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ISOLATION OF ENTOMOPATHOGENIC *Lysinibacillus sphaericus* FROM SEWAGE AT SOME HOUSING COMPLEX IN MATARAM CITY AND EVALUATION OF ITS TOXICITY AGAINST *Aedes aegypti* LARVAE IN LABORATORY

ISOLASI Lysinibacillus sphaericus ENTOMOPATOGENIK DARI SELOKAN DI BEBERAPA PERUMAHAN DI KOTA MATARAM DAN EVALUASI KEMAMPUANNYA DALAM MEMBUNUH LARVA Aedes aegypti DI LABORATORIUM

Novia DK Dewi[1)], Ernin Hidayati[1)], Sarkono Sarkono[1)], Eka S Prasedya[1)], Bambang F Suryadi[1*)]

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Authors affiliation:

[1)]Biology Department, Faculty of Mathematics and Natural Sciences, Mataram University, Indonesia

Correspondence email:

*bambangfajar@unram.ac.id

ABSTRACT

The aims of this study were to isolate Lysinibacillus sphaericus from sewage at some housing complex in Mataram city and evaluate its toxicity against third Aedes aegypti larvae. The bacteria collected from mud taken from diluted sewage and objected to heat shock procedure at 80ºC for 30 minutes. The microbiological culture was done using a Nutrient Agar solid medium and incubated at 30ºC for 72 hours. Bacterial characterization was done based on bacterial colony morphology, cell morphology, cell physiology, and cell biochemistry characteristics. Toxicity test on 3rd Aedes aegypti larvae was done for 24, 48, and 72 hours applying Nutrient Broth medium with various dilution. From this study total of 11 isolates of Lysinibacillus sphaericus were isolated, only three isolates showed a high killing rate against Ae. aegypti in 24-hour observation. They were isolate Bs9-2-3 (LC50 24-hour value was 1.75 x 10^4 cell/mL), isolate Bs9-1-5 (LC50 24-hour value was 6.23 x 10^4 cell/mL) and isolate Bs2-1-2 (LC50 24-hour value was 7.17 x 10^6 cell/mL). These local isolates of L. sphaericus had good potential to be developed for bacterial-based biopesticide/biolarvicide for battling Aedes mosquito larvae in the near future.

Keywords: *Lysinibacillus sphaericus, Mataram City, sewage, third instar Aedes aegypti larvae*

ABSTRAK

Penelitian ini bertujuan untuk mengisolasi Lysinibacillus sphaericus dari selokan di beberapa lokasi perumahan di Kota Mataram dan mengevaluasi toksisitasnya terhadap larva Aedes aegypti instar III. Bakteri L. sphaericus diisolasi dari lumpur dari selokan yang telah diencerkan dan diberi perlakuan panas pada suhu 80oC selama 30 menit. Pembiakan dilakukan secara aerobik pada media padat Nutrient Agar pada suhu 30oC. Karakterisasi bakteri dilakukan berdasarkan karakter morfologi koloni, morfologi sel dan uji fisiologis serta biokimia standar. Pengujian toksisitas pada larva Aedes aegypti instar III dilakukan secara in-vitro selama 24, 48 dan 72 jam menggunakan media cair Nutrient Broth dengan berbagai pengenceran. Dari penelitian ini didapatkan 11 isolat secara total. Dari beberapa isolat yang didapat tersebut, hanya 3 isolat L. sphaericus yang memiliki kemampuan yang baik membunuh larva Ae. aegypti dalam 24 jam, yaitu isolat Bs9-2-3 (dengan LC50 24 jam sebesar 1,75 x 104 sel/mL), isolat Bs9-1-5 (dengan LC50 24 jam sebesar 6,23 x 104 sel/mL) dan isolat Bs2-1-2 (dengan nilai LC50 = 7,17 x 106 sel/mL). Isolat L. sphaericus yang ditemukan pada penelitian ini berpotensi untuk dikembangkan menjadi pestisida (larvisida) biologis lokal.

Kata Kunci: *Kota Mataram, larva Aedes aegypti Instar III, Lysinibacillus sphaericus, selokan*

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INTRODUCTION

Dengue fever is one of the mosquito-borne diseases that still becomes a health problem in Indonesia and also West Nusa Tenggara. Dengue fever is caused by Dengue Virus, spread by *Aedes* mosquitoes. It was reported in 2021 that 95,833 people suffered from this disease in Indonesia, with 661 death known [1]. In West Nusa Tenggara alone, there were 3,304 people suffering from Dengue fever, with 13 death in the 2020 report [2].

One approach to deal with mosquito-borne diseases is by controlling the mosquito. Mosquito reproduction and spread can be handled in several ways, i.e., mosquito breeding habitat modification, adult mosquito control and mosquito larvae control (using chemical larvicide and biological agent) [3] [4].

There are several biological agents that can be applied in mosquito larvae control, one of them is bacteria. The first species used in mosquito control is *Bacillus thuringiensis*. It is used to control *Aedes*

(and other mosquito species). It is also used to control agricultural pests [5]. In 1977 some isolate of *Bacillus sphaericus* was found in Israel, and they showed high toxicity against *Culex* and *Anopheles* mosquito [6]. After this discovery, research in every aspect of *B. sphaericus* was developed in many countries [7].

In 2007 the name of *Bacillus sphaericus* was transferred to *Lysinibacillus sphaericus* due to the significant abundance of a lysine residue in the cell-wall peptidoglycan of the bacteria. This amino acid residue is unique and was not found in cell-wall peptidoglycan from any other species [8].

L. sphaericus was bacteria known to have a high ability to kill *Culex* and *Anopheles* larvae [9]. In some countries, the bacteria were routinely used to control mosquitoes, along with other procedures such as chemical pesticide spreading and other methods [10]. Besides *Culex* and *Anopheles*, *L. sphaericus* also showed the ability to kill other mosquito species, such as *Aedes*, although in a lower manner [11].

Since there is still little information about entomopathogenic isolate of *L. sphaericus* from various habitats in Lombok and its ability to kill *Aedes aegypti* larvae, we isolated *L. sphaericus* from sewage at some housing complexes in Mataram City and evaluated its toxicity against third instar *Aedes aegypti* larvae in the laboratory. Any information regarding the toxicity of the bacterial isolates will be used to develop our knowledge of this species and bacteria-based biopesticide/biolarvicide for controlling the mosquito.

METHODS

Location and time of the study. This study was carried out in Mataram City, from January to December 2021.

Sampling location. From previous preliminary research (unpublished data), there were some conditions of sewage that could be used as the target for mud collection that resulted in a toxic isolate of *L. sphaericus*. The condition was as follows.

1. The sewage located in the inside housing complex
2. The sewage hold standing water or slowly-flown water
3. Mosquito larvae must be seen live in sewage

Based on the condition/criterion, sewage was chosen at some housing complex in Mataram city. Those are:

1. Perumnas Ampenan Housing Complex,
2. Bumi Kodya Asri Housing Complex,
3. BTN Royal Housing Complex,
4. Pagutan Asri Housing Complex,
5. Taman Indah Housing Complex,
6. Lumba-Lumba Housing Complex,
7. Kota Mataram Asri Housing Complex,
8. Taman Baru Housing Complex,
9. Permata Indah Selagalas 01 Housing Complex,
10. Permata Indah Selagalas 02 Housing Complex,
11. Bumi Gora Permai Housing Complex

Those housing complexes were categorized as dense-populated housing complexes in Mataram.

Sampling method. From each housing complex, mud was collected from five sampling points, which was in sewage that met with three conditions for sampling location. Collected mud then combined in one place (composite sample). The mud separated from its water part was then put into a 200 mL sterile screw-cup container. All containers were then stored in 5ºC refrigerator before being used.

***L. sphaericus* isolation.** *L. sphaericus* bacteria was isolated applying standard procedure with slight modification [12]. Growth medium used in the procedure was Nutrient Agar (20 g/L concentration). Streptomycin was added to the medium before distributing to the Petri dish, with a final concentration was 30 µg/mL. The addition of streptomycin in the medium will inhibit the growth of other bacteria and facilitate bacterial colony purification more easily. Besides that, resistance against streptomycin was natural resistance characteristic of *L. sphaericus* [13].

Twenty-five grams of mud collected was suspended with a sterile physiological salt solution to form 250 mL of the final volume. The mixture was then homogenized using a vortex mixer and heated at 80ºC for 30 minutes in the water heater. The heated mixture was then serially diluted (10^{-1} to 10^{-5} dilution). One hundred microliters of the diluted mixture was then spread onto a Petri dish filled with Nutrient Agar added with Streptomycin.

The cultures then incubated aerobically at 33ºC for 3x24 hours. Observation of the culture was made every 24 hours (for three days) to evaluate the cell and endospore morphology of *L. sphaericus*.

L. sphaericus was positively isolated if Gram-positive rod cell and endospore were observed in the end/terminal of the cell. The endospore was rounded, with swollen sporangium enclosed the endospore [14].

Culture characterization. Culture characterization was made based on three aspects, i.e., colony morphology, cell morphology, and physiological-biochemical characteristics. Those characteristics obtained were then matched with a standard characteristic of *L. sphaericus* in *Bergey's Manual of Determinative Bacteriology* (profile matching method). Bacterial colony morphological characteristics observed were form, margin, surface, color, translucency, and size. Cell

morphological characteristics observed were cell form and size, endospore form, size and position, and reaction to Gram staining. Biochemical characterization was performed by testing some carbohydrate utilization, Methyl Red - Voges Proskauer, indole, catalase, and oxidase [15][16].

Larvae preparation. *Ae. aegypti* larvae used in this study were obtained from eggs provided by the Institute of Tropical Disease Airlangga University, Surabaya. Larvae rearing was performed by applying the rearing procedure as follows. *Ae. aegypti* egg, which was previously put on filter paper, was immersed under aquadest at room temperature and subjected to 12-hour dark and light conditions. The egg would be hatched within 8-10 hours of incubation. Larvae were fed with sterile oven-dried dog food [17]. The larvae used in the toxicity test were third instar larvae, reached within 9-10 days after hatching. This third instar larva had a body length of 5-6 mm [18].

***L. sphaericus* culture for toxicity test.** *L. sphaericus* culture in liquid medium was done by applying standard procedure with slight modification [19]. One full loop of *L. sphaericus* bacterial colony from solid medium (Nutrient Agar added with Streptomycin) was transferred to liquid medium (Nutrient Broth added with Streptomycin). The culture was incubated at 33ºC for 3x24 hours with 150-200 rpm shaking.

Selective toxicity test. Selective toxicity test aimed to select bacterial isolate for quantitative toxicity assay (bioassay). Bacterial isolate that showed 50% toxicity (or more) would be picked for quantitative assay [19]. A selective toxicity test was done as follows. Seventy-two-hour *Lysinibacillus sphaericus* cultures from all locations were prepared. Each culture was diluted with aquadest to form 10% diluted culture and distributed into three containers (with 200 mL diluted culture for each container). Twenty larvae were then put onto each culture-filled and negative control container (filled with aquadest only). Larval death was then observed every 24 hours in 72-hour observation. Selective toxicity test will provide larval mortality value (in %). The value can be calculated using this formula as follows [20].

$$\text{Observed mortality (\%)} = \frac{\text{Total Number of Dead Larvae}}{\text{Total Sample Size}} \times 100\%$$

If larval death in the control group was ≥20%, the experiment should be discarded and repeated. However, if larval death in control was <20%, the larval mortality value should be corrected using this formula [20].

$$\text{Corrected Mortality (\%)} = \frac{\text{\% Observed Mortality - \% Control Mortality}}{(100 - \text{\% Control Mortality})} \times 100\%$$

Quantitative toxicity assay (Bioassay). A quantitative toxicity test/bioassay was done using the procedure as follows. The *L. sphaericus* isolates showing ≥50% toxicity from the initial/selective toxicity test were grown in a liquid medium (Nutrient Broth + Streptomycin) for 72 hours. After incubation, cell concentration should be measured for a later assay. The bacterial cultured were then serially diluted with aquadest (forming 10^{-1} to 10^{-5} dilution), and each dilution was then transferred to the testing container (with 200 mL diluted culture for each container). Twenty larvae were then put onto each culture-filled and negative control container (filled with aquadest only). Larval death was then observed every 24 hours in 72-hour observation [21]. Using MINITAB statistical software applying probit analysis LC (Lethal Concentration) values (in cell/mL) were then calculated [22].

RESULTS AND DISCUSSION

Isolated *L. sphaericus* characteristics. Bacterial colony morphology of isolated *L. sphaericus* in this study was presented in Figure 1, and bacterial colony characteristics were presented in Table 1. Bacterial cell morphology of isolated *L. sphaericus* in this study is presented in Figure 2.

Bacterial cell morphology observed in the first 24-hour incubation showed a short rod/bacil form (almost 95% of the cell population), with a length of 2.5-3.5 µm. In 48-hour (day 2) incubation, 20-30% of cell population showed vegetative form with swollen endospore structure at the cell's end/terminal. Cell population with 90% vegetative and swollen endospores was seen at 72-hour (day 3) incubation. At 96-hour (day 4) incubation, all cell populations formed endospores, and some endospores were lysed.

Figure 1. Bacterial colony morphology of isolated *L. sphaericus* in Nutrient Agar + Streptomycin medium (24-hour culture)

Table 1. Bacterial morphological characteristics of isolated *L. sphaericus*

Morphological Characteristics	Results
Form	Circular
Margin	Entire
Surface	Smooth
Color	Cream
Translucency	Non-translucent
Single colony diameter in 24-hour culture	2-4 mm

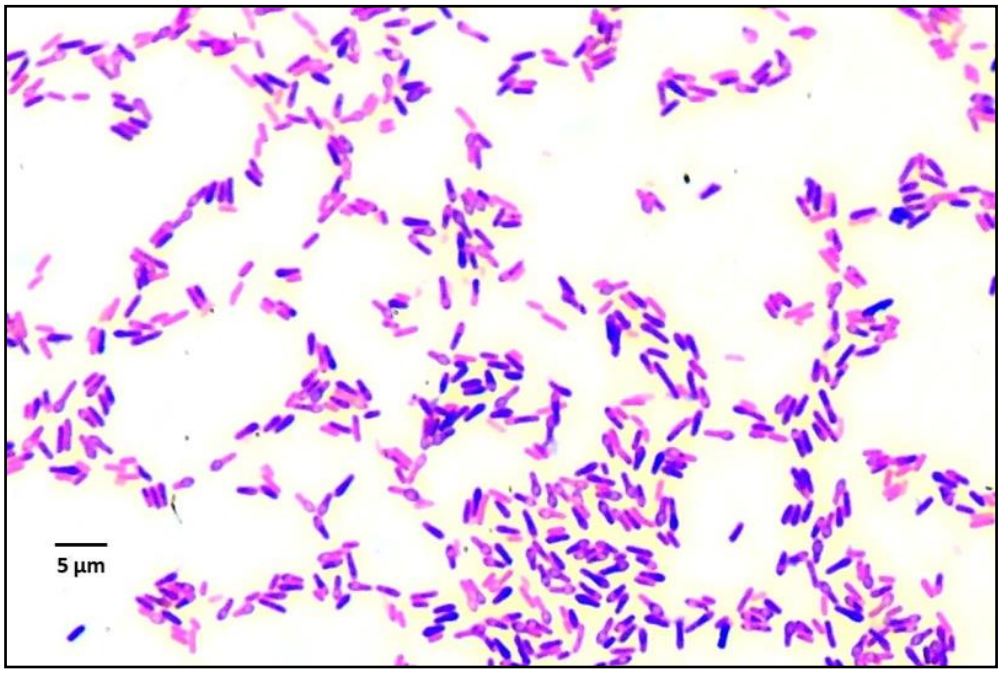

Figure 2. Bacterial cell morphology one of *L. sphaericus* isolated (Bs9-1-5) from 24-hour culture (Gram staining in 1,000x magnification)

The biochemical and physiological characteristics of isolated *L. sphaericus* were presented in Table 2. One unique characteristic for *L. sphaericus* was the inability of this species to utilize carbohydrates either as energy or carbon source [15]. *L. sphaericus* cannot utilize pentose, hexose, disaccharide, and polysaccharide as energy and carbon source. It was reported in some studies that *L. sphaericus* did not have a carbohydrate uptake system and some enzyme components in carbohydrate metabolism pathways (such as Embden-Meyerhof-Parnas and Entner Duodoroff pathways). Instead of sugars, *L. sphaericus* will use certain organic acids (such fatty acids), tricarboxylic acid intermediates, and amino acids [9, 23].

Toxicity of isolated *L. sphaericus*. Selective toxicity (initial toxicity) value of each isolated *L. sphaericus* was presented in Table 3 as follows. From 11 *L. sphaericus* isolated, only one isolate was unable to kill *Ae. aegypti* larvae in 3-day observation. Three *L. sphaericus* isolates showed the highest killing rate in 3-day observation, i.e., isolate Bs9-2-3, Bs9-1-5, and 36.67%. Seven other isolates showed a lower killing rate. Something interesting was seen in the killing rate of these three isolates, only isolate Bs9-1-5 showed the highest killing rate at the first 24-hour observation. Two other isolates, Bs9-2-3 and Bs2-1-2, showed the highest killing rate at 48-hour application.

The toxicity of *L. sphaericus* in certain isolates was mainly the effect of toxin type in the cell. There are two types of toxin protein known carried by a toxic strain of *L. sphaericus*. The first toxin was binary toxin (Bin/Btx), a potent toxin for killing mosquitoes, and the second one was a mosquitocidal toxin (Mtx). The mosquitocidal toxin was much weaker in toxicity compared to the binary toxin. Since it was more sensitive against bacterial protease of *L. sphaericus* [24]. In this study, the detection of those toxins was not carried out due to the time limitation of the study.

All sewages from which mud sampling was done, were not significantly different from one to other sewages. Therefore, it was unknown whether the sewage characteristics affected the toxicity difference.

Table 2. Biochemical and physiological characteristics of isolated *L. sphaericus*

Characteristics/ Test	Isolate										
	Bs1-2-1	Bs2-1-2	Bs3-1-2	Bs4-1-1	Bs5-2-3	Bs6-1-2	Bs7-1-1	Bs8-1-1	Bs9-1-5	Bs9-2-3	Bs10-1-6
Characteristics											
Glucose	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-
Manitol	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	-	+	+	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+
Growth on temperature …											
30ºC	+	+	+	+	+	+	+	+	+	+	+
40ºC	+	+	+	+	+	+	+	+	+	+	+
50ºC	-	-	-	-	-	-	-	-	-	-	-
Growth on NaCl concentration …											
3%	+	+	+	+	+	+	+	+	+	+	+
5%	-	+	+	-	+	-	-	-	-	+	-
10%	-	-	-	-	-	-	-	-	-	-	-

Table 3. Selective toxicity (initial toxicity) value of each isolated *L. sphaericus*

Isolate	Larval Death (%)			Housing Complex Location
	24 hours	48 hours	72 hours	
Bs1-2-1	0%	3.33%	19.97%	Perumnas
Bs2-1-2	36.67%	96.67%	96.67%	Bumi Kodya Asri
Bs3-1-2	0%	3.33%	6.66%	BTN Royal
Bs4-1-1	0%	0%	3.33%	Pagutan Asri
Bs5-2-3	3.33%	10%	13.33%	Taman Indah
Bs6-1-2	0%	0%	3.33%	Komplek Lumba-Lumba
Bs7-1-1	0%	3.33%	6.66%	Elit Kota Mataram Asri
Bs8-1-1	0%	0%	0%	Taman Baru
Bs9-1-5	73.33%	93.33%	93.33%	Permata Indah Selagalas (1)
Bs9-2-3	10%	80%	86.67%	Permata Indah Selagalas (2)
Bs10-1-6	0%	3.33%	3.33%	Bumi Gora Permai

Selective/initial toxicity test of isolated *L. sphaericus* is presented in Table 4. *L. sphaericus* isolate of Bs9-2-3 and Bs9-1-5 showed the lowest LC50 and LC90 values in 24-hour observation against *Ae. aegypti* larvae. This indicated that the isolates were very toxic to *Ae. aegypti* larvae. *L. sphaericus* had been isolated from the Lombok beach land area and tested against third *Ae. aegypti* in 2015. The LC value from previous study was 1.72 x 10^7 to 4.45 x 10^7 cell/mL [25]. *L. sphaericus* isolated in this study clearly demonstrated higher toxicity compared to *L. sphaericus* isolated in the previous study. We suggest that habitat type have great effect on the toxicity potential of *L. sphaericus*.

There are several possibilities allowing some *L. sphaericus* isolates from sewage to have such high toxicity. Firstly, contact with mosquito larvae was higher/more frequent in sewage/water habitats than inland. Secondly, organic contents in sewage/water were easily absorbed and utilized by the bacteria to synthesize its toxin protein, rather than those in the land habitat [26].

Table 4. Quantitative toxicity assay (in Lethal Concentration values) of each isolated *L. sphaericus*

L. sphaericus Isolate	Lethal Concentration Values	
	LC50-24 hour (cell/mL)	LC90-24 hour (cell/mL)
Bs2-1-2	7.17×10^6	1.18×10^8
Bs9-1-5	6.23×10^4	1.41×10^5
Bs9-2-3	1.75×10^4	1.07×10^9

Toxins and toxicity of *L. sphaericus*. The toxicity of *L. sphaericus* was contributed by some protein toxins owned by this species. There were two main toxin proteins, binary toxin (abbreviated by Bin/Btx) and mosquitocidal toxin (abbreviated by Mtx). The binary toxin consisted of two subunits, A/BinA (41.9 kDa) and B/BinB (51 kDa). These subunits were synthesized equitably, forming parasporal crystal that was clearly seen in stage III of sporulation event [9].

The mosquitocidal toxin consisted of three subunits, Mtx1 (100 kDa), Mtx2 (31.8 kDa), and Mtx3 (35.8 kDa). This protein was synthesized by *L. sphaericus* during vegetative growth. Compared to the binary toxin, Mtx toxin has lower toxicity to be able to kill larvae [27].

Most *L. sphaericus* strains that demonstrate high toxicity against some mosquito larvae, synthesize both binary (Bin/Btx) and mosquitocidal toxins (Mtx). Lower-toxicity strains of *L. sphaericus* only synthesized one of those toxins, mostly Mtx. However, Mtx toxin was prone to be degraded by proteases produced by *L. sphaericus* [28]. Besides toxins produced by the bacteria, the killing ability of *L. sphaericus* was affected by the existence of a-glucosidase receptor on microvillar brush border along midgut of targeted larvae. This receptor only existed in digestion tracts of *Culex* and *Anopheles* mosquito, but not or low number in *Aedes* [29].

The finding of new entomopathogenic isolates of *L. sphaericus* from sewage habitat in this study surely will enrich our knowledge on *L. sphaericus* toxicity characteristics on different habitats as well as add isolate collection to support the development of local biolarvicide in the near future.

CONCLUSION

In this study, we isolated 11 isolates of *L. sphaericus* from sewage at some housing complex in Mataram City. From these isolates, we have found 10 isolates were toxic against third instar *Ae. aegypti* larvae in the laboratory. Out of those ten isolates, three isolates were of the highest toxicity. They were Bs9-2-3, Bs9-1-5, and Bs2-1-2. This local isolate of *L. sphaericus* had good potential to be developed for bacteria-based biolarvicide for battling *Aedes* mosquito larvae in the near future.

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