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Research Article

Isolation of Bacillus subtilis from Several Locations in Mataram and its Toxicity to *Aedes aegypti* Mosquitoes in the Laboratory

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Abstract: One way to biologically control mosquitoes is by utilizing bacteria. Bacillus thuringiensis and Bacillus sphaericus are commonly used bacteria for this purpose. However, Bacillus subtilis has recently attracted the attention of researchers, as it is reported to have potential for use as a biopesticide. In this study, B. subtilis was isolated from several habitats in Mataram and West Lombok and tested on Aedes aegypti larvae to determine its potential toxicity. B. subtilis was isolated from the soil using a heating technique to separate it from other bacteria that do not have endospores and then grown at 33oC for 24 hours. The bacteria that grew were then tested on third-instar Ae. aegypti larvae. From several sampled locations, eight B. subtilis isolates with low toxicity levels were obtained. Toxic B. subtilis isolates were obtained from garden soil (isolate LT2C, 20%) and rhizosphere soil (isolate LB1A, 30%). The toxicity obtained is lower than the toxicity of B. thuringiensis and B. sphaericus, therefore further exploration is needed to be able to utilize B. subtilis as a biopesticide agent.

Keywords: Bacillus subtilis, Aedes aegypti Larvae, Mataram, Toxicity

Introduction

The use of microbes to control mosquito spread has been practiced since the 1970s in several countries. Two commonly used species are Bacillus thuringiensis and Bacillus sphaericus, with various commercially available preparations [1]. When compared to chemical pesticide agents, the use of microbes as biopesticide agents has some advantages compared to chemical pesticides such as more specific targets, leaving non-dangerous residues in the field and causing no health problems for humans [2].

One of the undesirable things in using microbes as biopesticide agents is the phenomenon of resistance that forms in mosquito larvae against the microbes used, since the presence of microbial resistance to target insects will reduce the effectiveness of the microbial-based insect control. Therefore, it is necessary to look for other microbes that can be used as biopesticide agents [1,3].

Several other microbes have been tested for their ability to control mosquitoes, one of which is Bacillus subtilis, which its research has become popular in the last ten years [4–6]. B. subtilis is a rod-shaped bacterium commonly found in soil and capable of forming endospores whenever it meets unfavorable conditions (like other members of the genus Bacillus). It is known for biofilms

production, which can be used for various purposes, such as organic matter decomposition, wastewater treatment, and bioremediation of the environment [6–8].

In this study, B. subtilis was isolated from various locations in Mataram Municipality and tested on instar III Aedes aegypti larvae maintained in the laboratory.

Material and Method

Sampling Locations. Samples were taken from several locations, including residential sewage (in Mataram), riverbanks (in Mataram), residential soil (in Mataram), terrestrial/garden soil (in Mataram), and mangrove habitats (on the coast of West Lombok).

Soil Sampling. The method used was based on the standard method with slight modifications [9,10]. Five hundred grams of soil/sediment from 1-10 cm above the surface was sampled using a sterile spoon. The soil/sediment was collected in a previously sterilized screw-cap bottle. The soil was then transported to the laboratory for research use. Unused soil was kept in the bottle and stored in a shaded area.

Bacterial Isolation. Isolation of B. subtilis was carried out using standard methods with slight modifications [9]. A total of 10 grams of soil from several locations (placed in separate tubes) was dissolved in sterile distilled water to reach 100 mL. The mixture was then heated at 60oC for 30 minutes. One hundred microliters of the mixture was then spread on solid Nutrient Agar media, then incubated at 33oC for 24 hours aerobically. From the growing colonies, samples were taken and Gram stained. Cells were observed under a microscope at 1,000x magnification. Colonies whose cell appearance matched the appearance of standard B. subtilis were purified until pure colonies and uniform cell appearance were obtained. [11,12].

Bacterial Characterization. The standard characteristics of B. subtilis based on several literature sources are as follows. Vegetative cells are short rod-shaped, measuring 2-3 x 7-8 µL with a Gram-positive reaction. Endospores are found in the central/subcentral part under unfavorable environmental conditions. The standard biochemical characteristics are as follows. Positive characteristics are: catalase, starch hydrolysis, Voges-Proskauer, fructose, glucose, and sucrose. Negative characteristics are: citrate, oxidase, lactose, and galactose [12,10]. However, for characterization purposes several other tests (that is not including standard characters) and resistance tests were carried out.

Bacterial Preparation for Toxicity Assay. After B. subtilis colonies were identified and purified, 1 full loop was inoculated with the prepared Nutrient Broth liquid media before used in toxicity assay. The culture was then incubated at 33oC for 72 hours aerobically with shaking at 120 rpm. Fermentation in liquid media aims to increase the number of cells and induce endospore formation. [13,14].

Ae. aegypti Rearing. Ae. aegypti mosquito eggs were soaked in well water and placed in a room with 12 hours of light and darkness. Eggs hatched in 1-2 days (first instar stage) and were reared until they reach third instar (or until their body length reaches 4-5 mm). During the larval rearing period, the larvae were fed with finely ground and sterilized dog food. Third instar stage was reached after 5-6 days of rearing at room temperature [14,15].

Toxicity Assay. The testing method uses the standard method issued by WHO [13,14]. For each isolate to be tested, three containers were prepared (for three replicates) containing 100 mL of a 10% test culture. The test culture was prepared by mixing 10 mL of a 72-hour-old B. subtilis culture with 90 mL of sterile distilled water. Ten larvae were placed in each container. In addition to the B. subtilis culture, a control containing 10 larvae and sterile distilled water was also used. Larval mortality was observed and recorded every 24 hours for 72 hours. The

following is a formula for determining the percentage of larval mortality. The formula is as follows.

……[16]

If the mortality of control larvae is greater than 20%, the experiment must be repeated. If the mortality of control larvae is less than or equal to 20%, the corrected mortality percentage formula will be used. The formula is as follows.

……[16]

Results

The following are the results of several B. subtilis isolates isolated from several sampling locations in Mataram and West Lombok are presented in Table 1. The colonies shown are colonies that have been characterized with standard characters from Bergey's Manual of Determinative Bacteriology [12].

Table 1. Colony characteristics of *B. subtilis* isolates that were isolated from several locations in Mataram and West Lombok

Isolate and Location	Bacterial Colony Morphology					
	Form	Margin	Surface	Colour	Texture	Transperency
LB1D – Plant Rhizosphere 1	Irregular	Undulate	Flat	White	Dry	Non transparent
LB1A – Plant Rhizosphere 2	Circular	Undulate	Umbonate	White	Dry	Non transparent
LM1C – Mangrove 1	Circular	Entire	Raised	White	Dry	Non transparent
LM2C – Mangrove 2	Circular	Entire	Raised	White	Dry	Non transparent
LT1B – Garden/terestrial 1	Irregular	Curled	Flat	Yellowish White	Dry	Non transparent
LT2C – Garden/terestrial 2	Circular	Undulate	Umbonate	Yellowish White	Dry	Non transparent
LS1A – Sewage 1	Circular	Rhizoid	Flat	White	Dry	Non transparent
LS1D – Sewage 2	Irregular	Undulate	Flat	White	Dry	Non transparent

Cell characteristics and cell reactions to Gram stain are presented in **Table 2** as follows.

Table 2. Cell characteristics and Gram stain reactions of several isolates of *B. subtilis* isolated

Isolate and Location	Cell Characteristics		
	Cell Form	Endospore position	Gram Reaction
LB1D – Plant Rhizosphere 1	Bacil	Subterminal	Positive
LB1A – Plant Rhizosphere 2	Bacil	Subterminal	Positive
LM1C – Mangrove 1	Bacil	Subterminal	Positive
LM2C – Mangrove 2	Bacil	Subterminal	Positive
LT1B – Land/terestrial 1	Bacil	Subterminal	Positive
LT2C – Land/terestrial 2	Bacil	Subterminal	Positive
LS1A – Sewage 1	Bacil	Subterminal	Positive
LS1D – Sewage 2	Bacil	Subterminal	Positive

The following are photos of colonies and cells of *B. subtilis* that were isolated from several sampling locations, presented in **Figure 1** as follows

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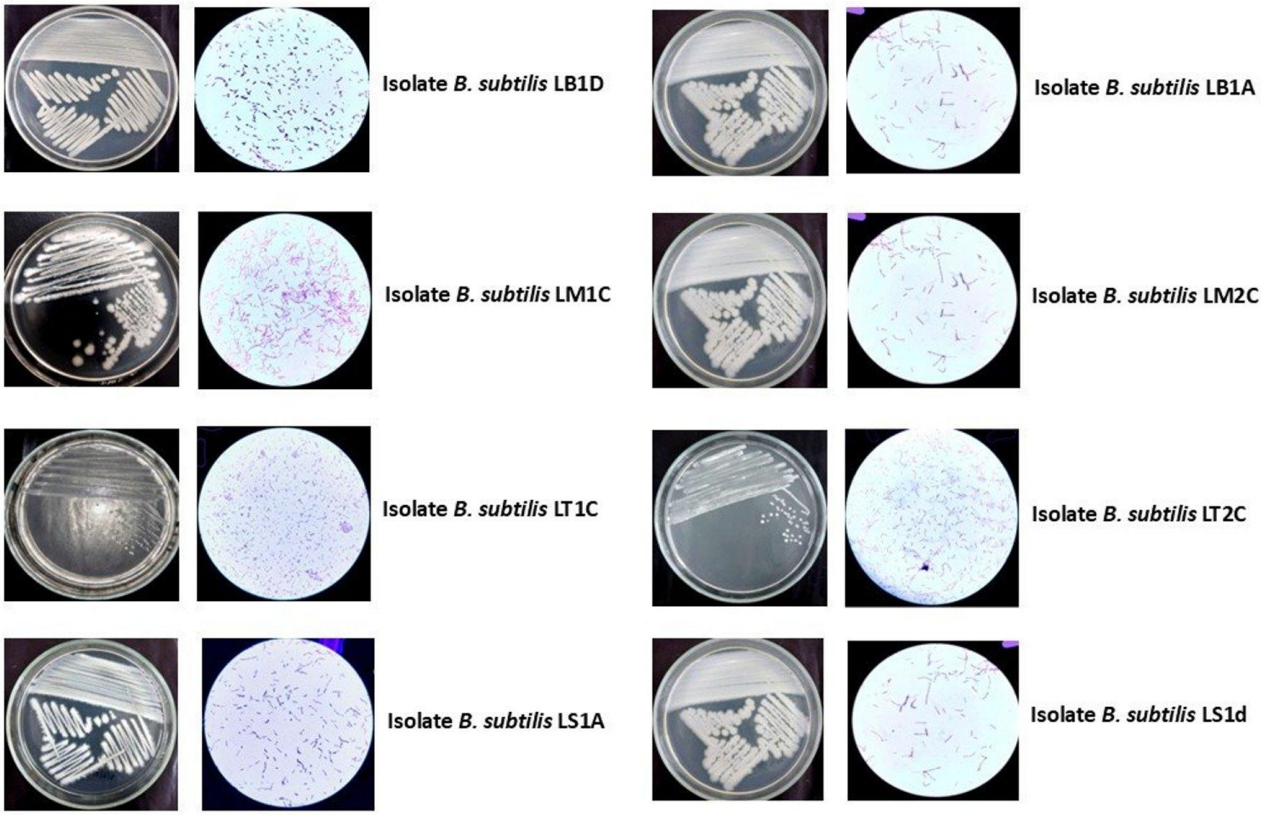

Figure 1. Photograph of B. subtilis isolate colonies and cells isolated from several locations in Mataram and West Lombok.

The results of biochemical and physiological character tests are presented in the following **Table 3**.

Table 3. Results of biochemical and physiological character of several isolates of *B. subtilis* isolate

No	Biochemical and Physiological Assay	*B. subtilis* Isolate								
		LT1B	**LT2C**	**LS1A**	**LS1D**	**LB1A**	**LB1B**	**LM1C**	**LM2C**	**Standard Characteristics**
1	TSIA (*Triple Sugar Iron Agar*)	Alkaline/ Alkaline	Alkaline/ Alkaline	Alkaline/ Acid	Alkaline/ Alkaline	Acid/Acid	Acid/Acid	Alkaline/ Alkaline	Alkaline/ Alkaline	Alkaline/ Acid
2	Cimmon Sitrate	+	+	+	+	+	+	+	+	+
3	Urease	+	+	+	+	+	+	+	+	-
4	Motility	+	+	+	+	+	+	+	+	+
5	Indole	-	-	-	-	-	-	-	-	-
6	Ornitin	-	+	-	-	+	+	-	-	nd
7	Glucose	+	+	+	+	+	+	+	+	+
8	Sukrose	-	+	-	+	+	+	+	-	+
9	Lactose	-	-	-	-	-	-	-	-	-
10	Mannitol	-	-	-	-	-	-	-	-	+
11	Maltose	-	+	+	+	+	+	+	+	+/-
12	*Methyl Red*	-	+	+	+	+	+	-	+	+
13	Voges- Proskauer	+	+	+	+	+	+	-	-	+
14	Starch Hydrolysis	+	+	+	+	+	+	-	-	+
15	Oxidase	+	-	+	+	+	+	+	+	-
16	Growth on 6,5 % NaCl	+	+	+	+	+	+	+	+	+
17	Growth on 55oC	-	-	-	-	-	-	-	-	-

Table description:(+) = Positive;(-) = Negative; (nd) = Not Determined

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The toxicity of *B. subtilis* isolates found against instar III *Ae. aegypti* larvae in the laboratory are presented in the following **Table 4**.

Table 4. Toxicity of *B. subtilis* isolates against instar III *Ae. aegypti* larvae in laboratory

Location	Toxicity (%)
LB1D – Plant Rhizosphere 1	0
LB1A – Plant Rhizosphere 2	30
LM1C – Mangrove 1	0
LM2C – Mangrove 2	0
LT1B – Land/terestrial 1	0
LT2C – Land/terestrial 2	20
LS1A – Sewage 1	0
LS1D – Sewage 2	0

Discussion

In this study, from the many colonies grown (as the temperature for isolation was only 60^{o}C, which was relatively low for Bacillus sp isolation), only eight isolates were obtained. Those isolates showed colony, cell and biochemical characteristics that were similar to the standard characteristics of *B. subtilis*. Of the eight isolates, only two isolates were toxic to the third instar larvae of *Ae. aegypti* tested. The toxicity of the two isolates reached 20% larval mortality (isolate LT-2C) and 30% (isolate LB-1A). The habitat where the LT-2C and LB-1A isolates were isolated had the same characteristics, only with different plants. The location that was loose soil, had 0% salinity, with a temperature of 27°C on the grass-covered soil and 25°C on the bamboo-covered soil (at the time of sampling). Water-containing habitats, both in sewage (LS-1A and LS-1D) and mangroves (LM-1C and LM-2C), produce *B. subtilis* isolates that are non-toxic to mosquito larvae. Habitat type cannot be used as a benchmark for obtaining *B. subtilis* isolates that are toxic to mosquito larvae.

According to research by Geetha and Manonmani in 2008 [17], *B. subtilis* can be isolated from mangrove habitats. Other studies also reported that this bacterium can be isolated from river sediments [18]. In addition, this bacterium was also successfully isolated from rhizosphere soil associated with a number of plants [10]. Many studies have also been found to successfully isolate this bacterium from standing water, such as that conducted by Das and Mukherjee in 2006 [19]. These studies were used as the basis for selecting the location for this study. The location was chosen in accordance with previous research but in a different place, namely in the area around Mataram City and West Lombok.

Unlike *B. thuringiensis* and *B. sphaericus*, which possess specific proteins as larvicidal toxins, *Bacillus subtilis* lacks these protein crystals. *B. subtilis*' toxicity is derived from its biofilm synthesis, a biosurfactant that has been shown to be effective against the larval and pupal stages of several mosquito species [17]. Biosurfactant is the primary secondary metabolite produced by *B. subtilis* and has

various functions, including assisting swarm motility, acting as a signaling molecule, and possessing antimicrobial activity [20].

Biosurfactants are composed of seven amino acids that form a cyclic peptide structure and are bound to a fatty acid chain [21]. According to Chen et al. [22], biosurfactants are amphiphilic, meaning they can insert into the membranes of microorganisms and disrupt their integrity, leading to increased permeability, leakage of cell contents, and disruption of protein synthesis and metabolic enzymes. Other researchers, Zhen et al. added that variations in biosurfactant structure, such as the length of the fatty chain and interactions with calcium ions, play a crucial role in determining how well these molecules can penetrate and damage the cell membranes of bacteria and other microorganisms [23].

However, not all *B. subtilis* strains are capable of producing biosurfactants, as the production of these compounds is highly dependent on the genetics of a *B. subtilis* strain. For example, the laboratory strain *B. subtilis* 168 is known to be unable to synthesize biosurfactants due to a mutation in the *sfp* gene, which plays a crucial role in *B. subtilis* biosurfactant production. The *sfp* gene activates the biosurfactant-making enzyme in *Bacillus subtilis*. In strain 168, this gene is mutated, so even though the production pathway exists, the strain cannot produce biosurfactants [24]. Furthermore, several isolates taken from nature also show variability in their production capacity, with some producing high amounts of biosurfactants, others low amounts, and some even none at all [25]. This confirms that biosurfactant production is not a universal trait of all *B. subtilis*, but rather varies between strains.

Conclusion

From this study, it can be concluded that *B. subtilis* isolated from several locations in Mataram showed low toxicity against *Ae. aegypti* larvae in the laboratory.

Declaration

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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