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Efficacy Test on Some Entomopathogenic Bacterial Isolates of the *Periplaneta americana* and *Blatella germanica* Cockroaches (Orthoptera) at Laboratory Scale

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Abstract

Cockroach is one of insects which plays a role as a disease vector, causing negative impacts to human health. Controlling cockroach by using excessive insecticide adds residue to the environment and strengthens its resistance toward insecticides. The objective of this research was to implement and evaluate the alternative use of biological control agent, such as entomopathogenic bacteria against two types of cockroach. In this study, three entomopathogenic bacterial isolates derived from dead *Spodoptera litura* pupa and *Bacillus thuringiensis* were employed. The test was performed by administering spraying and baiting methods at cell concentration of 10^8 CFU/ml. The results showed that three entomopathogenic bacterial isolates used in this study affected cockroach mortality. The result of morphological observation and Postulate Koch test showed that cockroach mortality was caused by the bacterial isolates. The spraying method on BLSP4 bacterial isolate contributed to the highest mortality rate by 80% to the *Blatella germanica*. Meanwhile, in the baiting method, the isolate of *B. thuringiensis* caused the highest mortality to the *Periplaneta americana* by 10%. Based on the mortality period, it can be recognized that BLSP4 treatment with spraying method affected the mortality of *B. germanica* effectively within 2 hours 30 minutes 46 seconds. An important finding in this study was that BLSP4 bacteria has been known potential for controlling cockroach using spraying method. The result of this study provides insights that BLSP4 bacteria can be used as a new alternative for controlling cockroach and generally in pest management.

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INTRODUCTION

The cockroach is one of vectors which can mechanically or biologically transmit disease to human in an urban environment. Cockroach is able to act as a harbor (habitat) as well as transmitter of pathogenic disease due to its ability to consume any food, including food waste in the kitchen and trash bin. It may transfer the pathogen by stepping on food and kitchen utensil (Ogg *et al.*, 2006). The most common diseases caused by its contamination include diarrhea, typhoid fever, dysentery, hepatitis A, polio and cholera. In some cases, few people suffer from allergies due to continuous exposure to the dirt (cases which lead to a risk of transmission). The excrement of cockroach contains carcinogenic compounds which might be harmful to a human when they consume carcinogenic-contaminated food.

Approximately, there are 3,500 species of cockroach, and two of them including *Periplaneta americana* and *Blattela germanica* are easy to be found in urban environment. People's awareness toward the negative impacts of cockroach increases once they discover that cockroach has a high rate of reproductive ability in which a cockroach is able to lay 30,000-40,000 eggs/year and also has a short life cycle (Barbara, 2005). The cockroach is one vector of diseases that should be under control to prevent it from harming human's health.

There are four different methods to control the insect pests including chemical, mechanical, physical and biological pest-control methods (Indriyanti *et al.*, 2017). The chemical method uses pesticides (Tarwotjo & Rahardian, 2017) and pheromones (Samudra, 2006). Mechanical and physical control can be done by collecting and killing the cockroach larvae in the soil. Biological control method can be done with the use of biological controlling agents.

Cockroach control strategy was issued by the Ministry of Health of the Republic of Indonesia (2002) including four ways of mechanical methods and the use of insecticides. Unfortunately, the mechanical methods are unable to control the presence of cockroach in the environment due to its short life cycle and high reproductive ability. Thus many people use insecticides to control it. The use of insecticide has been proven to have some advantages. However, excessive amount and continuous application of the substance will lead to stronger resistance of the insect. Its chemical residue may also contaminate other organisms and the environment as well. Regarding those negative impacts caused by the use of insecticides, an environment-friendly alternative pest-control solution and disease vector solution is needed.

Cockroach control activity can be performed by utilizing the entomopathogenic bacteria. Different from the chemical control using insecticides that may be persistent in the environment, the use of entomopathogenic bacteria is considered environmentally friendly as it has no negative effects on the environment. The biological agents used in this method also offer several advantages, such as high selectivity, wide distribution in the nature, ability to actively search and locate the host, easy to be multiplied and dispersed, do not make the target become resistant in case the process occurs slowly, and can also operate itself (Arifin, 2011).

Entomopathogenic bacteria have a specified effect on the target insects. However, recent information related to entomopathogenic bacteria as a potential biological agent in controlling cockroach is not yet widely available, especially the ones about *P. americana* and *B. germanica* which can be easily found in residential areas in Indonesia. Hence, more studies on entomopathogenic bacterial isolates as a potential biological agent in controlling cockroach are still required. This study aims to understand the result of the efficacy test of some entomopathogenic bacterial isolates as a biological control agent against cockroach as it is given both contact and baiting toxins. The highly potential bacterial isolates in this study are expected to develop as biopesticide products for control cockroachs especially species of *P. americana* and *B. germanica*.

METHODS

Study Site

The study site was conducted at Microbiology Laboratory, Research Center for Biomaterials – Indonesian Institute of Sciences (LIPI) Cibinong, Bogor.

Methods of Study

*Preparation of isolates and samples***.** Bacterial isolates used consists of three kinds of isolates, such as *B. thuringiensis* (Bt) derived from IPB Culture Collection (IPBCC) and two bacterial isolates coded as BLSP4 and BLSP3 derived from the results of isolated dead *Spodoptera litura* pupa at Cibinong, Bogor (Krishanti *et al.*, 2017). Samples used in this study were two types of cockroach *P. americana* and *B. germanica* at the phase of imago, as the collection of Research Center for Biomaterials LIPI, Cibinong-Bogor.

*Gram coloring and bacterial growth curve making***.** Bacterial gram coloring was based on Narasimhulu (2010). The curve of bacterial growth is made to estimate the number of cells and the speed of growth by the generation period, or time needed by bacteria to self-multiply. The procedure of growth curve making was performed by Handayani (2006).

*Efficacy test on spraying method***.** The highest density concentration of bacterial cell used was 10^8 CFU/ml, namely bacterial culture aged 15-24 hours. The efficacy test was based on Wege *et al*. (1999) with modification of a number of cockroach sample used. A total of 5 ml bacterial culture was sprayed evenly on each cockroach imago. Then, the imago was inserted into a plastic jar with a diameter of 15 cm, each jar was filled with 5 cockroach imagos that have been sprayed. Pellet and drink were given to the imago every 2 days. The treatments consisted of bacterial isolates BLSP3, BLSP4, *B. thuringiensis*, and sterile distilled water (control). Each treatment used 6 replications. In the first 4 hours after treatment, the observation was done once in 1 hour and the observation was performed once a day for 14 days (Lee, 1999).

*Efficacy test on baiting method***.** The feed composition of the test consisted of 5.2 g of agar, 166.7 g of white sugar, 8 g of medium Nutrient Broth (NB) in 1 liter of sterile distilled water. A total of 1.5 ml of bacterial liquid culture was inoculated into the baiting medium and poured into a sterile petri dish and was incubated for 48 hours. The imago tested has been set for 7 days to eliminate the influence of food that has been consumed. Each of five *P. americana* and *B. germanica* was inserted into a plastic jar with a diameter of 15 cm, each treatment used 6 replications. The semi-solid medium that has been overgrown with bacteria was dissolved and smeared onto bread, then weighed, and put into a jar containing cockroaches as well as wet cotton as a drink media for cockroach. After 2 days of treatment, the food was taken out and weighed to determine the weight loss during the test. Furthermore, cockroaches were fed with baiting method without bacteria where food and drink were replaced regularly every 2 days. The observation was conducted in 14 days based on the study by Lee (1999).

*Koch Postulates Test***.** Each of dead *B. germanica* and *P. americana* cockroaches in each treatment was inserted into 9 ml of physiological salt solution and homogenized. Koch Postulates Test was performed by isolating bacteria using serial of dilution method on Nutrient agar growth medium. Observation of bacterial colony growth was performed after 24 hours incubation.

*Data analysis***.** Data analysis was performed using Randomized Group Design. Data percentage of cockroach mortality was obtained by transforming data of square root and arc sin. Then, it was analyzed by using one way ANOVA and followed by an advanced test of DMRT. In the baiting method, the data percentage of cockroach mortality obtained was initially transformed. Two types of data transformation used are square root data transformation for baiting method of *B. germanica* and data transformation of arc sin for *P. americana*. Furthermore, the data were analyzed by one-way ANOVA and followed by DMRT advanced test. A number of cockroach mortality per hour observed on each treatment was analyzed by using probit analysis to determine the LT-50 of some entomopathogenic bacterial isolates in detailed and clearly, thus another expert can do the similar study (implementation of study must be repeatable and reproducible).

RESULTS AND DISCUSSIONS

Entomopathogenic Bacterial Isolates

The isolation results of dead *S. litura* pupa indicated infections of entomopathogenic bacteria obtained in soybean plantation in Cibinong, Bogor West Java Province which showed that there were 122 isolates of bacteria found (data not shown). The result of screening test selected three isolates (BLSP3, BLSP4, *B. thuringiensis*) to be tested on the cockroach. Gram staining character test was performed to determine the characteristics of those bacterial isolates (Table 1).

According to Krishanti *et al*. (2017), BLSP4 and BLSP3 were identified as *Serratia marcescens* (similarity 94%) and *Staphylococcus sciuri* (similarity 99%) respectively through molecular identification of the gene encoding 16S rDNA. *Serratia*

Table 1. Characteristics of BLSP3, BLSP4, and *B. thuringiensis* bacterial isolates

Bacterial Isolates	Gram	Form	Edge of Colony	Elevation of Colony	Form of Colony	Color of Colony
BLSP4	-	Oval	Smooth	Convex	Circular	Red
BLSP3	-	Coccus	Smooth	Convex	Circular	Yellow
Bt	+	Stem	Rhizoid	Flat	Irregular	White

marcescens is a Gram-negative bacterium (Enterobacteriaceae) often associated with infection of insects such as *Phyllophaga blanchardi* (Monica *et al.*, 2015), *Plodia interpunctella* and *Ephestia kuehniella* fifth instar larvae (Bidari *et al.*, 2017), and also *Spodoptera litura* (Aggarwal *et al.*, 2015). Meanwhile, the *Staphylococcus sciuri* species group consists of five species that are most often regarded as commensal animal-associated bacteria. This bacteria has been detected to have genes implicated in biofilm formation or coding for toxins which trigger toxic shock syndrome and multi-resistance, similar to those carried by *Staphylococcus aureus*. The *S. sciuri* species group is often considered harmless and, as such, not as well documented as, for example, *S. aureus* (Nemeghaire *et al.*, 2014).

Cockroach Mortality After Treatment

*Spraying Method***.** The observation results showed that BLSP4 bacterial isolate treatment contributed to the greatest effect of both *P. americana* (26.67%) and *B. germanica* (80%) mortalities (Figure 1). It was respectively followed by the results of BLSP3 and Bt bacterial isolates treatments. Meanwhile, the control treatment of sterile distilled water did not give any effect on mortality for both types of cockroaches. Based on the results of statistical analysis, it was found that the treatment done using bacterial isolate BLSP4 toward *P. americana* and *B. germanica* cockroaches showed significantly different results from the control (Table 2).

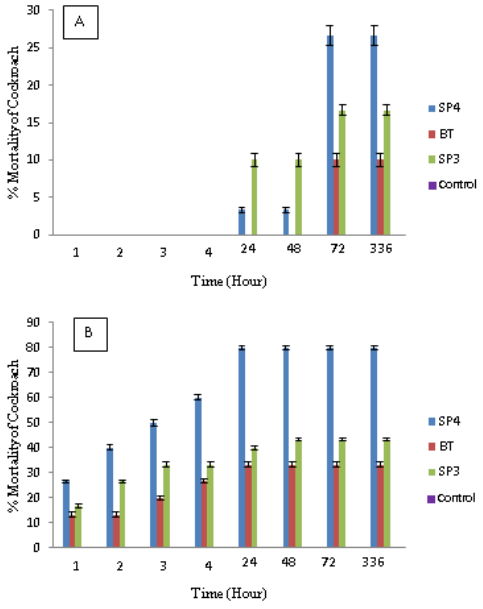

Figure 1. The mortality percentage of *P. americana* (A) and *B. germanica* (B) within14 day observation using spraying method.

Table 2. The mortality of *P. americana* and *B. germanica* treated with entomopathogenic bacteria isolates using spraying method within 14 days of observation. Caption: Numbers followed by the same letter in the same column indicate insignificantly different result.

Treatment	Mortality (%) ± SD	
	P. Americana	*B. germanica*
Control	0.00 ± 0.00 a	0.00 ± 0.00 p
Bt	10.00 ±14.25 ab	33.33 ± 13.40 q
BLSP3	16.67 ±12.10 ab	43.33 ± 9.04 q
BLSP4	26.67 ±19.86 b	80.00 ± 10.55 r

*Statistically significant correlation at $p<0.05$

The red pigment generated by BLSP4 bacterial isolate is a secondary metabolite which is able to become toxin that can kill cockroaches. This result supports Priyatno *et al.* (2011), who stated that the red pigment produced by Serratia marcescens is a secondary metabolite known as prodigiosin which has insecticidal properties. Pathogenicity or the ability of pathogenic microorganisms to transmit the disease to the target insect that is influenced by several factors, such as how bacteria penetrates the target insect body, the bacteria penetration on the insect, and the resistance of the target insect.

Optimum mortality time of target insect after the treatment is one of the efficacy test indicators of biopesticide test. The results of the probit analysis showed that the bacterial isolate of BLSP4 treated by spraying method against *P. americana* cockroach (10 hours 20 minutes 38 seconds) and *B. germanica* (2 hours 30 minutes 46 seconds) obtained the fastest Lethal Time (LT-50) than other entomopathogenic bacterial isolates (Table 3).

Table 3. LT-50, entomopathogen bacterial isolate concentrated at 10^8 CFU/ml was treated using spraying and baiting methods against *P. americana* and *B. germanica* cockroaches.

Treatment	*P. Americana*		*B. germanica*	
	Spraying (hour)	Baiting (hour)	Spraying (hour)	Baiting (hour)
BLSP4	10.344	19.679	2.513	36.914
Bt	11.712	16.665	19.298	23.281
BLSP3	12.700	19.679	19.298	36.914

Symptoms of *P. americana* cockroach death are shown by the presence of 3 entomopathogenic bacterial isolates on caput and dorsal areas,

and the bright red color is visible in the treatment of BLSP4 isolate and bright yellow appears in the treatment of BLSP3 isolate, while Bt isolates shows dark and black color (Figure 2). Koch Postulates test results showed that the treatment of bacterial isolates of BLSP4, BLSP3, and Bt was pathogenic to cockroaches. The bacteria that were successfully isolated from the dead cockroaches in each treatment using spraying method indicated the presence of BLSP4, BLSP3, and Bt bacterial colonies.

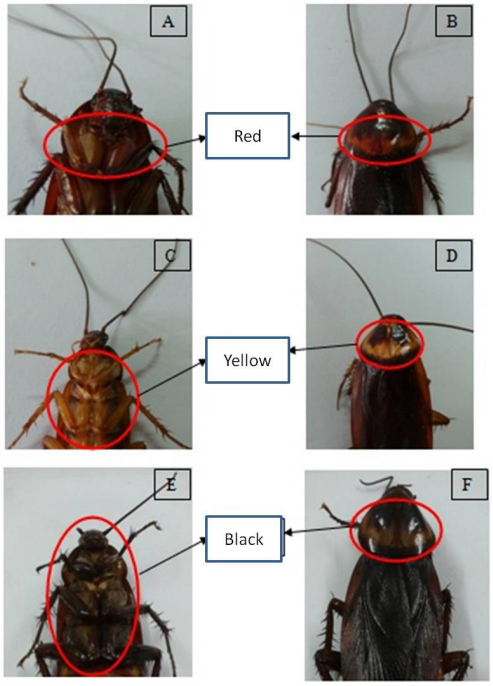

Figure 2. Part of *P. americana* which died due to entomopathogenic bacterial treatment using spraying method with pictures scaled 1: 0,18 (A) BLSP4 ventral part; (B) BLSP4 dorsal part; (C) BLSP3 ventral part; (D) BLSP3 dorsal part; (E) Bt ventral part; (F) Bt dorsal part.

In this study, spraying method created the isolate of BLSP4 bacteria to penetrate through the opened hole or the naturally-opened part of the body or directly thorough cockroach's mouth. The target insect will die when bacteria are directly contacting and colonizing the target insect (Djojosumarto, 2008). The death of insect is caused by bacterial proliferation in its body or toxin/enzyme secreted by bacteria which can damage insect's body by invading hemocoel and causing septicemia (blood poisoning) in the end. According to the Guidelines of the Use of Insecticides (Pesticides) issued by the Ministry of Health of the Republic of Indonesia (2012), cockroach control as a vector can be done using spraying method as it is rather smooth and creates thin integument of cockroach that allows liquid insecticides to penetrate into the body. Cockroach's caput and legs have a similar color to the bacterial isolate, because its body is exposed directly during the spraying treatment. The cuticle that protects its legs and caput is not as thick as the ones in the abdomen and the thorax on the dorsal part. Therefore, colors of bacterial isolate colonies are invisible in these parts.

On the contrary to the case of the death of cockroaches by BLSP4 spraying, the death of *P. americana* and *B. germanica* is indicated by pale yellow color on caput and thorax as the effect of BLSP3 treatment. Before the death, cockroaches experience to knock down. The BLSP3 isolate attacks the nervous system, reflected by symptoms of seizures experienced by a cockroach. The average duration of seizures ranges from 1 hour to the death of cockroaches. The neural toxin is toxic to the nervous system (neurotoxins) which works in the nervous system of both insects and mammals. This attack can cause tremor and seizures (Tanada & Kaya, 1993). Meanwhile, dead cockroaches from the Bt treatment show darker color in the bodies than other treatments and negative control. It indicates the presence of toxin in the stomach, which attacks the epithelial cells of target insect's digestive tract.

Overall, bacterial isolate treatment is able to kill the cockroaches of *P. americana* and *B. germanica*, yet it is not effective in killing the whole *P. americana* as the mortality is below 50%. This phenomenon happens because entomopathogenic bacterial isolates have specific target properties in infecting particular insects.

Baiting Method. The results showed that Bt isolate treatment gave the highest mortality effect to P. americana (10%) and B. germanica (6.67%) (Figure 3). However, based on the statistical analysis, the baiting method treatment seen from the percentages of the mortality of both types of cockroaches showed the insignificantly different result. Besides observing the rate of cockroach mortality, another observation was done to calculate the weight of feed loss after 48 hours of observation (Table 4).

The morphological characteristics of dead *P. americana* and *B. germanica* caused by baiting method treatment are wet, mushy, crumbly, releases bad odor, and has a darker color. It happens due to the influence of stomach toxin caused by Bt bacterial isolate that damages the middle abdomen and interferes with the metabolic process of cockroaches. This is similar to the statement from Lacey & Undeen (1986), the death

process of the insect sample occurred due to the consumption of protein crystals contaminated by entomopathogenic bacteria of *B. thuringiensis* as it will dissolve in the digestive system of insects. Moreover, protease enzymes in the insect will facilitate the protein crystal to break the crystal. Then, the bacteria spore will germinate inside the insect sample and harm the intestinal membrane of the insect.

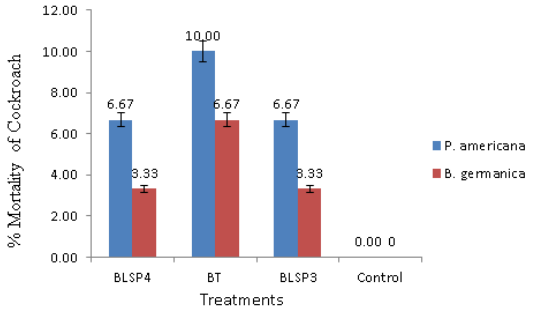

Figure 3. The percentage mortality of *B. germanica* and *P. americana* treated with entomopathogenic bacteria using baiting method.

Table 4. The weight of the feed loss after 48 hours of entomopathogenic bacteria treatment against *P. americana* and *B. germanica* by baiting method

Treatment	Average Feed Loss (%)	
	P. Americana	*B. germanica*
Control	65.24	65.77
BLSP4	52.75	50.14
BLSP3	53.28	48.22
Bt	55.00	56.73

The results of baiting method treatment showed that *B. thuringiensis* has the highest mortality effect on cockroach than other treatments. According to Palma *et al*. (2014), the targeted insect should be inappropriate growth and the number of bacteria consumed should be sufficient enough. Death can occur within a few hours or several days. Most of the contact toxin acts as stomach toxin as well. Therefore, a bacterial isolate of BLSP4 which gives the greatest cockroach mortality within the use of spraying method also gives a great effect on the number of cockroach mortality when it is applied in baiting method, even the result is smaller than the result of spraying method. A similar statement is also mentioned by Klein (2008), that some pesticides may act as both contact and stomach toxins at the same time.

The spraying method has a significant effect on the mortality of *P. americana* and *B. germanica* cockroaches compared to the use of baiting method. The mortality time required in the spraying method is shorter than baiting method. Morphological symptoms that appear are also different, in which the color of pathogenic bacterial colony isolate is shown by morphological symptoms of a dead body in spraying method, while using the baiting method, the dead color is black and smells bad due to the attack of pathogenic bacteria toward the cockroach's digestive system.

The use of entomopathogenic bacterial isolates in the baiting method is able to kill the cockroach, yet it is still considered ineffective as the percentage of death is below 50%, besides the time of death (LT-50) is more than 6 hours. An effective bioinsecticide requirement for cockroach is proposed by Bestari *et al*. (2014) and Jauharlina & Hendrival (2003) that a bioinsecticide is said to be effective if the time of the death of the sample reaches at least 6 hours after exposure and can kill target insect by more than 50%. Therefore, more advance studies on the potential entomopathogenic bacteria application are highly required.

CONCLUSION

The spraying method used in the treatment of BLSP4 bacterial isolate has shown the greatest effect on *P. americana* mortality and effective to the *B. germanica*, because it triggers mortality up to 80% rate with the value of LT-50 within 2 hours 30 minutes 46 seconds. In the baiting method, the greatest mortality of *P. americana* and *B. germanica* is found in the treatment using *B. thuringiensis* isolate.

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