International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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Insecticidal potency of entomopathogenic bacterium *Bacillus subtilis* on cockroach (*Periplaneta americana*)

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International Journal of Science and Research Archive, 2024, 12(01), 934–939

Publication history: Received on 01 April 2024; revised on 21 May 2024; accepted on 24 May 2024

Article DOI: https://doi.org/10.30574/ijsra.2024.12.1.0839

Abstract

The effect of exposing cockroaches to entomopathogenic bacteria isolated from the diseased specie was examined based on established parameters. Cockroaches were collected from residential areas in Akure metropolis in Nigeria using a bread and beer trap, brought to the laboratory, housed in wooden cages with wire nettings, provided with starch-based food and sterile water. Collected cockroaches were observed for the onset of possible disease symptoms amongst the population for several weeks. Resulting moribund and dead cockroaches were aseptically picked, surface sterilized and homogenized inside a sterile mortal with a buffer. Bacteria were isolated from the homogenate using various general and specialized media. Isolated bacteria include; *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Bacillus licheniformis, Proteus mirabilis* and *Enterobacter aerogenes.* The bacterial isolates were used to re-infect healthy cockroaches out of which only *Bacillus subtilis* was able to cause disease in the cockroaches. The bacteria were then inoculated into another set of healthy cockroaches at varying concentrations to obtain the minimum lethal concentrations capable of causing disease and it was discovered that the organism was able to cause disease in cockroaches at concentration of 10^8 cfu/ml.

Although *Bacillus subtilis* is yet to enjoy a global recognition as a biocontrol agent to be used in the formulation of biopesticides compared to other organisms such as *Bacillus thuringiensis*, its insecticidal properties have many potential applications especially in integrated pest management (IPM) programs. Incorporation of pesticidal products - formulated using *Bacillus subtilis* - into IPM strategies, farmers can significantly reduce the usage of chemical insecticides thus minimizing its toxic environmental impact.

Keywords: Entomopathogens; Integrated pest management; *Bacillus subtilis; Periplaneta americana*

1. Introduction

The term entomopathogenic comes from the Greek word *'entomon'* meaning insect, and 'pathogenic' which means causing disease. Thus, entomopathogenic bacteria refer to those category or types of bacteria which are able to cause diseases in insects (Azizoglu *et al.,* 2020). Entomopathogenicity is an important phenomenon in biological sciences especially in the area of biological control (Burges, 1998). Many insects are considered as nuisances to man, animals and crops. The *Periplaneta americana* is an example of such insect. They are a fascinating and ancient species. They belong to the class Insecta with their ancestors originating from the carboniferous period, emerging approximately 300–350 million years ago (Tinker and Ottesen 2021; Wang *et al.* 2017). Cockroaches are one of the "hardiest" insects. They are difficult to totally eradicate once they are established in a particular habitat. They are able to survive without food for up to a month, without air for around 45 min and being submerged under water for 30 min (Lee *et al.* 2012). In addition, they can withstand high doses of radiation – an amount that is 15 times higher than what the average human can tolerate (Zhao *et al.* 2017).

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Several attempts have been made to control them using chemical insecticides and a host of other techniques. However, most of these insecticides are synthetic, they are toxic to man and have detrimental effects on his environment and other non-target organisms. The insect also become resistant to the insecticide overtime. Thus, effective and more environmental-friendly ways are required to better control the insect pest population. Therefore, other ways like the use of insecticidal organisms and their products which are harmful to the insects but less toxic to non-target living cells becomes imperative in the control and decimation of target insect population.

2. Materials and methods

2.1. Collection of cockroaches

Periplaneta americana were collected from residential buildings in Akure metropolis, Ondo State, Nigeria using a beer trap. The beer trap was designed using a shallow container or bowl, white bread, cheap beer, rubber bands, petroleum jelly or cooking oil. The white bread was cut into small pieces and place at the bottom of the container. The beer was added until it covers the bread pieces. The container was placed in areas where cockroaches are present at night. The trap was secured from being knocked over using the rubber bands to attach it to a stable surface. A thin layer of petroleum jelly was applied to the inner rim of the container. This makes it difficult for the cockroaches to climb out.

The trapped roaches were then taken to the laboratory with an ambient temperature between 25-27^{o}C and were supplied with a source of starchy food of properly cooked rice and water and kept in a wooden cage. The cage was covered with wire nettings to prevent their escape and also allow for adequate air passage. Enclosed areas were also provided for hiding.

2.2. Observation of the insect population for diseased individuals

Insects collected were subjected to no treatment under laboratory conditions. They were left to acclimatize to the new environmental conditions. The population was closely watched for over 4 weeks after which individuals showing morbid and mortal symptoms probably induced by the combine effect of environmental stress and pathogenic microbes acquired while still in the fields. Symptoms shown were in the form of lethargy, reduced activities, colour change, reduced feeding rate and subsequent death.

2.3. Isolation of bacteria from cockroach cadavers

Each of the morbid cockroaches were picked aseptically and surface sterilized by rinsing in sterile phosphate-buffered saline (PBS) solution for 2 minutes. After rinsing, they were surface sterilized by immersing in 70% ethanol for 1 minute. The insects were fully homogenized by grinding all body parts inside sterile mortar. Serial dilutions of the homogenate were carried out and the appropriate diluents using sterile saline buffer were plated on both general and selective bacterial media. The media were prepared according to manufacturer's specification and used to isolate the microbes present.

2.4. Purification and identification of isolated bacteria cells

After incubation, visible colonies obtained were further re-streaked on fresh plates to obtain them in pure forms. Identification of cultures was performed using different standard cultural and biochemical means. The identified cultures were then streaked on double strength nutrient agar slants and kept for further analysis to be carried out on them. Most of the cultures were identified using their growth pattern on the different specialized culture media use. The *Bacillus* sp were further confirmed by allowing the culture to sporulate and heating the suspension spores to 80°C for 10 minutes which ultimately kill the non-sporulating bacteria. Spore suspension is plated on LB agar and colonies checked.

2.5. Infection of cockroaches

Tests insects were divided into batches and each batch was infected with each of the bacteria obtained. The bacteria were first grown in liquid media. After incubation, they were separated by centrifugation and washed twice with saline buffer. The cells were then reconstituted using PBS buffer to make a suspension. The cockroaches were infected by spraying with the cell suspensions of each isolated microbe. Sterile buffer was sprayed on a separate population of the cockroaches as control. This is in accordance with the methods of Bracke *et al.* (1977).

2.6. Selection of entomopathogens from the isolated bacteria

Selection of entomopathogens was done by observing the bacteria able to cause infection in the insects in forms of morbidity and mortality characterized by death, reduced activities, lethargy and reduced feeding. Bacteria which were able to exhibit morbid and mortal effects on the insects after pathogenicity were selected as suspected entomopathogens.

2.7. Determination of the minimum bacterial concentration needed for pathogenicity

The minimum bacterial concentration needed for pathogenicity was determined by infecting the insects with various concentrations of the recorded entomopathogens. The least concentration showing pathogenic activity was calculated by multiplying the colony forming unit in the suspension by the dilution factor and recorded after observing for 120 hours.

2.8. Statistical analysis

All data were expressed as mean ± SEM. Analysis of variance (ANOVA) was performed on the data and the means compared by Duncan Multiple Range Test using SPSS version 15. Differences were judged to be statistically significant at $p < 0.05$.

3. Results

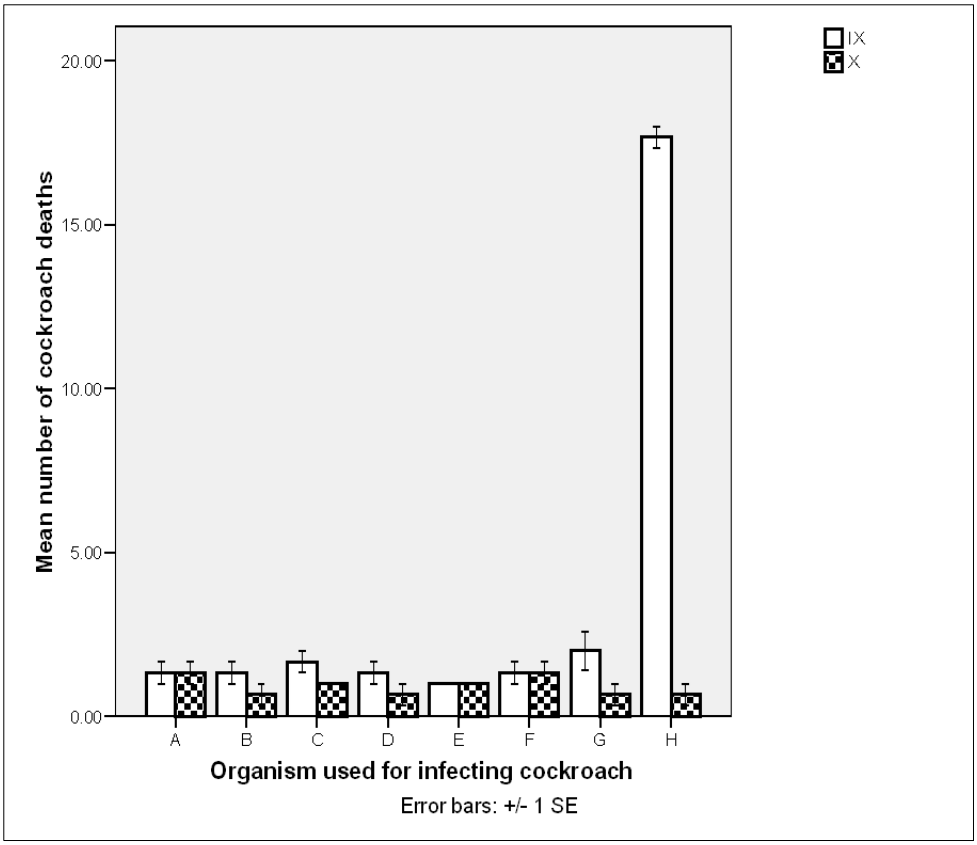
A total number of eight bacteria were obtained from cockroach homogenate and these include *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Bacillus cereus, Bacillus subtilis, Citrobacter freundii* and *Enterobacter aerogenes* (Table 1). After the infection of cockroaches with the bacterial cells isolated from the homogenates, only *Bacillus subtilis* showed marked signs of pathogenicity after 120 hours (Figure 1). The signs were first noticed after 48 hours of infection in some of the insects and these increased with increase in the number of hours. Some of the cockroaches exhibited lethargic signs and subsequently died.

Table 1 Bacterial isolates obtained from cockroach, *Periplaneta americana*

Organism	***E. coli***	***S. aureus***	***P. aeruginosa***	***S. marcescens***	***Bacillus cereus***	***B. subtilis***	***Citrobacter freundii***	***E. aerogenes***
Cultural Characteristics								
Pigment	White	White	Green	Red	Yellowish white	White	Yellowish white	White
Shape	Circular	Circular	Irregular	Circular	Undulate	Irregular	Circular	Circular
Elevation	Raised	Raised	Flat	Raised	Flat	Flat	Raised	Raised
Surface	Moist	Moist	Moist	Moist	Dry	Dry	Moist	Moist
Morphological Characteristics								
Gram reaction	-	+	-	-	+	+	-	-
Cell Shape	Rods	Cocci	Rods	Rods	Rods	Rods	Rods	Rods
Spore formation	-	-	-	-	+	+	-	-
Biochemical Characteristics								
Catalase	+	+	+	+	+	+	+	+
Coagulase	+	+	-	-	-	-	+	+
Oxidase	-	-	+	-	-	-	-	-
Sugar Fermentation Test								
Glucose	AG	AG	AG	AG	AG	AG	AG	AG

Galactose	-	AG	-	-	AG	AG	-	-
Sucrose	AG	AG	AG	AG	AG	AG	-	AG
Lactose	AG	AG	-	-	-	AG	AG	AG
Mannitol	AG	AG	AG	-	AG	AG	-	AG

Key: AG = Acid and Gas production, A = Acid production, - = Negative, + = Positive



Keys: A - *Bacillus cereus*, B - *Citrobacter freundii*, C – *Escherichia coli*, D – *Enterobacter aerogenes*, E – *Pseudomonas aeruginosa*, F – *Serratia marcescens*, G – *Staphylococcus aureus*, H – *Bacillus subtilis*, IX – Infected cockroaches, X – Control cockroaches

Figure 1 Mortality of cockroaches infected with isolated bacteria after 120 hours

The minimum number of *Bacillus subtilis* cells required for significant pathogenicity in cockroach was 10^8 cfu/ml. Other concentrations were unable to give clear signs of true pathogenicity (Figure 2)

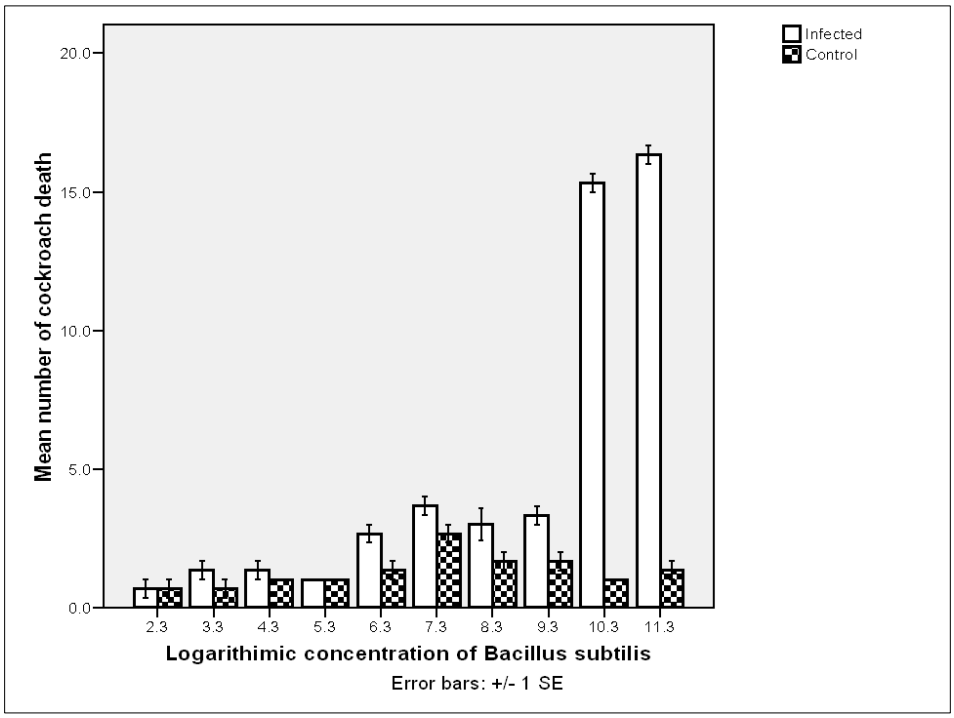


Figure 2 Minimum lethal concentration of *Bacillus subtilis* against cockroaches

4. Discussion

The bacteria isolated from cockroaches used in this study (*Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis*) were also reported by various researchers (Roth & Wlillis, 1957; Cornwell & Mendes, 1981; Guyader *et al.* 1989). Tatfeng *et al.* 2005 also reported the isolation of *Staphylococcus aureus, Citrobacter freundii, Serratia marcescens* as well as *Enterobacter* sp. from roaches collected from households in Nigeria.

This study suggests that *Bacillus subtilis* is a potential entomopathogen as a result of the lethargic effects it caused among the cockroaches used in this study which subsequently results to their death. This is in accordance with the findings of Backman *et al.* (1997) who described some naturally occurring strains of *Bacillus subtilis* as having innate insecticidal properties which is useful in biocontrol activities for the control of insect populations in the environment. Other studies have also investigated the lethal activity of *Bacillus subtilis* against certain insect pests. For example, a study by Tounsi *et al.* (2016) evaluated the insecticidal activity of *Bacillus subtilis* strain against larvae of the tomato leaf miner, *Tuta absoluta*. Results obtained showed significant insecticidal effects, suggesting the potential of *Bacillus subtilis* as a biocontrol agent against this pest. A similar study by Su *et al*. (2010) equally showed the lethal activity of *Bacillus subtilis* strain NSRS 89-24 against larvae of the Asian corn borer, *Ostrinia furnacalis*. The inferred from the study that the *Bacillus subtilis* strain produced a crystal protein toxic to the larvae thus making it a candidate for the formulation of biological insecticides against the agricultural pest

The insecticidal activity of *Bacillus subtilis* as demonstrated in this study can be attributed to different factors which include the production of metabolites and toxins that are lethal to the insects. *Bacillus subtilis* are known to produce lipopeptides includng surfactins and fengycins. These compounds have been shown to have insecticidal properties (Liu *et al.,* 2017). Furthermore, some strains of *Bacillus subtilis* are known to produce proteases and chitinases that can degrade insect cuticle and inhibit insect growth (Pathak and Keharia, 2013). The degradation of the chitinous cuticle will lead to the creation of openings and loss of integrity of the insect's exoskeleton and as a result, secondary bacterial or fungal infection might set in which may result to morbid or mortal conditions. The Recovery of the bacteria used for infecting cockroach during the re-isolation of bacteria from cockroach cadavers is in conformity with the findings of Bracke *et al.* (1977) who recovered the organisms which was incorporated into sterilized food substances fed to cockroaches captured under laboratory conditions.

The emergence of marked lethal signs in insects after 48 hours of infection by bacterial entomopathogens especially in the case of cockroach further confirms that bacterial pathogens of insects requires at least 48 hours of incubation period before the onset of morbid manifestations. This is in agreement with the works of Hajek (2004).

This research has indicated that *Bacillus subtilis* possesses insecticidal activities against *Periplaneta americana*. They can therefore be employed in biocontrol activities against their proliferation by formulating them into future bioinsecticides. In addition, ways through which these potential biocontrol agents can be engineered to enhance their entomopathogenicity should be further researched into. Ways by which these entomopathogens can be mass produced cheaply probably on industrial effluents or byproducts must be investigated.

Compliance with ethical standards

Acknowledgements

The author wishes to acknowledge and appreciate Prof. Omoya F. O. of the Microbiology Department of the Federal University of Technology Akure, Nigeria for her technical support in the methodology used in this work.

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