20	17.11	100	82.89
25	0.00	100	100

* 3 replicates, 10 ootheca of *P. americana* in each.

Insecticidal activity of *B. subtilis* free-cells on ootheca treated for two times was recorded in Table 3 and Fig. 3. There was positive correlation between concentrations and treatment times. Spraying twice causing decreasing in hatchability and high significant of 0.1 % to 19.56%. Using concentrations range of 0.05-10% of *B. subtilis* free-cells against *P.*

americana ootheca for three times showed highly significant decrease in hatchability concentrations increased. The lowest concentration was recorded at 0.05% which caused 21.78 %. The highest concentration of 10% caused 0% of hatchability (Table 4). Fig. 4, showed the LC₅₀ value and slope of treated *P. americana* ootheca for three times with *B. subtilis* free-cells was 0.48%.

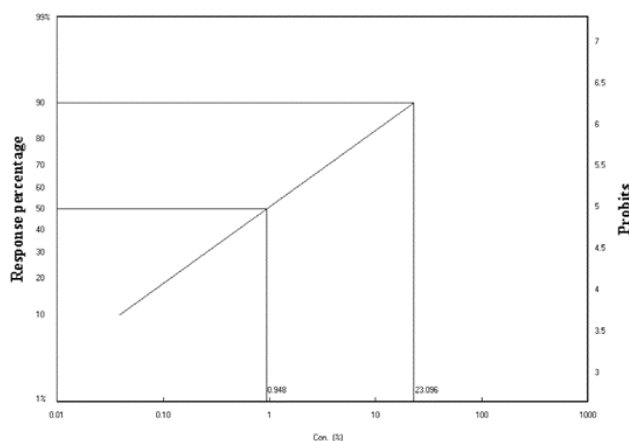


Figure 3 LC₅₀ of *P. americana* ootheca treated with bacteria free-cells of *B. subtilis* for two times in a month

Table 3 The effect of treatment of *P. americana* ootheca with *B. subtilis* bacterial free-cells on hatching when treated two times in a month

Concentrations (%)	Ootheca hatchability (%)*		Decrease in hatchability (%)
	Bacterial free-cells treated	Control	
0.05	88.88	100	11.11
0.1	80.44	100	19.56
0.5	60.22	100	39.78
1	47.11	100	52.89
3	36.88	100	63.12
5	28.44	100	71.56
7	15.77	100	84.23
10	0.00	100	100

* 3 replicates, 10 ootheca of *P. americana* in each.

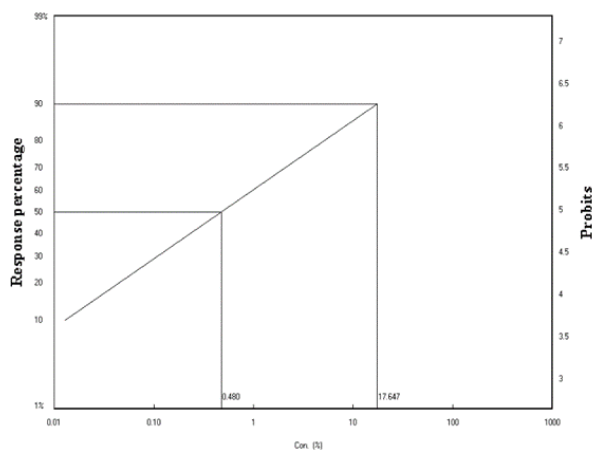


Figure 4 LC₅₀ of *P. americana* ootheca treated with bacteria free-cells of *B. subtilis* for three times in a month

Table 4 The effect of treatment of *P. americana* ootheca with *B. subtilis* bacterial free-cells on hatching when treated three times in a month

Concentrations (%)	Ootheca hatchability (%)*		Decrease in hatchability (%)
	Bacterial free-cells treated	Control	
0.05	78.22	100	21.78
0.1	66.88	100	33.12
0.5	58.44	100	41.56
1	40.22	100	59.78
3	24.88	100	75.12
5	17.11	100	82.89
7	0	100	100

* 3 replicates, 10 ootheca of *P. americana* in each.

3.3. Comparison between the susceptibility of *P. americana* ootheca to bacteria free-cells of *B. subtilis* treated with different times

Susceptibility of *P. americana* ootheca to *B. subtilis* bacteria free-cells treated for different times in a month is illustrated by figure 5. *P. americana* ootheca treated for three times was more affected to *B. subtilis* bacteria free-cells by 7.4%, than *P. americana* ootheca treated for two times by 0.9%, followed by *P. americana* ootheca treated for one time by 0.4%.

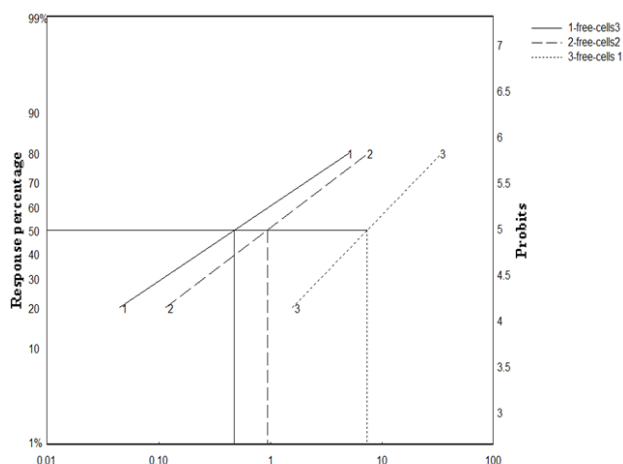


Figure 5 Comparison between the susceptibility of *P. americana* ootheca to bacteria free-cells of *B. subtilis* treated with different times

In respect to the slope values, it can be observed from Table 5 that *P. americana* ootheca treated for one time had higher degree of homogeneity for the susceptibility to bacteria free-cells of *B. subtilis* by slop values (7.442), than those resulted from ootheca treated for two times (0.948). *P. americana* ootheca treated for four times exhibited the lowest degree of homogeneity by the lowest slope (0.819).







Table 5 Comparison between the susceptibility of *P. americana* ootheca treated with different times of *B. subtilis* bacterial free-cells in a month

Treated times	Slope	LC ₅₀ (%)	95% Confidence	
			Lower	Upper
1	1.253	7.442	4.764	13.246
2	0.924	0.948	0.734	1.224
3	0.819	0.48	0.351	0.649

3.4. Effect of *B. subtilis* on *P. americana* ootheca morphology










Comparison between the susceptibility of different treatment times of *P. americana* ootheca with different concentrations of *B. subtilis* bacterial and free-cells indicated in Table 6 and 7. We investigated that high shrinkage, dehydrated and rigid were typically highest when the bacterial cells and free-cells reached the stationary phase and high concentration, respectively.

Table 6 Comparison between the susceptibility of different treatment times of *P. americana* ootheca with *B. subtilis* bacterial cells

Treatment time	Negative Control	Positive Control	1	2	3	4
Oothecal morphology						

In one treatment, high concentrations caused more effects than low concentrations and in one concentration, treatment time effect more than one time. Treated *P. americana* ootheca with 0.05% of *B. subtilis* bacterial free-cells for one time were less shrinkage, dehydrated and rigid. On the other hand, treated *P. americana* ootheca with bacterial free-cells with 0.05% for three times caused more effects more than ootheca treated for one time (Table 7). Hatching percentage in positive and negative control were 100% for each ootheca.

Table 7 Comparison between the susceptibility of *P. americana* ootheca with *B. subtilis* bacterial free-cells treated for different times

Concentrations	Oothecal morphology		
	After one treatment	After two treatments	After three treatments
0.05			
1			
7			

4. Discussion

The pathogenic ability of many insecticidal bacteria depends on toxins that is present in their genome [19]. However, the mode of action of these toxins are not understood [20]. Not much is known about *B. subtilis* effects and their mode of action as a biocontrol agent on insects and especially on oothecal phase. In our study, we found that isolated bacteria *B. subtilis* from different area of *P. americana* ootheca and their supernatant were the reason for the ootheca mortality. Higher effects through spraying mode might be due to the penetration of bacteria and their toxins into the ootheca. Mortality in *P. americana* ootheca might be because that, the toxins action of bacteria and their supernatant secreted by the bacteria during the treatment infection of egg cases. Reactive oxygen species (ROS) induces oxidative stress by causing cellular damage, which are acted upon by the enzymes of the antioxidant system [21]. Similar results have been

observed by [22], who suggest that *H. thompsonii* causes oxidative damage in cockroaches, probably by generating reactive-oxygen stress in their bodies, similar findings for reaction oxygen species has also been observed by [23]. In the present study, an increased concentration of isolated bacterial cells and their supernatant activity has been observed in the mortality of *P. americana* ootheca which might be an effect of the high resistance of free radicals. In the present study, bacterial cells and their supernatant activity has declined in hemolymph as compared to control which may be one of the reasons that causes the high mortality rate of the ootheca treated with *B. subtilis*. Similar finding was investigated by [18], who found that solutions containing high concentrations of bacterial cells of *Xenorhabdus nematophila* and free-cells caused high mortality to *Spodoptera exigua*, *Plutella xylostella*, *Otiorhynchus sulcatus* and *Schistocerca gregaria*.

5. Conclusion

The present study revealed that the isolated bacteria *B. subtilis* is pathogenic and can be used as a biological control against ootheca of *P. americana*. The mortality occurs through the cuticle of the ootheca and the pathogenic action culminates their toxins.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

It has to be declared that the authors of the study have no conflict of interest among them.

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