INDIGENOUS *BACILLUS* SPECIES ISOLATED FROM *AEDES AEGYPTI* LARVAE: ISOLATION, LARVICIDAL TOXICITY SCREENING, PHENOTYPIC CHARACTERIZATION, AND MOLECULAR IDENTIFICATION

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ABSTRACT

Vector-borne diseases transmitted by mosquitoes are considered a significant public health problem worldwide. *Aedes aegypti* is one of the mosquito species responsible for transmitting these diseases. One environmentally friendly method of vector control is the use of microbial agents such as *Bacillus* species. This study aimed to explore investigate indigenous entomopathogenic bacteria of *Bacillus* species isolated from *A. aegypti* larvae. Larvae samples were collected from breeding sites of *A. aegypti*. All isolates underwent screening and affirmation confirmation tests to assess their larvicidal toxicity against *A. aegypti* larvae. Phenotypic characterizations and molecular identifications were conducted to determine the species of the *Bacillus* isolates based on similarity index and percent identity (%ID). Phylogenetic trees were used to compare the isolates with other *Bacillus* species. The results revealed 120 isolates of *Bacillus* species from *A. aegypti* larvae samples. Among them, three isolates (LS3.3, LS9.1, and LSD4.2) exhibited the highest larvicidal toxicity in the confirmation test, resulting in larval mortality rates of 100%, 96.7%, and 100%, respectively, after 48 hours of exposure. Molecular identifications, showed that LSD4.2 had a 99.16% ID with *Bacillus velezensis*, LS3.3 had a 98.22% ID with *Bacillus mojavensis*, and LS9.1 had a 99.93% ID with *Bacillus subtilis*. These three bacteria from the *Bacillus* genus have been reported to offer significant benefits to humans.

Keywords: *Aedes aegypti*, *Bacillus mojavensis*, *Bacillus subtilis*, *Bacillus velezensis*, Dengue vector, Larvicidal toxicity

INTRODUCTION

Dengue Fever (DF) is a vector-borne infection transmitted by mosquitoes, which is considered a significant public health problem worldwide (Dahmana *et al.* 2020). *Aedes aegypti* is the mosquito species responsible for transmitting this disease. Various attempts have been made to address the issue of DF, but the outcomes have fallen short of expectations. Extensive research has been conducted on developing vaccines to prevent this disease; however, satisfactory results have yet to be achieved. One alternative to combatting this disease is controlling the population of the vector (Melanie *et al.* 2018). Several measures have been taken to suppress the population

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of *A. aegypti*, including the use of chemical insecticides. However, the use of chemical insecticides has negative implications for environmental quality and is toxic to non-target organisms present in breeding sites for *A. aegypti* larvae (Dahmana *et al.* 2020).

Experts have suggested the development of bioinsecticides as biocontrol agents for disease vectors in response to the DF problem (Thomas 2018). Bioinsecticides are known to possess advantages such as specificity and safety for non-target organisms and the environment. One of the biocontrol agents being developed is entomopathogenic bacteria from the genus *Bacillus*. *Bacillus* sp. has been proven to be effective and highly specific, particularly toxic to the *A. aegypti* mosquito. Certain *Bacillus* species are capable of producing protein crystals along with spores during sporulation (Evdokimov *et al.* 2014). Numerous studies have demonstrated that multiple bacterial strains within the *Bacillus* genus have the potential to eliminate *A. aegypti* larvae, including *B. thuringiensis* and *B. sphaericus* (Boyce *et al.* 2013). These species exhibit high toxicity towards mosquito larvae while being safe for other parasites, predators, and mammals, in addition to causing no environmental pollution (Melanie *et al.* 2018). In general, *Bacillus* sp. can form endospores when confronted with unsuitable growth conditions that compromise their survival structure (Zeigler & Perkins 2015). The isolation and characterization of indigenous strains of *B. thuringiensis* from Saudi Arabia have been carried out (El-Kersh *et al.* 2016). Sixty-eight isolates have demonstrated larvicidal potential against the Malaria disease vector, *Anopheles gambiens* (El-Kersh *et al.* 2016). Similarly, *B. sphaericus* was isolated and characterized on Lombok Island, showing potential as a bio-insecticide for controlling the Malaria vector *A. aconitus* (Suryadi *et al.* 2016). Salamun *et al.* (2021) recently isolated a *Bacillus* species, *Bacillus thuringiensis* BK5.2, from Baluran National Park, East Java, Indonesia, which displayed high toxicity against *A. aegypti* larvae.

B. thuringiensis strains isolated and characterized from Lebanese soils have also proven to be effective (Fayad *et al.* 2019). These strains have been developed as bioinsecticides targeting agricultural pest insects (Kumar *et al.* 2021). Numerous scientific studies have explored the role of biocontrol agents and their potential in disease vector control (Thomas 2018). Toxins produced by *Bacillus* sp. exhibit specific activity against target insects (Schünemann *et al.* 2014). Microbial larvicides can be employed as environmentally friendly biological agents for disease vector control (Benelli *et al.* 2016). Building on previous studies utilizing natural soil samples collected in Baluran National Park, East Java, Indonesia (Salamun *et al.* 2021), our research aims to identify the diversity of *Bacillus* species isolated from *A. aegypti* larvae in DF endemic areas.

This study aims to isolate indigenous entomopathogenic *Bacillus* sp. from samples of *A. aegypti* larvae in their breeding sites in DF endemic areas, conduct screening and affirmation tests to determine the larvicidal toxicity of the isolates against *A. aegypti* larvae, perform phenotypic characterizations, and conduct molecular identification. The findings are expected to contribute to the development of diverse entomopathogenic *Bacillus* species as potential agents for the biocontrol of disease vectors, plant diseases, and pests.

MATERIALS AND METHODS

Materials

The materials and tools utilized in this study were employed for the isolation, larvicidal toxicity screening, phenotypic characterization, and molecular identification of *Bacillus* sp. from the aforementioned isolation. Samples of *A. aegypti* larvae were collected from water reservoirs serving as breeding sites for *A. aegypti* in Gresik, Surabaya, and Sidoarjo, East Java, Indonesia. Screening and affirmation of the larvicidal toxicity of *Bacillus* sp. were performed using third-instar *A. aegypti* larvae. The *A. aegypti* larvae were obtained from the Tropical Disease Institute, Universitas Airlangga, Surabaya, Indonesia.

Sampling of *Aedes aegypti* Larvae

Samples of *A. aegypti* larvae were collected from mosquito breeding sites, specifically water reservoirs. Identification of *A. aegypti* larvae samples was conducted following the Identification Key of *A. aegypti* larvae (Bar &

Andrew 2013). Previous studies have shown that *Bacillus* can be isolated from various sources, including soil, aquatic environments, herbivorous droppings, forest soil, dead insects, and mosquito breeding sites (Paul 2007; Zeigler & Perkins 2015; Suryadi *et al.* 2016). Five larvae per sample were extracted using a pipette and placed in sterile glass bottles.

Isolation of *Bacillus* sp.

Bacillus sp. was isolated from the Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, as conducted by Suryadi *et al.* (2016). Five *A. aegypti* larvae were sampled from a suspected location infected with entomopathogenic bacteria, where the larvae exhibited minimal or slow movement on the water's surface. All larvae were placed in a sterile test tube and macerated, followed by the addition of 9 mL of 0.85% NaCl solution. The mixture was allowed to sit for 5 minutes. A 10^1-10^2 dilution of the sample was prepared, heated at 70°C for 30 minutes, and then inoculated with 1 mL of nutrient agar (NA) using the pour plate method onto a sterile Petri dish. The solidified media was incubated at 30°C for 48 hours. The resulting colonies were subjected to spore stain. The *Bacillus* colonies were isolated on NA media using the streak method and stored at 4°C (Suryadi *et al.* 2016).

Larvicidal Toxicity Screening of *Bacillus* sp.

A pure *Bacillus* sp. isolate was inoculated into a sterile glass container containing 10 mL of Nutrient Yeast Salt Medium (NYSM) and incubated on a rotary shaker incubator at room temperature (35°C) for 48 hours (Suryadi *et al.* 2016). The absorbance value of the *Bacillus* sp. suspension was measured using a spectrophotometer at a wavelength of 600 nm (OD_{600nm}). For the screening of larvicidal toxicity, ten third-instar larvae of *A. aegypti* reared at the Entomology Laboratory of the Institute of Tropical Diseases, Airlangga University, were inoculated with a 5 mL suspension of *Bacillus* sp. in a bottle containing 45 mL of tap water. The control group consisted of 45 mL of well water, 5 mL of NYSM, and 10 *A. aegypti* larvae (Suryadi *et al.* 2016). The percentage of larvae that died after 24 and 48 hours of exposure was calculated. The screening for larvicidal toxicity was conducted with three replicates, using a mortality range of 60-100 larvae, and the absorbance was set to 0.8.

Phenotypic Characterizations

Morphological characterizations were conducted to determine the macroscopic and microscopic characteristics, such as the colony shape and the spore location. Three *Bacillus* sp. isolates with the highest potential were cultured on Petri dishes containing 8 mL of NA media using the streak method. The plates were then incubated for 48 hours and stained using the spore staining method. Physiological characterization included testing for indole production, motility, oxidase activity, starch hydrolysis, and salinity tolerance. Additionally, the Microbact 12A/12B kit was used for an additional test. For this test, 225µL of bacterial suspension was taken and added to each well of the kit. One drop of immersion oil was added to each well, and the results were observed after incubation at 37°C for 24 hours.

Molecular Identification

Molecular identification of bacterial isolates was conducted through the 16S rRNA gene (Kumar *et al.* 2016; Johnson *et al.* 2019). Initially, an isolated culture in 20 mL of NB media was incubated at 120 rpm and room temperature (35°C) for 48 hours. DNA extraction was performed using the CTAB method. The concentration and purity of the DNA were determined at 280 nm and 260 nm using the Multiskan GO. The 16S rRNA gene was amplified using the Eppendorf Mastercycler tool and the PCR method. The process involved adding GoTaq Green Master Mix and primers 16S rRNA, P0 (5'-GAG AGT TTG ATC CTG GCT CAG-3') and P6 (5'-CTA CGG CTA CCT TGT TAC GA-3'). The steps included denaturation at 94°C for 2 minutes, denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for one minute, and final elongation at 72°C for 5 minutes, repeated for 35 cycles of polymerase chain reaction (PCR). Amplicons were sequenced, and similarity analysis was conducted by comparing the data in GenBank using NCBI's BLASTn. The PCR visualization results were obtained by

electrophoresis of a 1% agarose gel stained with ethidium bromide and observed under UV light. Bacterial relationship analysis was performed by constructing a phylogenetic tree using the MEGA 6.0 application (Tamura *et al.* 2013).

Data Analysis

The results of the isolation and larvicidal toxicity screenings were analyzed using descriptive analysis. Bergey's Manual of Systematic Bacteriology (Paul *et al.* 2009) was utilized to obtain data on morphological and physiological properties of the local *Bacillus* sp. The similarity index percentage was calculated based on the positive and negative similarity of the characters of each isolate to determine the bacterial species of *Bacillus* sp. (Paul *et al.* 2009). Based on phenotypic characteristics, the similarity percentage pointed towards *Bacillus thuringiensis* and *Bacillus sphaericus*, bacteria that have demonstrated larvicidal activity against *A. aegypti* larvae.

The 16S rRNA gene, which had been amplified by PCR and confirmed by electrophoresis, was further purified and sequenced to determine the sequence of the 16S rRNA gene in bacterial isolates. The PCR results were then submitted to Malaysia's First Base DNA Sequencing Service. The sequencing results were edited using BioEdit Sequence Alignment Editor software version 7.2.5, and the similarity of the isolated 16S rRNA gene of *Bacillus* sp. with the gene data of bacteria in GenBank was determined using the Basic Local Alignment Search Tools (BLAST). The nucleotide BLAST analysis was conducted by the National Center for Biotechnology Information at the National Library of Medicine in Washington, DC and can be accessed at https://blast.ncbi.nlm.nih.gov/.

RESULTS AND DISCUSSION

Sampling and Isolation of *Bacillus* sp.

In the isolation of 30 samples (150 larvae) of *A. aegypti* larvae from Surabaya, Gresik, and Sidoarjo, East Java, Indonesia, and the map of sampling locations shown in Figure 1, there were 120 isolates of *Bacillus* sp. (Table 1). Larvicidal toxicity screening results with varying OD_{600nm} values were obtained for isolates of *Bacillus* sp., which exhibited potential diversity as entomopathogenic *Bacillus* sp. (Table 1 and Figure 2A).

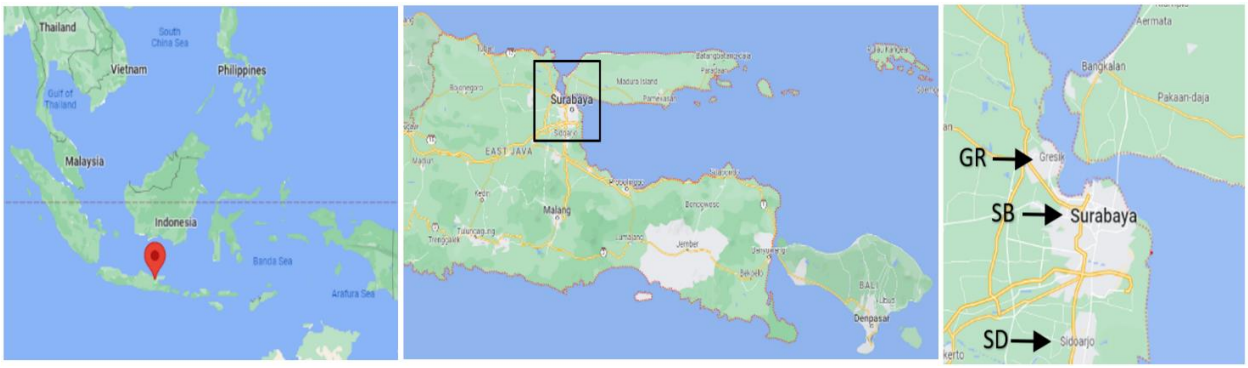

Figure 1 Map of Sampling Locations: Gresik (GR), Surabaya (SB), and Sidoarjo (SD), East Java, Indonesia

Table 1 Potency of Indigenous *Bacillus* sp. Isolates (OD_{600nm} varies) Based on the Results of Larvicidal Toxicity Screening Against *Aedes aegypti* Third Instar Larvae at 48-Hour Exposure

Sampling location (City)	Global positioning systems (GPs) of sampling locations	Sample codes	Number of isolates collection	Screening results of larvicidal toxicity				Culture turbidity of *Bacillus* sp. isolates (OD_{600nm})
				Up	Lp	Mp	Hp	
Surabaya	S07'03.293É112'42.460'	LS1	2	0	1	1	0	1.50 – 1.50
	S07'03.293É112'42.452'	LS2	4	1	1	2	0	1.00 – 1.25
	S07'03.293É112'42.447'	LS3	5	0	1	1	3	0.80 –1.50
	S07'03.293É112'42.434'	LS4	6	0	4	0	2	0.80 – 1.30
	S07'03.293É112'42.438'	LS5	4	2	1	1	0	1.00 – 1.20
	S07'03.293É112'42.455'	LS6	4	1	2	1	0	1.10 –1.50
	S07'03.293É112'42.446'	LS7	4	3	1	0	0	0.80 – 1.15
	S07'03.293É112'42.445'	LS8	3	0	3	0	0	1.00 – 1.35
	S07'03.293É112'42.452'	LS9	3	1	1	0	1	0.85 – 1.40
	S07'03.293É112'42.443'	LS10	4	2	1	0	1	0.95 – 1.50
Gresik	S07'03.293É112'34.459'	LG1	2	0	2	0	0	1.10 –1.10
	S07'03.293É112'34.471'	LG2	4	3	1	0	0	1.00 – 1.50
	S07'03.293É112'34.437'	LG3	4	0	2	2	0	0.40 – 1.30
	S07'03.293É112'34.436'	LG4	4	0	2	2	0	0.85 – 1.40
	S07'03.293É112'34.484'	LG5	4	4	0	0	0	0.95 – 1.50
	S07'03.293É112'34.515'	LG6	6	2	4	0	0	0.80 – 1.50
	S07'03.293É112'34.536'	LG7	5	0	3	1	1	0.50 – 1.20
	S07'03.293É112'34.530'	LG8	2	1	0	0	1	1.00 – 1.40
	S07'03.293É112'34.948'	LG9	5	1	2	2	0	0.80 – 1.50
	S07'03.293É112'34.965'	LG10	6	0	4	1	1	1.00 – 1.50
Sidoarjo	S07'03.293É112'45.472'	LSD1	6	3	3	0	0	0.75 – 1.40
	S07'03.293É112'45.578'	LSD2	4	2	2	0	0	0.55 – 1.50
	S07'03.293É112'45.491'	LSD3	3	0	1	2	0	1.10 – 1.30
	S07'03.293É112'45.264'	LSD4	2	0	0	0	2	0.90 – 1.10
	S07'03.293É112'45.624'	LSD5	3	1	1	1	0	0.80 – 1.35
	S07'03.293É112'45.623'	LSD6	5	4	0	1	0	1.10 – 1.40
	S07'03.293É112'45.432'	LSD7	4	2	0	0	2	0.40 – 0.95
	S07'03.293É112'45.536'	LSD8	3	0	1	2	0	0.90 – 1.40
	S07'03.293É112'45.542'	LSD9	3	0	2	0	1	1.10 – 1.15
	S07'03.293É112'45.541'	LSD10	6	2	4	0	0	0.65 – 1.50
			120	35	50	20	15	0.40 – 1.50

Descriptions: Up = Un-potential, larval mortality 0%; Lp = Low-potential, larval mortality <30%; Mp = Medium-potential, larval mortality 30-50%; Hp = High-potential, larval mortality >50%.

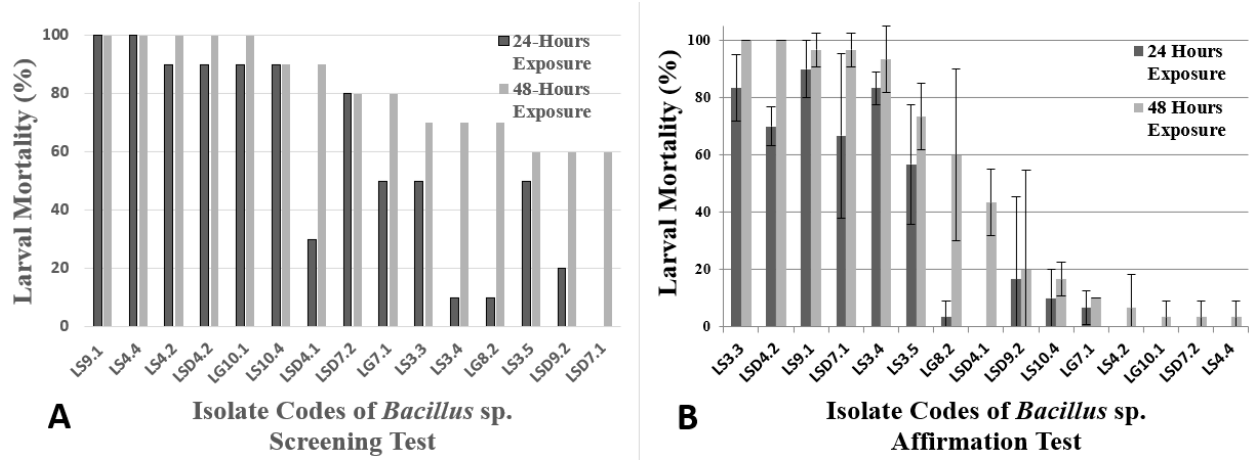

Figure 2 Results of the Larvicidal Toxicity Screening (A) (OD_{600nm} varies) with One Replication and the Affirmative Toxicity Test (B) (OD_{600nm} = 0.80) with Three Replications, Performed on 15 Isolates of Indigenous *Bacillus* sp. from Gresik (LG), Surabaya (LS), and Sidoarjo (LSD) Against *Aedes aegypti* Third-Instar Larvae at 24- and 48-Hour Exposure

Larvicidal Toxicity Screening of *Bacillus* sp.

The results of the Affirmative Toxicity Test (Fig. 2B) were conducted at turbidity of 0.80 (OD_{600nm}) from cultures of *Bacillus* sp. isolates. The correlation between turbidity and the concentration of *Bacillus* sp. (CFU/mL) yielded a regression line of Y=151.5+17.6, with a coefficient of determination (R2) of 0.9525, as depicted in Figure 3. Based on calculations, turbidity of 0.8 in *Bacillus* sp. cultures is equivalent to a bacterial cell count of 13.8x107 CFU/mL. Following the Affirmative Toxicity Test (Fig. 2B), the three isolates with the highest potential underwent phenotypic characterizations. The results of the phenotypic characterizations for these three isolates are presented in Figure 4 and Table 2.

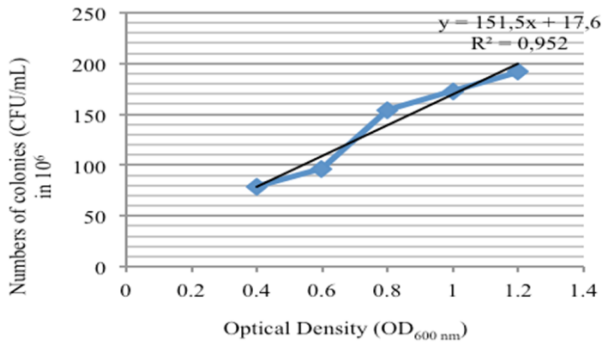

Figure 3 Standard curve for quantifying *Bacillus* sp. cell count (CFU/mL) in LSD4.2 isolate culture using optical density (OD_{600nm}) variation

Phenotypic Characterizations

The LS3.3 and LS9.1 isolates exhibited colonies with irregular shapes and flat elevations, while the LSD4.2 isolate had circular colonies with raised elevations. The size of the colonies for all three isolates was moderate. The margins of LS3.3, LS9.1, and LSD4.2 isolates were lobate, serrate, and entire, respectively. Microscopic characterization using spore staining (Fig. 4) revealed that LS3.3 and LSD4.2 isolates had spherical spores located at the terminal end, while the LS9.1 isolate had oval-shaped spores located at the subterminal end. Detailed phenotypic characterizations are provided in Table 2.

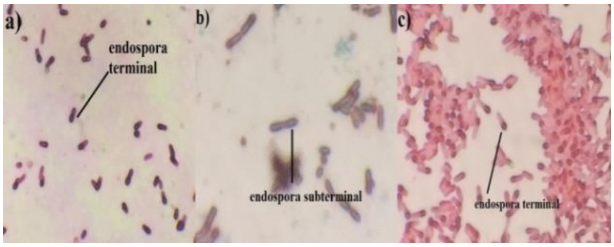

Figure 4 Spore location of endospores in *local Bacillus sp.* isolates using spore staining. Descriptions: a) LSD4.2 isolate; b) LS9.1 isolate; c) LS3.3 isolate

Table 2 Phenotypic characterizations based on the physiological tests of *Bacillus* sp. isolates coded LSD4.2, LS9.1, and LS3.3

No.	Physiological Tests	Characteristics of *Bacillus* sp.		
		LSD4.2	LS9.1	LS3.3
1.	Lysine	-	-	+
2.	Ornithine	-	-	-
3.	H_2S	-	-	-
4.	Glucose	-	-	-
5.	Mannitol	-	-	-
6.	Xylose	-	+	+
7.	ONPG	-	+	+
8.	Indole	-	-	-
9.	Urease	+	-	-
10.	VP	+	+	+
11.	Citrate	-	-	-
12.	TDA	-	-	-
13.	Gelatin	+	+	+
14.	Malonate	-	+	-
15.	Inositol	-	-	-
16.	Sorbitol	-	-	-
17.	Rhamnose	-	-	-
18.	Sucrose	-	-	-
19.	Lactose	-	-	-
20.	Arabinose	-	+	+
21.	Adonitol	-	-	-
22.	Raffinose	-	-	-
23.	Salicin	-	-	-
24.	Arginine	-	-	-
25.	Motility	+	+	+
26.	Katalase	+	+	+
27.	Oksidase	+	-	-
28.	Salinity 5%	-	+	+
29.	Salinity 10%	-	-	-
30.	Hidrolysis of Amylum	+	+	+

Molecular Identification

The results of PCR amplification of the 16S rRNA gene for three *Bacillus* sp. isolates, confirmed by electrophoresis, are shown in Figure 5. The third band of *Bacillus* sp. appeared at approximately 1500 bp.

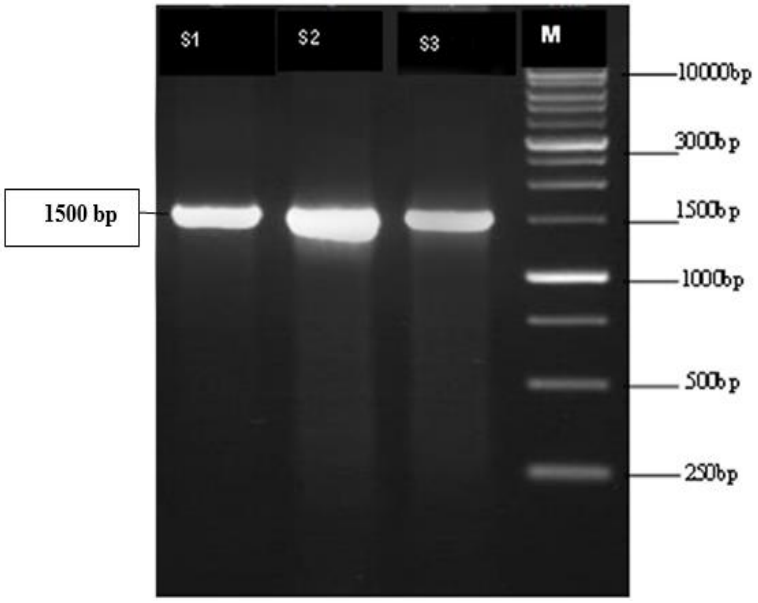

Figure 5 Confirmation of the 16S rRNA gene in three *Bacillus* sp. isolates using electrophoresis methods. (Descriptions: S1 = LSD4.2; S2 = LS3.3; S3 = LS9.1; M = Marker)

Table 3 shows the results of sequencing to identify the similarity of the 16S rRNA gene for *Bacillus* sp. using BLAST. Isolate code LSD4.2 had a 99.16% identity with *Bacillus velezensis*, LS3.3 had a 98.22% identity with *Bacillus mojavensis*, and LS9.1 had a 99.93% identity with *Bacillus subtilis*, respectively. The results of constructing the phylogenetic tree of *Bacillus* sp. on GenBank are shown in Figure 6.

Table 3 Similarity of *Bacillus* sp. based on sequencing of the 16S rRNA gene using the Basic Local Alignment Search Tools (BLAST) program

Isolates Code	Spesies Name	Accession No.	E value	% ID	Query Cover (%)
LSD4.2	*Bacillus velezensis* strain CBMB205	NR_075005.2	0.0	99.16	99
	Bacillus velezensis strain FZB42	NR_116240.1	0.0	99.02	99
LS9.1	*Bacillus subtilis* subs. inaquosorum strain BGSC 3A28	NR_104873.1	0.0	99.93	100
	Bacillus subtilis strain JCM 1465	NR_113265.1	0.0	99.86	100
LS3.3	*Bacillus mojavensis* strain IFO15718	NR_024693.1	0.0	98.22	99
	Bacillus halotolerans strain LMG 22477	NR_115931.1	0.0	98.11	99

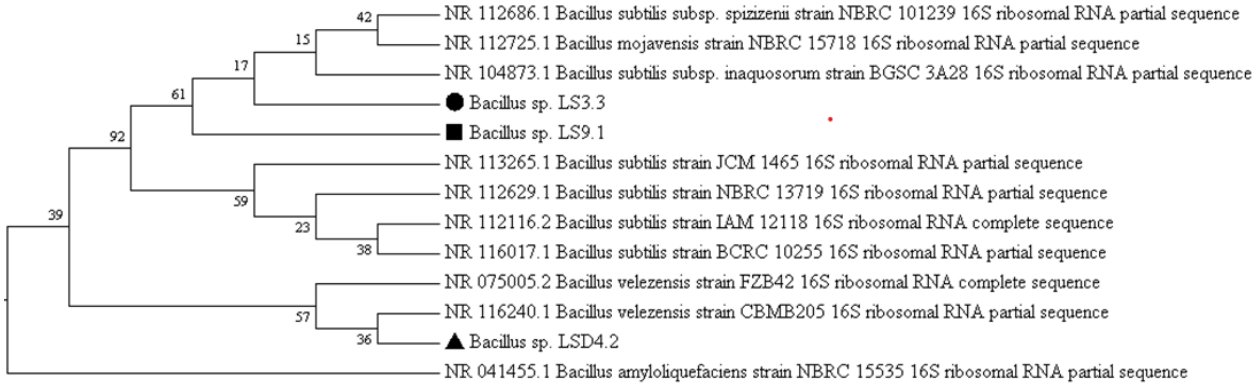

Figure 6 Phylogenetic tree of *Bacillus* sp. isolates coded LSD4.2, LS9.1, LS3.3, and their relationship to other *Bacillus* sp. in the GenBank database

In this study, the initial objectives were to isolate *B. thuringiensis* or *B. sphaericus* and screen their toxicity to *A. aegypti* larvae. Variations in the mortality rate of *A. aegypti* larvae due to exposure to *Bacillus* sp. were observed. Third-instar larvae of *A. aegypti* were used for both screening and confirming the larvicidal toxicity of *Bacillus* sp. (Table 1; Fig. 2A and 2B). A total of 120 isolates could be isolated from 150 samples of *A. aegypti* larvae collected from Surabaya, Sidoarjo, and Gresik cities in East Java, Indonesia. Among them, 15 isolates showed high potency in the larvicidal toxicity screening. The affirmation test of larval toxicity (Fig. 2B) revealed that three isolates exhibited the highest toxicity. The larvicidal toxicity screening using third-instar *A. aegypti* larvae was based on their sensitivity to entomopathogenic bacterial toxins (Kim *et al.* 2017). The older the larval instar, the lower their sensitivity to the bacterial toxin. Additionally, fourth-instar larvae exhibit less feeding habits compared to younger larvae, resulting in reduced consumption of bacterial toxins. Furthermore, during the pupal phase, feeding activity ceases (Aynalem 2022). In the affirmation test of *Bacillus sp.* LSD4.2 (Fig. 3), a concentration of 13.8 x 10^7 CFU/mL, caused 100% larval mortality after 48 hours of exposure, categorizing it as highly toxic. *B. thuringiensis* PWR4.32, isolated in Malang, Indonesia, exhibited a lethal concentration 50% (LC50) value of 22.79 x 10^7 cells/mL after 72 hours of exposure- (Gama *et al.* 2010). Similarly, *B. thuringiensis* W.Swh.S.K2, isolated in Nganjuk, Indonesia, had an LC50 value of 3.53 x 10^7 cells/mL after 48 hours of exposure (Pratiwi *et al.* 2013). *B. thuringiensis* BK5.2, isolated from Baluran National Park in East Java, Indonesia, showed an LC50 value of 8.3 x 10^6 cells/mL after 48 hours of exposure (Salamun *et al.* 2021). The results of this study indicate differences in larvicidal toxicity among different *Bacillus* sp. isolates, suggesting that these isolates may belong to different species or strains.

Bacillus sp. larvicidal toxicity can be identified through two mechanisms of action. During sporulation, bacteria produce an insecticidal toxin stored in parasporal inclusions. During the vegetative stage, bacteria produce secondary metabolites, such as enzymes or other chemical compounds, that are also insecticidal. The entomopathogenic action of *Bacillus* sp. involves the toxin produced during sporulation, which binds to intestinal cell receptors, causing pores to form in the intestinal cell membrane. This leads to the entry of ions to balance intracellular and extracellular fluids. Consequently, intestinal cells experience rapid damage, resulting in the lysis of epithelial cells. Infected larvae stop feeding for several hours, ultimately leading to their death (Polenogova *et al.* 2022). The endotoxin in the parasporal inclusion of the entomopathogenic *Bacillus* sp. also reduces the blood's acidity (pH), leading to larval death due to septicemia (Poopathi *et al.* 2013).

Other actions of entomopathogenic *Bacillus* sp. as bioinsecticides have also been reported. *Bacillus sp.* produces secondary metabolites, including biosurfactants, during bacterial growth in suitable media. The biosurfactant produced by the *B. subtilis* strain is composed of a mixture of molecules, some of which are toxic to arthropods and vectors (Sachdev & Cameotra 2013). Biosurfactant-producing bacteria have been found to be effective in controlling diseases in plants and insects (Zhao *et al.* 2014). Biosurfactants can affect the cuticle of insects due to their amphiphilic nature, which includes hydrophobic and hydrophilic molecules. This can damage cell membranes and epithelial cells and ultimately cause death (Zhao *et al.* 2014).

Based on the phenotypic characteristics (Table 2 and Fig. 4) and identification using Bergey's Manual of Systematic Bacteriology, the isolates coded LSD4.2 and LS3.3 showed similarity indices of 82.6% and 63.3%, respectively, with *B. sphaericus*. Isolate LS9.1 had a similarity index of 62.50% with *B. thuringiensis*. However, based on molecular identification using the 16S rRNA gene, these three *Bacillus* sp. isolates showed different results. They were identified as *B. velezensis*, *B. mojavensis*, and *B. subtilis* (Table 3; Fig. 6).

B. velezensis FZB42T, previously classified as part of the *B. subtilis* group due to its 99% genetic similarity, was later included in a different phylogenomics category based on additional genetic characteristics. This strain of *B. velezensis* produces unique intracellular biomolecules that have the potential for development through genetic engineering in various industries, including health, pharmaceuticals, environment, and food, particularly in agriculture (Adeniji *et al.* 2019).

Studies have shown that *B. velezensis* NKG-2 is useful as a potential biocontrol agent and promoter of plant growth (Myo *et al.* 2019). *B. velezensis* strain WLYS23 has great potential as a biocontrol agent for disease control in freshwater aquaculture (Zhang *et al.* 2021). *B. velezensis* 33RB is a potential alternative to chemical pesticides as a biological control agent for phytopathogens, offering environmentally friendly and sustainable properties (Dawwam & Sehim 2022).

The search for new biocontrol agents focuses on *Bacillus subtilis* and its related species, including *Bacillus mojavensis*. The metabolites produced by the *B. mojavensis* PS17 isolate from wheat germ inhibit the growth of the plant pathogen *Fusarium spp.*, indicating its potential as a biocontrol agent for agriculture (Diabankana *et al.* 2021). *B. mojavensis* shares similarities with *B. subtilis* but differs in fatty acid composition, DNA sequences, and resistance to genetic transformation (Bacon & Hinton 2002). *B. mojavensis* produces surfactin, iturin, and fengycin, which belong to an antimicrobial and antifungal lipopeptide group (Mounia *et al.* 2014; Blacutt *et al.* 2016). According to Jasim *et al.* (2016), the lipopeptide compounds surfactin and fengycin in *B. mojavensis* have antimicrobial activity against pathogenic bacteria, including both Gram-negative and Gram-positive strains. Hmidet *et al.* (2017) reported that *B. mojavensis* produces surfactin and fengycin, with optimal production occurring in media containing glucose. *B. mojavensis* demonstrated hemolytic activity on blood agar, suggesting the production of biosurfactants (Berekaa & Ezzeldin 2018). *B. mojavensis* BTCB15 is capable of producing 2.3 nm AgNPs and exhibits antibacterial activity against numerous drug-resistant pathogens (Iqtedar *et al.* 2019). In their study, Fanaei *et al.* (2021) discovered that *B. mojavensis* HF produces three types of lipopeptides: surfactin, fengycin, and kurstakin. They identified a wide variety and number of surfactin and fengycin isomers compared to previous reports and claimed to be the first to report the presence of kurstakin in *Bacillus mojavensis* species. Further research is needed to determine whether kurstakin is stored in parasporal inclusions or excreted as secondary metabolites.

B. subtilis also produces biosurfactant as a mosquitosidal toxin (Kumar *et al.* 2022). Mosquitosidal toxin activity has also been reported from *B. cereus* (Mani *et al.* 2017). Biosurfactants, synthetic compounds produced by several strains of *Bacillus* sp., have been used as biocontrol agents against insects (Mani *et al.* 2017). For example, *B. subtilis* isolated from soil has been introduced as a biological control agent for insects due to its production of surfactin (Kumar *et al.* 2022). *B. subtilis* (MW644765) mediated silver nanoparticles (AgNP) have shown promising larvicidal activity against mosquito larvae, making them a potential biocontrol agent for reducing mosquito populations (Wilson *et al.* 2022). *B. subtilis* is considered a universal cell factory for various industries such as agriculture, biomaterials, pharmaceuticals, and industry (Su *et al.* 2020).

Molecular identification results have identified three high-potential *Bacillus* species: *B. subtilis* (LS9.1), *B. velezensis* (LSD4.2), and *B. mojavensis* (LS3.3). Commercial products derived from *B. thuringiensis* and *B. sphaericus* have been used for the control of *A. aegypti* larvae (Boyce *et al.* 2013). The discovery of *B. velezensis*, *B. mojavensis*, and *B. subtilis* in this study is highly significant. These bacteria have been reported as multifunctional bacteria in various industries, including health, pharmaceuticals, environment, and food, and as biocontrol agents for disease vectors, plant pests, and disease control in freshwater aquaculture.

CONCLUSION

The results of the isolation and larvicidal toxicity screenings of *Bacillus* sp. against *Aedes aegypti* larvae revealed a range of potential larvicidal toxicity levels, varying from low to high. Screening 120 isolates of *Bacillus* sp. for larvicidal toxicity identified 15 isolates with high potency. The confirmation test identified three isolates with the highest potential. The larval mortality rates due to exposure to isolates LS3.3, LS9.1, and LSD4.2 were 100%, 96.7%, and 100%, respectively, after 48 hours of exposure. Molecular identification using the 16S rRNA gene revealed the diversity of the isolates, with isolate LSD4.2 sharing 99.16% identity with *Bacillus velezensis*, LS3.3 sharing 98.22% identity with *Bacillus mojavensis*, and LS9.1 sharing 99.93% identity with *Bacillus subtilis*. These three bacteria, belonging to the *Bacillus* genus, offer significant benefits for humans.

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