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Researchers found that the indigenous *Bacillus* species isolated from *Aedes aegypti* breeding sites, namely *Bacillus mojavensis* (EG6.4) had 97.89% similarity with *Bacillus mojavensis* strain NBRC 15718 and *Bacillus mojavensis* IFO 15718. The results of the larvicidal bioassay showed that the bacteria could kill larvae of *A. aegypti* in the category of high toxicity. Ultrastructural observations of bacterial cells did not Cry-toxin, but the hemolytic activity test showed positive results, so that the bacteria have produced biosurfactants with proven surfactin coding genes from these bacteria. The results of this study which are supported by the discussion of the literature review have shown that *Bacillus* sp. EG6,4 has the potential to be developed as a biocontrol agent for vector-borne diseases and plant pathogens or plant pests.

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Salamun

Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

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Abstract: Molecular identification, as well as the determination and mechanism of actions of the larvicidal toxicity of *Bacillus* sp. EG6.4 isolated from breeding-sites of *Aedes aegypti* was conducted from Gersik City, East Java, Indonesia. Previous studies of *Bacillus* sp. EG6.4 is a Gram-positive endospore-forming bacteria. Molecular species identification using 16S rRNA gene sequencing showed that the isolate had 97.89% similarity with *Bacillus mojavensis*. The isolate showed larvicidal toxicity against *A. aegypti* larvae. The Lethal Concentration 50% (LC_{50}) values at 24- and 48-hours exposure were 8.99±1.01 $x10^7$ cells/mL and 8.43±1.01 $x10^7$ cells/mL, respectively. Meanwhile, the Lethal Time 50% (LT_{50}) value was 11.9±1.1 hours. To determine the larvicidal mechanisms, whether due to the production of chitinolytic enzymes or biosurfactants, chitinolytic and hemolytic assay was conducted, respectively. As a result, *Bacillus* sp. EG6.4 showed hemolytic but not chitinolytic activity, indicating its potency to produce biosurfactants. The detection using Transmission Electron Microscopy (TEM) showed that the isolate has oval-shaped endospores that were located subterminal with massive-shape parasporal inclusions. The detection of srfA-D gene showed that isolate produced surfactin biosynthesis thioesterase. Thus, *Bacillus* sp. EG6.4 produced biosurfactant that potentially to be developed as a biocontrol agent for disease vectors and plant pathogens.

Keywords: *Aedes aegypti*, *Bacillus mojavensis*, Biosurfactants, Larvicidal toxicity, parasporal inclusion, srfA-D gene

Running title: Potential biocontrol agent of indigenous *Bacillus mojavensis* EG6.4

INTRODUCTION

The biodiversity of microorganisms is one of the abundant renewable biological resources in the world, including in Indonesia. The use of *Bacillus* in the biocontrol of vectors, pests, and plant diseases has grown rapidly (Benelli et al. 2016). *Bacillus thuringiensis* var. *israelensis* (Bti) used as a dengue vector biocontrol agent (BCAs) for the controlling of *A. aegypti* larvae (Boyce et al. 2013). During sporulation, *B. thuringiensis* produce parasporal inclusions and the resulting Cry toxin causes the death of several insect species (Aramideh et al. 2016).

The group of spore-forming bacteria will stop their growth under abnormal conditions and begin to increase their metabolic activity (Shahcheraghi et al. 2015). Sporulation is an effort to defend bacteria against an unfavorable environment. Several *Bacillus* species are reported to produce a protein toxin (Cry toxin) and also secondary metabolites that act as self-defense to survive. In the vegetative phase, bacteria grow and develop, but in unfavorable conditions will produce secondary metabolites such as biosurfactants, enzymes, or exotoxins to maintain their lives. Biosurfactant synthesis is associated with hemolytic activity, according to Carrillo et al. (1996). The hemolytic activity assay is a typical screening method for detecting biosurfactant activity. Biosurfactant generated by *Bacillus* sp. was found to be effective as a plant pathogenic and insecticidal agent in several tests (Bais et al. 2004). Nafidiastri et al. (2021) reported that indigenous *Bacillus velezensis* ES4.3 resulted biosurfactant activity was indicated by the formation of clear zones, emulsions, and a decrease in surface tension and the surfactin gene showed a 100% ID with the surfactin biosynthesis thioesterase SrfA-D gene.

Isolation and characterization of indigenous *Bacillus* entomopathogenic species have been isolated from the breeding sites of *A. aegypti* in Surabaya, Gresik, and Sidoarjo, East Java, Indonesia (Salamun et al. 2020). According to preliminary test, about 133 *Bacillus* sp. is entomopathogenic against larvae of *A. aegypti*. The majority of *Bacillus* sp. isolates showed mild to moderate toxicity, but 16 isolates were highly toxic, including *Bacillus* sp. EG6.4, which causes 100% larval mortality. Salamun et al. (2020) identified microscopic, macroscopic, and biochemical phenotypic similarities indicating that *Bacillus* sp. EG6.4 had a similarity level of 80.60% with *B. thuringiensis*.

Further research is needed for molecular identification to determine species names of *Bacillus* sp. EG6.4 and genetic relationships with other bacilli, bioassays to determine larval mortality, and to detect the mechanism of action by detecting the presence of paraspore inclusions, surfactin coding genes, and hemolytic and chitinolytic activities, so it is hoped that the mechanism of action of *Bacillus* sp. EG6.4 as larvicidal can be identified. This study aimed to molecular identification and larvicidal toxicity and larvicidal mechanism of the indigenous *Bacillus* sp. EG6.4. The Identification of the species name is conducted with the 16S rRNA gene and traces genetic affinity through a phylogenetic tree. The larvicidal activity to determine the LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} values. To determine the larvicidal

mechanisms of action, with detection of endospore by TEM and SEM, surfactin coding genes (srfA-D) and detection of secondary metabolite activity by hemolytic and chitinolytic activity assay.

MATERIALS AND METHODS

Molecular Identification

The DNA isolation

The genomic DNA of *Bacillus* sp. EG6.4 was isolated using the Promega DNA Purification Kit (Promega, 2018). The isolated DNA was visualized by electrophoresis. The results of genomic DNA electrophoresis of *Bacillus* sp. EG6.4 was photographed under a UV Transilluminator. Ratio of absorbance at 260 nm and 280 nm is use assessed Purity of genomic DNA by Thermo Scientific Multiskan GO Microdroplet Spectrophotometer.

The analysis of 16S rRNA gene

The isolated DNA of *Bacillus* sp. EG6.4 was amplified using the Eppendors Mastercycler with the PCR method and universal primers 27F and 1492R at 1st Base Sequencing Service in Singapore. The Bio Edit Sequence Alignment Editor software version 7.2.5 was used to evaluate the data from the sequencing results. The 16S rRNA gene nucleotide sequence from *Bacillus* sp. EG6.4 was matched with 16S rRNA gene sequences from other bacteria reported to GenBank. The Basic Local Alignment Search Tool (BLAST) at The National Library of Medicine, National Center for Biotechnology Information in Washington, DC (http://blast.ncbi.nml.nih.gov/) was used to find homology of the 16S rRNA gene.

Analysis of phylogenetic tree

The results of genomic DNA sequencing of *Bacillus* sp. EG6.4 which has been compared with other bacteria through BLAST was then compiled to determine the relationship through a phylogenetic tree was created by Mega 7 program. A phylogenetic tree was created by including FASTA from other species. All of these species were selected based on nucleotide sequence of 16S rRNA gene, because relationships of a species with other species can be seen from the sequence.

Bioassay larvicidal toxicity

Determination of the LC of *Bacillus* sp. EG6.4 against *A. aegypti* larvae was prepared by inoculating on NYSM broth were incubated on a rotary shaker (130 rpm) at 30°C for 72 hours. Then, bacterial density was determined by spectrophotometer at A_{600nm}. Bioassays were conducted by Suryadi et al. (2016). The final culture concentration was adjusted to variations concentration of (v/v) NYSM. Approximately of 20 *A. aegypti* larvae was tested at six concentrations. each treatment in triplicate. The larvae were seen to die after 24- and 48-hours exposure. Lethal Time of *Bacillus* sp. EG6.4 of *A. aegypti* larvae was determined at a concentration of LC_{90}, as many as 20 larvae were exposed, each treatment had three replications. Mortality of the larvae was scored after 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48-hours exposure along with NYSM (10% v/v) without inoculum as negative controls.

Bioassay of larval mortality (%) used to determine LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} of *Bacillus* sp. EG6.4 against *A. aegypti* larvae. Probit analysis using Minitab Version 17 was used to calculate the toxicity of *Bacillus* sp. EG6.4 against *A. aegypti* (Postelnicu, 2018).

Larvicidal Mechanisms

Detection of parasporal inclusion

The process of detecting *Bacillus* sp. EG6.4 parasporal inclusions included multiple phases, including rejuvenation, purification, and growth on NYSM broth. At 30 ^{0}C, cultures were cultured in a shaker incubator (130 rpm). After producing a pure liquid culture of *Bacillus* sp. EG6.4, TEM examination was performed at the Eijkman Institute's TEM and Histology Laboratory in Jakarta, while SEM study was performed at LPPT Gadjah Mada University in Yogyakarta using an SEI 10 kV WD10mm SS30.

Hemolytic and chitinolytic activity

Hemolytic activity was tested using blood agar media acquired from the Surabaya Health Laboratory Center and utilized to screen *Bacillus* sp. EG6.4 for haemolytic activity. Colony of isolates cultured for two days at room temperature. The formation of distinct clean zones surrounding a colony. Chitinolytic activity was carried out by growing *Bacillus* sp. EG6.4 on colloidal chitin agar media by the streak method. Observation of the clear zone around the colonies was carried out after an incubation period of 4 days, by adding Congo red dye to Petri and then washing it with NaOH.

Detection of surfactin-coding gene

Researchers used the DNA obtained from the identification of the 16S rRNA gene stage in this stage. The approach for detecting the biosynthetic surfactin gene is similar to that for identifying the 16S rRNA gene, with the exception of the primer employed. On the page ThermoFisher Scientific Oligo Perfect Primer Designer cloning application, the srfA-D gene primers are self-designed.

RESULTS AND DISCUSSION

Results

Molecular identification

Molecular identification (**Figure 1; Table 1**) showed that the *Bacillus* sp. EG6.4 had 97.89% similarity with *Bacillus mojavensis* strains NBRC 15718 and *Bacillus mojavensis* IFO 15718 and was closely related to *Bacillus halotolerans* strains DSM 8802 and *Bacillus halotolerans* LMG 22476 (**Figure 2**).

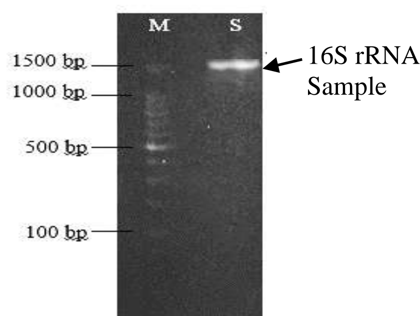

Figure 1. The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M=100bp DNA marker; S= sample of 16S rRNA gene.

Table 1. The name of species of indigenous *Bacillus* sp. EG6.4 based on 16S rRNA gene analysis by Basic Local Alignment Search Tool (BLAST)

No.	Species Name with 16S rRNA Gene	Accession	E value	%ID	Query Cover
1	*Bacillus mojavensis* strain NBRC 15718	NR 112725.1	0.0	97.89	100%
2	*Bacillus mojavensis* strain IFO 15718	NR 024693.1	0.0	97.89	100%
3	*Bacillus halotolerans* strain DSM 8802	NR 115063.1	0.0	97.89	100%

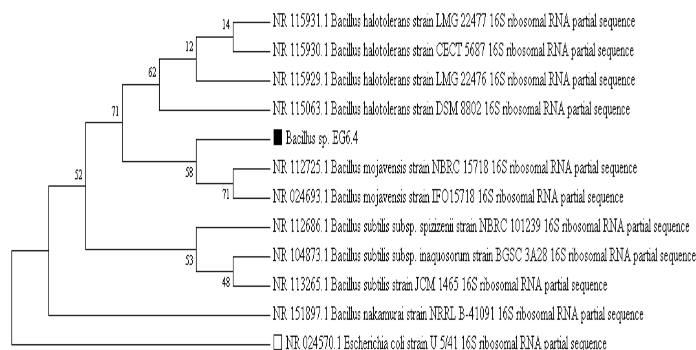

Figure 2. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application

Bioassay

The results of bioassay of *Bacillus* sp. EG6.4 against third instar larvae of *A. aegypti* larvae (**Table 2**). The results of the Probit analysis, the LC_{50} values at 24- and 48-hours exposure were 8.99±1.01 x10^7 cells/mL and 8.43±1.01 x10^7 cells/mL, respectively (**Figure 3**). Meanwhile, the LT_{50} value was 11.9±1.1 hours (**Figure 4**).

Table 2. The effect of indigenous *Bacillus* sp. EG6.4 concentrations (OD_{600nm} and CFU/mL) on mortality of *Aedes aegypti* third instar larvae (%) after 24- and 48-hours exposure.

Treatments (Concentration Series)	Culture (per-10 mL NYSM)	OD_{600nm}	CFU/mL	24 h exposure, larval mortality = %	48 h exposure, larval mortality = %
C_1	0.5 mL	0.07	09.8 x 10^7	6.7 ± 5.8	6.7 ± 5.8
C_2	1.0 mL	0.15	2.29 x 10^7	20 ± 10	26.7 ± 5.8
C_3	2.5 mL	0.37	5.90 x 10^7	43.3 ± 15.3	43.3 ± 15.3
C_4	5.0 mL	0.75	12.1 x 10^7	73.3 ± 5.8	76.7 ± 5.8
C_5	10 mL	1.50	24.4 x 10^7	93.3 ± 5.8	93.3 ± 5.8

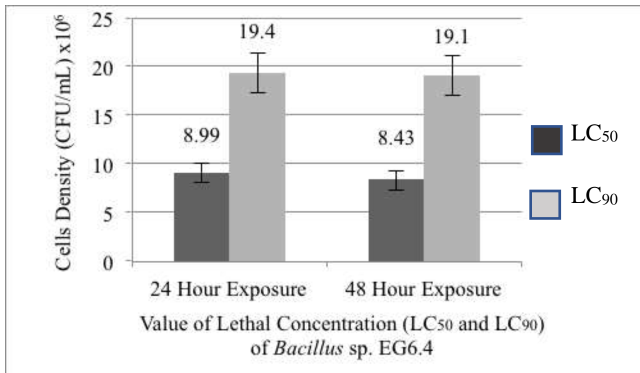

Figure 3. Value of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48 hours of exposure

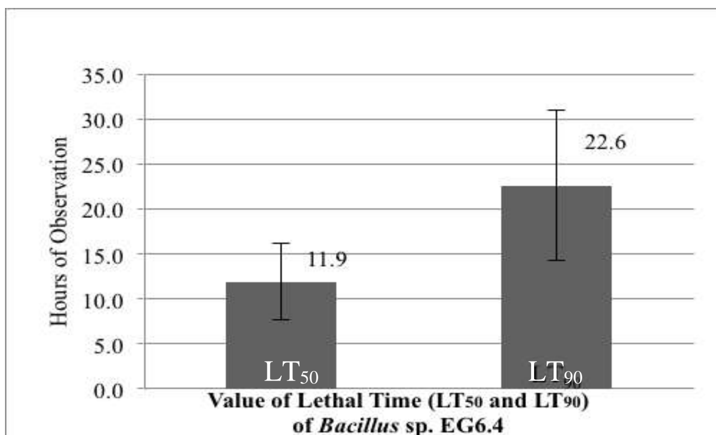

Figure 4. The Lethal Time 50% (LT_{50}) and Lethal Time 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae on observation: 0; 0.5; 1; 2; 4; 8; 10; 20; 24; 48-hours exposure

Larvicidal Mechanism

Detection parasporal inclusion

Bacillus sp. EG6.4 is a Gram-positive and endospore-forming bacteria. Detection using TEM showed subterminal oval-shaped endospores (Salamun et al. 2020). Detection using TEM showed massive paraspore inclusions (Figure 5). Detection using SEM only showed spores and did not the Cry toxin (Figure 6).

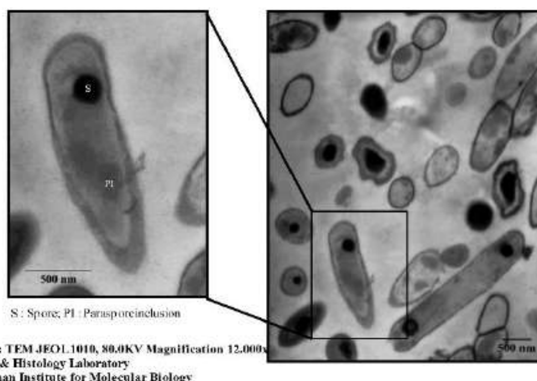

Figure 5. The organelles of indigenous *Bacillus* sp. EG6.4 endospore cells were visible using transmission electron microscopy (TEM). TEM JEOL1010 80.0 kV, 12.000x magnification, S=Forespore, PI=Parasporal Inclusion

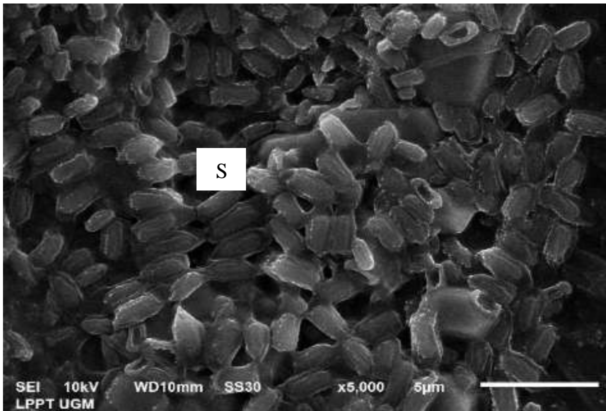

Figure 6. Scanning Electron Microscope (SEM) showed only visible spore of indigenous *Bacillus* sp. EG6.4, in SEI 10kV WD10mm SS30 x5,000 magnification (5µm), by LPPT UGM, Yogyakarta.

Hemolytic and chitinolytic activity

The results of hemolytic and chitinolytic activity assay, showed that hemolytic but not chitinolytic activity (**Figure 7**), indicating its potential to produce biosurfactants.

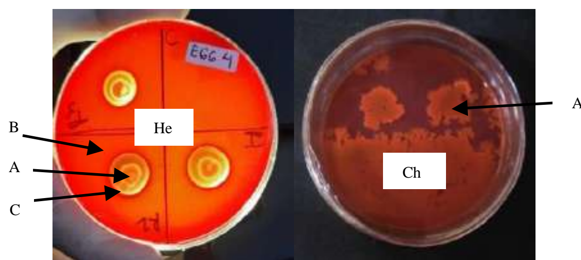

Figure 7. Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal chitin plate media (Ch). Note: A=Colony of isolate; B=Blood agar plate, C=Clear zone around colony of isolate

Detection of surfactin-coding gene

The electrophoresis results (Figure 8) showed a band of about 700 bp. Based on data in GenBank CP0514641, the srfA-D gene of *B. mojavensis* was 729 bp. The srfA-D gene has encoded a thioesterase enzyme that is important in the surfactin biosynthetic pathway. Band position on electrophoretic visualization showed that *B. mojavensis* EG6.4 had the srfA-D gene.

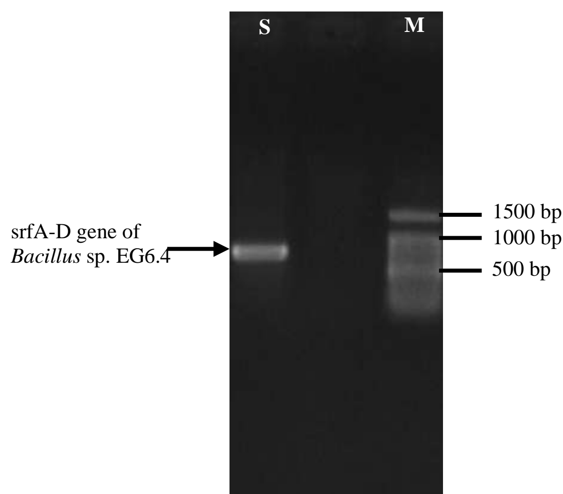

Figure 8. Electrophoresis results of the srfA-D gene *Bacillus mojavensis* EG6.4 which was successfully amplified. Description M=500bp DNA marker; S=Sample of srfA-D gene.

Table 3. Results of the Basic Local Alignment Search Tool (BLAST) nucleotide analysis the srfA-D gene of *Bacillus mojavensis* EG6.4

Species Name with srfA-D Gene	No. Accession	E. value	QC	% ID	Description
Bacillus mojavensis strain PS17	CP066516.1	0,0	99%	98,35%	Complete genome
Bacillus mojavensis strain UCMB5075	CP051464.1	0,0	100%	94,92%	Complete genome

The nucleotide BLAST results from the srfA-D gene of *B. mojavensis* EG6.4 showed a similarity value of 98.35% with the gene in *B. mojavensis* strain PS17 and had a similarity value of 94.92% with *B. mojavensis* strain UCMB5075 (**Table 3**). Further studies, the results of protein BLAST against the amino acid gene srfA-D *B. mojavensis* EG 6.4 obtained the highest similarity of 98.35% with surfactin biosynthesis thioesterase SrfA-D from the bacterium *B. mojavensis* strain PS17.

Discussion

The results of the isolation of *Bacillus* sp. EG6.4 has DNA purity = 1.84 (A260/A280), DNA concentration 44.7 (ng/µl), has been confirmed by electrophoresis showing DNA size is well above 1500bp. The DNA migration during electrophoresis is influenced by agarose concentration, DNA molecule size, voltage, and temperature (Fatchiyah et al. 2011; Brown, 2016). The results of the 16S rRNA gene amplification were visualized by electrophoresis (**Figure 1**), showed that a band measuring about 1550bp and about 500 bases at the end of the sequence is a hypervariable region. Pearson (2014) reported that the 16S rRNA gene can be used for the molecular identification of microorganisms. The results of purification and sequencing of the 16S rRNA gene of *Bacillus* sp. EG6.4 showed a nucleotide sequence with a size of 1424 bp. Based on BLAST analysis, *Bacillus* sp. EG6.4 is similar to *B. mojavensis* (**Table 1**) and is related to other bacteria (**Figure 2**).

Bacillus sp. EG6.4 has a close relationship with *Bacillus mojavensis* and *Bacillus halotolerans*. *B. mojavensis* and *B. halotolerans* are two species of bacteria that are closely related to *Bacillus subtilis*, however it differs significantly in terms of fatty acid content, DNA sequences, and transgenic species resistance (Bacon and Hinton 2002). *B. mojavensis* have been known that produce secondary matabolites such as oxygenated monoterpenes and lipopeptides, group of biosurfactants, that have promising applications in agricultural, food industry, and clinical fields. In agriculture, *B. mojavensis* as antimicrobial agent against pathogenic bacteria and fungi (Camele *et al.,* 2019). *B. halotolerans* has been known that produces biosurfactants. Based on research reported by Wang et al. (2002), biosurfactants from *B. halotolerans* can be used as agent to remediation in polluted environment petroleum.

The values of LC_{50} and LC_{90} are the concentrations of microorganisms that cause 50% and 90% mortality of the target organism population. The lower the LC value of microorganisms, the higher the toxicity. Toxicity of *Bacillus* sp. influenced by the type of toxin or secondary metabolites produced by microorganisms, the species and age of the target larvae, and the media used for bacterial growth (Suryadi et al. 2016). Determination of LC_{50} and LC_{90} as well as LT_{50} and LT_{90} was carried out to determine the concentration and length of time required by *Bacillus* sp. EG6.4 to kill 50% and 90% and the length of time to kill 50% and 90% of third instar *A. aegypti* larvae. Based on the mortality rate of *A. aegypti* larvae in the treatment, *Bacillus* sp. EG6.4 in the category of high toxicity. Based on the mortality rate of *A. aegypt*i larvae by *Bacillus* sp. EG6.4, indicates that the isolate has the potential to be developed as a biolarvacidal agent.

The value of LC_{50} and LC_{90} values of *Bacillus* sp. EG6.4 has high potential (Figure 3), when compared to previous studies. Gamma et al. (2010) reported that *B. thuringiensis* PWR4 32 isolated from Malang, Indonesia had an LC_{50} value of 22.79 x 10^7 cells/mL at 72-hour exposure. Pratiwi et al. (2013) have reported that *B. thuringiensis* W.Swh.S.K2 isolated from Nganjuk Indonesia, had an LC_{50} value of 3.53x10^7 cells/mL at 48-hour exposure. The number of spores consumed by the larvae affects the rate of larval death. Gamma et al. (2010) found that as B. thuringiensis spores developed, more Cry was released to kill A. aegypti larvae. The two isolates have different strains or species, so there is a difference in toxicity between them. One of the characteristics of *Bacillus* sp. can produce endospores and protein crystals (parasporal inclusions) at the time of cell sporulation.

Bacillus sp. EG6.4 shows a complete endospore with sections including a spore layer, spore, and paraspore inclusions (**Figure 5; Figure 6**). *Bacillus* sp. EG6.4 produces paraspore inclusions, but the shape is massive and has not been proven as a protein toxin that has larvicidal activity against *A. aegypti* larvae, so it is necessary to further investigate whether the components of paraspore inclusions cause larval death. Iftikhar et al (2018) reported that *B. mojavensis* BTCB15 synthesized silver nanoparticles as nanosides against the larvae of *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, important vectors of disease transmission. The LC_{50} and LC_{90} for third instar larvae of the three species

were 0.80, 0.75, and 1.2 ppm, and 0.80, 0.67, and 0.86 ppm, respectively. Iftikhar et al (2018), recommended the development of silver nanoparticles produced by *B, mojavensis* BTCB15, which may play a role in combating mosquito populations, thereby controlling vector-borne diseases. The larvicidal activity of silver nanoparticles (Ag/AgCl NPs) synthesized using Bti supernatant showed that LC_{50}=0.133 g/mL, higher toxicity than that synthesized using insecticidal protein, LC_{50}=0.148–0.217 g/mL (Chimkhan et al. 2022). Possible mechanism of action of Ag/AgCl NPs against *A. aegypti* larvae is through mitochondrial dysfunction, DNA and protein damage, inhibition of cell proliferation, and cell apoptosis, and recommended that Ag/AgCl NPs are an alternative approach to control *A. aegypti* larvae, a mosquito-borne disease vector (Chimkhan et al. 2022). Further research needs to be done, whether *Bacillus* sp. EG6.4 can produce silver nanoparticles that have a larvicidal effect on *A. aegypti* larvae.

Hemolytic activity assay of *Bacillus* sp. EG6.4 on blood agar showed a positive result (**Figure 7**). Biosurfactants have been utilized as an alternative to chemical pesticides in the past. Previous studies found that biosurfactant-producing bacteria are suitable for controlling plant pathogenic and insecticidal (Bais et al. 2004; Zhao et al. 2014). *B. subtilis* has been reported as bacterial agent have producting mosquitocidal toxin (Das and Mukherjee 2006; Manonmani et al. 2011). The toxicity of biosurfactants or their potential as biocontrol agents has not been well investigated. Biosurfactants, which are surfactin produced by *Bacillus* strains, have been offered as a potential alternative to mosquito-borne disease control. Biosurfactants produced by *Bacillus* strains have been shown to have mosquitocidal activity in adult mosquitos, killing both immature and adult mosquitos (Geetha et al. 2012). The mosquito's pupal phase relies solely on its trumpet for breathing, but the surfactant-induced decrease in surface tension of the water prevents the trumpet from keeping up with its position on the water's outer layer. As a result, the pupa loses contact with the air and dies from respiratory arrest. The low oxygen concentration causes the insect spiracles to continue to open and can cause the insect to die. Other studies have shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects (Zhao et al. 2014). From these studies, biosurfactants are known to be able to affect the cuticle of insects, due to their amphiphilic nature in the presence of hydrophobic and hydrophilic molecules, and damage cell membranes, damage epithelial cells, and cause death (Zhao et al. 2014).

B. mojavensis has been shown to create lipopeptides, which have a unique structure and are important antibacterial and antifungal substances derived from surfactin, iturin, and fengysin (Mounia et al. 2014; Blacutt et al. 2016). According to Jasim et al. (2016), lipopeptide in *B. mojavensis* has antiviral, antitumoral, antifungal, and antimycoplasmatic properties. The production of lipopeptide compounds by *B. mojavensis* is influenced by environmental factors, especially the carbon source is an important parameter in the formation of lipopeptides. Based on the research of Hmidet et al. (2017), *B. mojavensis* can produce surfactin and fengysin on all carbon sources used and the best production occurs in media with glucose as a carbon source and the least production occurs in media with starch carbon sources. *B. mojavensis* also showed α-hemolytic activity during its growth in blood agar (Berekaa and Ezzeldin 2018). These investigations revealed that bacteria-produced biosurfactant can be employed as a biocontrol for plant pathogens and as an effective bioinsecticide. Insecticidal metabolites with an amphiphilic structure comprising hydrophobic and hydrophilic molecules were created by biosurfactants.

Melo et al. (2016) discovered that the larvicidal paraspore toxin produced by *B. thuringiensis* also produces chitinase enzymes for agroindustrial use. The chitinolytic activity assay result are negative (**Figure 7**). This chitinolytic activity assay is also a screening effort to determine the potentials of a bacterium to produce chitinase. Chitinolytic microorganisms have a lot of potential to be applied as biocontrol agents (Wang et al. 2006). The growth and development of insects is highly dependent on the construction and structure of chitin Merzendorfer and Zimoch (2003). Chitinase induces peritrophic membrane damage in the insect intestine which causes a decrease in digestive activity and interferes with insect growth (Tera and Tera 2005). In a study conducted by Arora et al. (2003), purified chitinase from *Bacillus* spp. and has been analyzed as an insecticide. However, the results of this study did not show a clear zone formed around the colony, thus indicating a negative result.

The results of nucleotide BLAST analysis and BLAST protein srfA-D gene *B. mojavensis* EG 6.4 showed the highest similarity to *B. mojavensis* strain PS17 with access number CP066516.1 and surfactin biosynthesis thioesterase srfA-D from *B. mojavensis* bacteria with access number QQF62274.1. SrfA-D from *B. mojavensis* EG6.4 has been known that play a role in biosynthesis thioesterase enzyme to produce biosurfactants.

The indigenous *Bacillus* sp. EG6.4 is similar to *Bacillus mojavensis* and produced massive shape parasporal inclusion. Bioassay results in the category of high toxicity against *A. aegypti* larvae. *Bacillus* sp. EG6.4 showed hemolytic activity and be detected the srfA-D gene to produce surfactin, indicating its potency to produce biosurfactants. *Bacillus* sp. EG6.4 showed potential to be developed as a biocontrol agent for disease vectors and plant pests and plant diseases.

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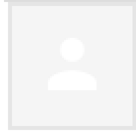

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2 Attachments • Scanned by Gmail

Abstract

Lines 12-13: it is not easy to read.

Line 18: change “assay was” to “assays were”.

Line 21: srfA-D are four genes, please change “gene” to “genes”, and change “isolate” to “the isolate”.

Line 25: from the running title, it could be referred that strain EG6.4 was a member of *Bacillus mojavensis*, however, it was not in lines 1-2.

Introduction

Line 28: change “*A. aegypti*” to “*Aedes aegypti*”

line 43: “…been reported by Carrillo et al. since 1996…”, it seems not to be a formal style.

Line 47: change “*Bacillus velezensis*” to “*B. velezensis*”

Line 52: change “is” to “are”

Line 59: change “names” to “name”

Lines 59-62: the content of this paragraph seems like a part of discussion, it needs to be revised.

Line 66: please describe the full names of “TEM”: and “SEM”

Line 67: change “assay” to “assays”

Materials and methods

Line 71-75: this paragraph seems unnecessary.

Line 77: "ES4.3"?

Line 90-91: this sentence is just a repeat, and where is 1st base sequencing service was made? In Malaysia or Singapore?

Lines 76-88 and lines 89-95 are two different methods for 16s rRNA analysis, please describe the difference.

Line 99: "Mega 7"? it is different from the above mentioned "Mega 6".

Line 104: please show more details of NYSM broth.

Line 107: "20"?

Line 133: please show the related primers.

Results and discussion

Line 133-134: change "*Bacillus mojavensis*" to "*B. mojavensis*", change "*Bacillus halotolerans*" to "*B. halotolerans*"

Line 175: delete the last "larvae"

Line 246: please figure out the site of "A" "B" "C".

Discussion

Lines 279-282: delete theses sentences.

This part should be carefully revised.

The authors need pay more attention to the writing of this manuscript.

COVERING LETTER

Dear **Editor-in-Chief,**

I herewith enclosed a research article,

Title:

Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

Author(s) name:

Salamun, Rizky Danang Susetyo, Farah Aisyah Nafidiastri, Rizki Amaliah Zain, Rossy Permata Sari, Almando Geraldi, Fatimah, Ni'matuzahroh

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Researchers found that the indigenous *Bacillus* species isolated from *Aedes aegypti* breeding sites, namely *Bacillus mojavensis* (EG6.4) had 97.89% similarity with *Bacillus mojavensis* strain NBRC 15718 and *Bacillus mojavensis* IFO 15718. The results of the larvicidal bioassay showed that the bacteria could kill larvae of *A. aegypti* in the category of high toxicity. Ultrastructural observations of bacterial cells did not Cry-toxin, but the hemolytic activity test showed positive results, so that the bacteria have produced biosurfactants with proven surfactin coding genes from these bacteria. The results of this study which are supported by the discussion of the literature review have shown that *Bacillus* sp. EG6.4 has the potential to be developed as a biocontrol agent for vector-borne diseases and plant pathogens or plant pests.

Statements:

This manuscript has not been published and is not under consideration for publication to any other journal or any other type of publication (including web hosting) either by me or any of my co-authors. Author(s) has been read and agree to the Ethical Guidelines.

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Place and date:

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Sincerely yours,

(fill in your name, no need scanned autograph)

Salamun

Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

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Abstract. This research was carried out for the molecular identification, as well as the determination and mechanism of action of the larvicidal toxicity of *Bacillus* sp. EG6.4 was isolated from the breeding sites of *Aedes aegypti* from Gresik City, East Java, Indonesia. Previous studies of *Bacillus* sp. EG6.4 is a Gram-positive endospore-forming bacteria. Molecular species identification using 16S rRNA gene sequencing showed that the isolate had 97.89% similarity with *Bacillus mojavensis*. The isolate showed larvicidal toxicity against *A. aegypti* larvae. The Lethal Concentration 50% (LC_{50}) values at 24- and 48-hours exposure were 8.99±1.01 x10^7 cells/mL and 8.43±1.01 x10^7 cells/mL, respectively. Meanwhile, the Lethal Time 50% (LT_{50}) value was 11.9±1.1 hours. To determine the larvicidal mechanisms, whether due to the production of chitinolytic enzymes or biosurfactants, chitinolytic and hemolytic assays were conducted, respectively. As a result, *Bacillus* sp. EG6.4 showed hemolytic but not chitinolytic activity, indicating its potency to produce biosurfactants. The detection using Transmission Electron Microscopy (TEM) showed that the isolate has oval-shaped endospores that were located subterminal with massive-shape parasporal inclusions. The detection of srfA-D genes showed that the isolate produced surfactin biosynthesis thioesterase. Thus, *Bacillus* sp. EG6.4 produced biosurfactant that potentially to be developed as a biocontrol agent for disease vectors and plant pathogens.

Key words: *Aedes aegypti*, *Bacillus mojavensis*, Biosurfactants, Larvicidal toxicity, parasporal inclusion, srfA-D gene

Running title: Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4

INTRODUCTION

Dengue fever (DF), a vector-borne disease and a serious public health problem in the world, this disease transmitted by the *Aedes aegypti* mosquito (Dahmana and Mediannikov, 2020; Falqueto et al., 2021). Vaccine development has been carried out, but the results have not been satisfactory. To suppress mosquito vector populations, including the use of chemical insecticides, it has had a negative impact on the environment and is toxic to non-target organisms (Dahmana and Mediannikov, 2020). So that one alternative to overcome this disease with integrated vector control (Melanie *et al*. 2018). Biological control experts suggest developing bioinsecticides as biocontrol agents for disease vectors (Thomas 2017)

The biodiversity of microorganisms is one of the abundant renewable biological resources in the world, including in Indonesia. The use of *Bacillus* in the biocontrol of vectors, pests, and plant diseases has grown rapidly (Benelli et al. 2016). *Bacillus thuringiensis* var. *israelensis* (Bti) used as a dengue vector biocontrol agent (BCAs) for the controlling of *A. aegypti* larvae (Boyce et al. 2013). During sporulation, *B. thuringiensis* produce parasporal inclusions and the resulting Cry toxin causes the death of several insect species (Aramideh et al. 2016).

The group of spore-forming bacteria will stop their growth under abnormal conditions and begin to increase their metabolic activity (Shahcheraghi et al. 2015). Sporulation is an effort to defend bacteria against an unfavorable environment. Several *Bacillus* species are reported to produce a protein toxin (Cry toxin) and also secondary metabolites that act as self-defense to survive. In the vegetative phase, bacteria grow and develop, but in unfavorable conditions will produce secondary metabolites such as biosurfactants, enzymes, or exotoxins to maintain their lives. Biosurfactant synthesis is associated with hemolytic activity, has been reported by Carrillo et al. (1996) and also applied by Colonna et al. (2017) as a rapid screening technique for assesing the toxicity of native surfactin. The hemolytic activity assay is a typical screening method for detecting biosurfactant activity. Biosurfactant generated by *Bacillus* sp. was found to be effective as a plant pathogenic and insecticidal agent in several tests (Bais et al. 2004). Nafidiastri et al. (2021) reported that indigenous *B. velezensis* ES4.3 resulted biosurfactant activity was indicated by the formation of clear zones,

emulsions, and a decrease in surface tension and the surfactin gene showed a 100% ID with the surfactin biosynthesis thioesterase SrfA-D gene.

Isolation and characterization of indigenous *Bacillus* entomopathogenic species have been isolated from the breeding sites of *A. aegypti* in Surabaya, Gresik, and Sidoarjo, East Java, Indonesia (Salamun et al. 2020). According to preliminary test, about 133 *Bacillus* sp. are entomopathogenic against larvae of *A. aegypti*. The majority of *Bacillus* sp. isolates showed mild to moderate toxicity, but 16 isolates were highly toxic, including *Bacillus* sp. EG6.4, which causes 100% larval mortality. Salamun et al. (2020) identified microscopic, macroscopic, and biochemical phenotypic similarities indicating that *Bacillus* sp. EG6.4 had a similarity level of 80.60% with *B. thuringiensis*. Mechanism of action of larvicidal toxicity of *Bacillus* spp. against *A. aegypti* in two ways, namely in the sporulation phase the bacteria produce endotoxins which are stored in the paraspore inclusions (pellet fraction) and produce secondary metabolites (supernatant fraction) which are secreted out of the cells. (Dahmana et al. 2020; Falqueto et al., 2021; Katak et al. 2021).

Further research is needed for molecular identification to determine species name of *Bacillus* sp. EG6.4 and genetic relationships with other bacilli, bioassays to determine larval mortality, and to detect the mechanism of action by detecting the presence of paraspore inclusions, surfactin coding genes, and hemolytic and chitinolytic activities, so it is hoped that the mechanism of action of *Bacillus* sp. EG6.4 as larvicidal can be identified.

This study aimed to molecular identification and larvicidal toxicity and larvicidal mechanism of the indigenous *Bacillus* sp. EG6.4. The Identification of the species name is conducted with the 16S rRNA gene and traces genetic affinity through a phylogenetic tree. The larvicidal activity to determine the LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} values. To determine the larvicidal mechanisms of action, with detection of endospore by Transmission Electron Microscopy (TEM) and Scanning Electron Microsopy (SEM), surfactin coding genes (srfA-D) and detection of secondary metabolite activity by hemolytic and chitinolytic activity assays.

MATERIALS AND METHODS

Molecular Identification

The DNA isolation

The genomic DNA of *Bacillus* sp. EG6.4 was isolated using the Promega DNA Purification Kit (Promega, 2018). The isolated DNA was visualized by electrophoresis. The results of genomic DNA electrophoresis of *Bacillus* sp. EG6.4 was photographed under a UV Transilluminator. Ratio of absorbance at 260 nm and 280 nm is use assessed Purity of genomic DNA by Thermo Scientific Multiskan GO Microdroplet Spectrophotometer.

Identification of 16S rRNA gene and phylogenetic tree

Identification of 16S rRNA gene was initiated by culturing 2-3 ose isolates of *Bacillus* sp. EG6.4 into 20 mL of Luria Bertani media, incubated at room temperature with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out using the CTAB method (Ausubel et al. 2003). DNA purity and concentration values were measured using Multiskan GO on λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene amplification was carried out using Eppendorf Mastercycler. This process begins by adding GoTaq Green Master Mix and 16S rRNA primers, in the form of primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was conditioned as follows: initial denaturation of 94ºC for 2 minutes, denaturation of 92ºC for 30 seconds, annealing 55ºC for 30 seconds, elongation of 72ºC for 1 minute, final elongation of 72ºC for 5 minutes, 35 cycles. The PCR samples were sent to the 1st Base DNA Sequencing Service Malaysia. Amplikon was sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The results of PCR were visualized through an electrophoresis process using 1% agarose gel followed by Ethidium Bromide staining and observed in ultraviolet light. The data were also analyzed for their relation by building a phylogenetic tree using MEGA 6.0 (Tamura et al. 2013).

The analysis of 16S rRNA gene

The isolated DNA of *Bacillus* sp. EG6.4 was amplified using the Eppendors Mastercycler with the PCR method and universal primers 27F and 1492R at 1st Base Sequencing Service in Singapore. The Bio Edit Sequence Alignment Editor software version 7.2.5 was used to evaluate the data from the sequencing results. The 16S rRNA gene nucleotide sequence from *Bacillus* sp. EG6.4 was matched with 16S rRNA gene sequences from other bacteria reported to GenBank. The Basic Local Alignment Search Tool (BLAST) at The National Library of Medicine, National Center for Biotechnology Information in Washington, DC (http://blast.ncbi.nml.nih.gov/) was used to find homology of the 16S rRNA gene.

Analysis of phylogenetic tree

The results of genomic DNA sequencing of *Bacillus* sp. EG6.4 which has been compared with other bacteria through BLAST was then compiled to determine the relationship through a phylogenetic tree was created by Mega 7 program. A phylogenetic tree was created by including FASTA from other species. All of these species were selected based on

101 nucleotide sequence of 16S rRNA gene, because relationships of a species with other species can be seen from the
102 sequence.
103
104 **Bioassay larvicidal toxicity**
105 Determination of the LC of *Bacillus* sp. EG6.4 against *A. aegypti* larvae was prepared by inoculating on Nutrient Yeast
106 Salt Medium (NYSM) broth were incubated on a rotary shaker (130 rpm) at 30°C for 72 hours. Then, bacterial density was
107 determined by spectrophotometer at A_{600nm}. Bioassays were conducted by Suryadi et al. (2016). The final culture
108 concentration was adjusted to variations concentration of (v/v) NYSM. Samples of 20 third-instar larvae of *A. aegypti*
109 were tested at six concentrations each treatment in triplicate. The larvae were seen to die after 24- and 48-hours exposure.
110 Lethal Time of *Bacillus* sp. EG6.4 of *A. aegypti* larvae was determined at a concentration of LC_{90}, as many as 20 larvae
111 were exposed, each treatment had three replications. Mortality of the larvae was scored after 0; 0.5; 1; 2; 4; 8; 10; 20; 24;
112 and 48-hours exposure along with NYSM (10% v/v) without inoculum as negative controls.
113 Bioassay of larval mortality (%) used to determine LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} of *Bacillus* sp. EG6.4
114 against *A. aegypti* larvae. Probit analysis using Minitab Version 17 was used to calculate the toxicity of *Bacillus* sp. EG6.4
115 against *A. aegypti* (Postelnicu, 2018).
116
117 **Larvicidal Mechanisms**

118 **Detection of parasporal inclusion**

119 The process of detecting *Bacillus* sp. EG6.4 parasporal inclusions included multiple phases, including rejuvenation,
120 purification, and growth on NYSM broth at 30 ^{0}C 72 hours, in a shaker incubator (130 rpm). After producing a pure liquid
121 culture of *Bacillus* sp. EG6.4, TEM examination was performed at the Eijkman Institute's TEM and Histology Laboratory in
122 Jakarta, while SEM study was performed at LPPT Gadjah Mada University in Yogyakarta using an SEI 10 kV WD10mm
123 SS30.

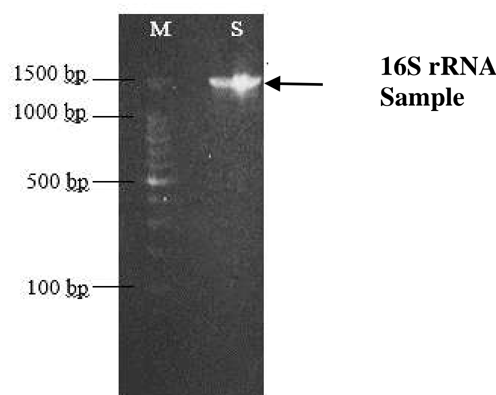
124 **Hemolytic and chitinolytic activity**

125 Hemolytic activity was tested using blood agar media acquired from the Surabaya Health Laboratory Center
126 and utilized to screen *Bacillus* sp. EG6.4 for haemolytic activity. Colony of isolates cultured for two days at room
127 temperature. The formation of distinct clear zones surrounding a colony. Chitinolytic activity was carried out by
128 growing *Bacillus* sp. EG6.4 on colloidal chitin agar media by the streak method. Observation of the clear zone
129 around the colonies was carried out after an incubation period of 4 days, by adding Congo red dye to Petri and
130 then washing it with NaOH.

131 **Detection of surfactin-coding gene**
132 Researchers used the DNA obtained from the identification of the 16S rRNA gene stage in this stage. The
133 approach for detecting the biosynthetic surfactin gene is similar to that for identifying the 16S rRNA gene, with
134 the exception of the primer employed. On the page ThermoFisher Scientific Oligo Perfect Primer Designer
135 cloning application, the srfA-D gene primers are self-designed. The surfactin gene primers that have been
136 designed are forward primer (5'- ATGAGCCAACTGTTCAAATCATTTG -3') and reverse primer (5'-
137 TCAGGAACTGGAAATCGGATGC -3').
138

139 **RESULTS AND DISCUSSION**

140 **Molecular identification**
141 Molecular identification (**Figure 1; Table 1**) showed that the *Bacillus* sp. EG6.4 had 97.89% similarity with *B.*
142 *mojavensis* strains NBRC 15718 and *B. mojavensis* IFO 15718 and was closely related to *B. halotolerans* strains DSM
143 8802 and *B. halotolerans* LMG 22476 (**Figure 2**).
144



145
146 **Figure 1.** The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M=100bp DNA marker; S= sample of
147 16S rRNA gene.
148
149 **Table 1**. The name of species of indigenous *Bacillus* sp. EG6.4 based on 16S rRNA gene analysis by Basic Local Alignment Search
150 Tool (BLAST)
151

Description	Accession	E value	Query Cover	% ID
Bacillus mojavensis strain NBRC 15718	NR 112725.1	0.0	100%	97.89
Bacillus mojavensis strain IFO 15718	NR 024693.1	0.0	100%	97.89
Bacillus halotolerans strain DSM 8802	NR 115063.1	0.0	100%	97.89

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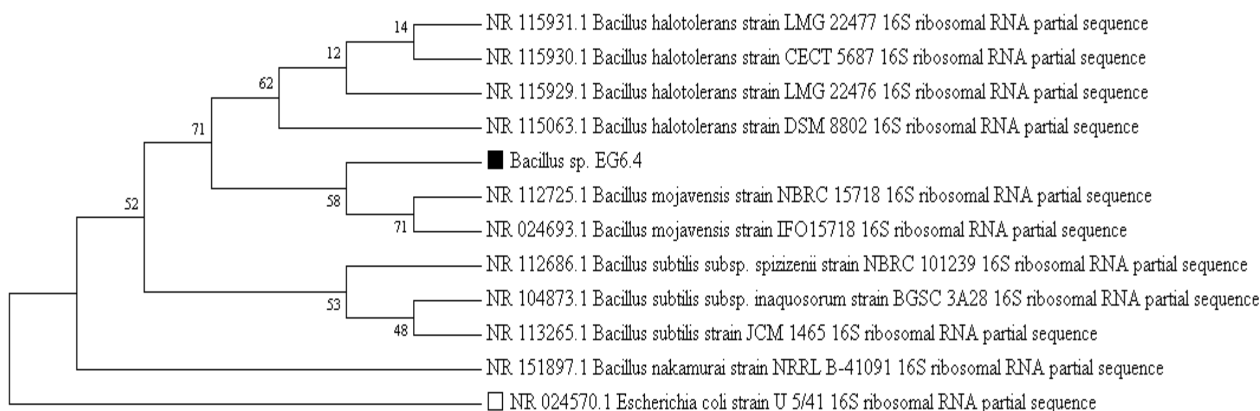

154
155
156 **Figure 2**. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application
157

158 **Bioassay**

159 The results of bioassay of *Bacillus* sp. EG6.4 against third instar larvae of *A. aegypti* in **Table 2**. The results of the
160 Probit analysis, the LC_{50} values at 24- and 48-hours exposure were 8.99±1.01 x10^7 cells/mL and 8.43±1.01 x10^7 cells/mL,
161 respectively (**Figure 3**). Meanwhile, the LT_{50} value was 11.9±1.1 hours (**Figure 4**).
162
163 **Table 2.** The effect of indigenous *Bacillus* sp. EG6.4 concentrations (OD_{600nm} and CFU/mL) on mortality of *Aedes aegypti* third instar
164 larvae (%) after 24- and 48-hours exposure.

Treatments (Concentration Series)	Culture (per-10 mL NYSM)	$OD_{600\,nm}$	CFU/mL	24 h exposure, larval mortality = %	48 h exposure, larval mortality = %
C_1	0.5 mL	0.07	09.8 x 10^7	6.7 ± 5.8	6.7 ± 5.8
C_2	1.0 mL	0.15	2.29 x 10^7	20 ± 10	26.7 ± 5.8
C_3	2.5 mL	0.37	5.90 x 10^7	43.3 ± 15.3	43.3 ± 15.3
C_4	5.0 mL	0.75	12.1 x 10^7	73.3 ± 5.8	76.7 ± 5.8
C_5	10 mL	1.50	24.4 x 10^7	93.3 ± 5.8	93.3 ± 5.8

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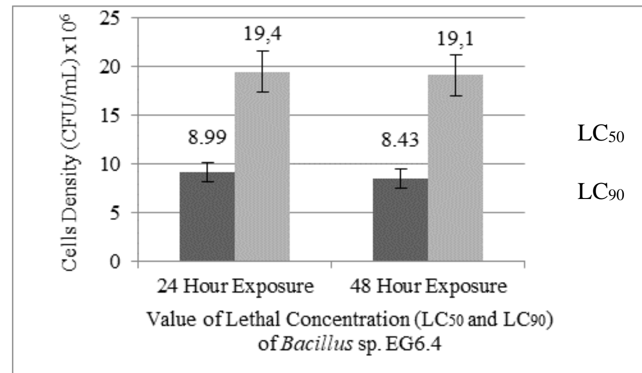
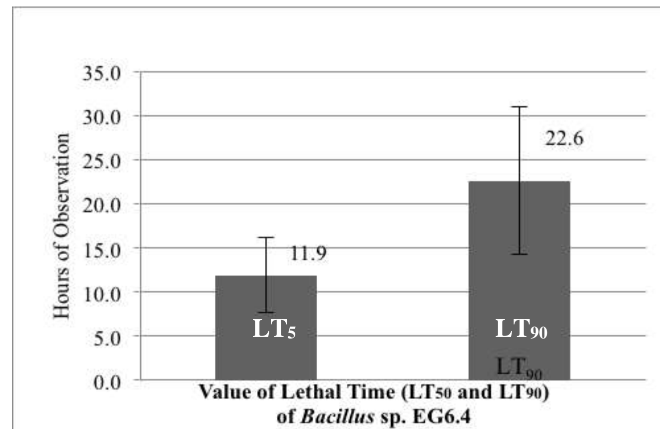


167
168

169 **Figure 3.** Value of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48
170 hours of exposure

171



172
173
174 **Figure 4**. The Lethal Time 50% (LT_{50}) and Lethal Time 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae on
175 observation: 0; 0.5; 1; 2; 4; 8; 10; 20; 24; 48-hours exposure

Larvicidal Toxicity Mechanism

Detection parasporal inclusion

Bacillus sp. EG6.4 is a Gram-positive and endospore-forming bacteria. Detection using TEM showed subterminal oval-shaped endospores (Salamun et al. 2020). Detection using TEM showed massive paraspore inclusions (**Figure 5**). Detection using SEM only showed spores and did not the Cry toxin (**Figure 6**).

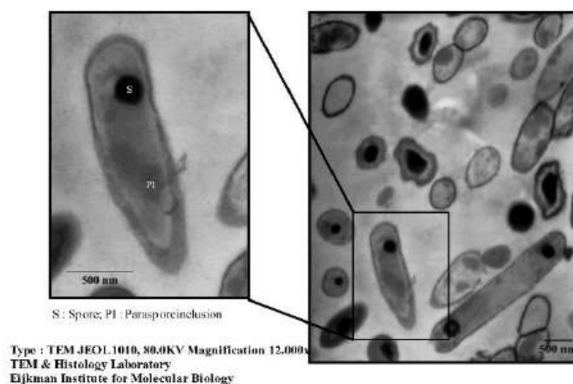


Figure 5. The organelles of indigenous *Bacillus* sp. EG6.4 endospore cells were visible using transmission electron microscopy (TEM). TEM JEOL1010 80.0 kV, 12.000x magnification, S=Forespore, PI=Parasporal Inclusion

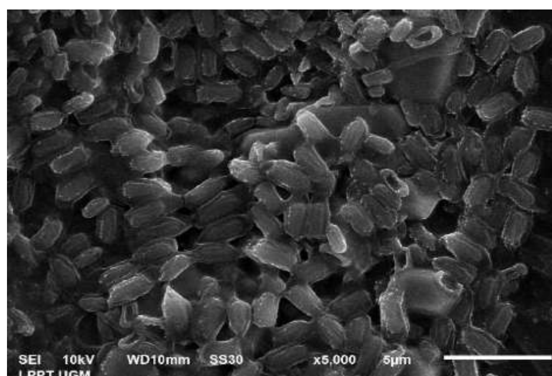
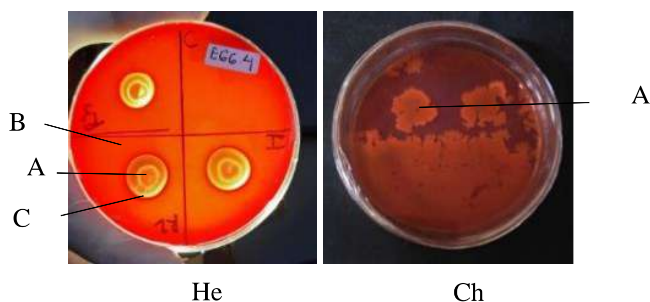


Figure 6. Scanning Electron Microscopy (SEM) showed only visible spore of indigenous *Bacillus* sp. EG6.4, in SEI 10kV WD10mm SS30 x5,000 magnification (5µm), by LPPT UGM, Yogyakarta.

Hemolytic and chitinolytic activity

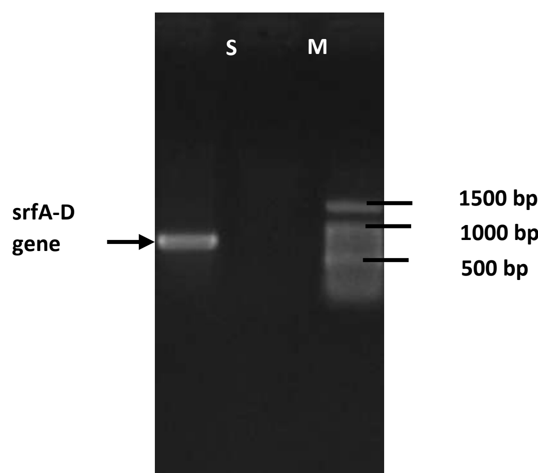
The results of hemolytic and chitinolytic activity assays, showed that hemolytic but not chitinolytic activity (**Figure 7**), indicating its potential to produce biosurfactants.



195 **Figure 7.** Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal
196 chitin plate media (Ch). Note: A=Colony of isolate; B=Blood agar plate, C=Clear zone around colony of isolate

197 **Detection of surfactin-coding gene**

198 The electrophoresis results (**Figure 8**) showed a band of about 700 bp. Based on data in GenBank CP0514641, the
199 srfA-D gene of *B. mojavensis* was 729 bp. The srfA-D gene has encoded a thioesterase enzyme that is important in the
200 surfactin biosynthetic pathway. Band position on electrophoretic visualization showed that *B. mojavensis* EG6.4 had the
201 srfA-D gene.
202
203



204
205
206 **Figure 8.** Electrophoresis results of the srfA-D gene *Bacillus mojavensis* EG6.4 which was successfully amplified. Description
207 M=500bp DNA marker; S=Sample of srfA-D gene.
208
209 **Table 3**. Results of the Basic Local Alignment Search Tool (BLAST) nucleotide analysis the srfA-D gene of *Bacillus mojavensis* EG6.4

Species Name with srfA-D Gene	No. Accession	E. value	QC	% ID	Description
Bacillus mojavensis strain PS17	CP066516.1	0,0	99%	98,35%	Complete genome
Bacillus mojavensis strain UCMB5075	CP051464.1	0,0	100%	94,92%	Complete genome

210
211 The nucleotide BLAST results from the srfA-D gene of *B. mojavensis* EG6.4 showed a similarity value of 98.35% with
212 the gene in *B. mojavensis* strain PS17 and had a similarity value of 94.92% with *B. mojavensis* strain UCMB5075 (**Table**
213 **3**). Further studies, the results of protein BLAST against the amino acid gene srfA-D *B. mojavensis* EG 6.4 obtained the
214 highest similarity of 98.35% with surfactin biosynthesis thioesterase SrfA-D from the bacterium *B. mojavensis* strain
215 PS17.

216 **Discussion**

217 The results of the isolation of *Bacillus* sp. EG6.4 has DNA purity = 1.84 (A260/A280), DNA concentration 44.7
218 (ng/µl), has been confirmed by electrophoresis showing DNA size is well above 1500bp. The DNA migration during
219 electrophoresis is influenced by agarose concentration, DNA molecule size, voltage, and temperature (Fatchiyah et al.
220 2011; Brown, 2016). The results of the 16S rRNA gene amplification was visualized by electrophoresis (**Figure 1**),
221 showed that a band measuring about 1550bp and about 500 bases at the end of the sequence is a hypervariable region.
222 Pearson (2014) reported that the 16S rRNA gene can be used for the molecular identification of microorganisms. The
223 results of purification and sequencing of the 16S rRNA gene of *Bacillus* sp. EG6.4 showed a nucleotide sequence with a
224 size of 1424 bp. Based on BLAST analysis, *Bacillus* sp. EG6.4 is similar to *B. mojavensis* (**Table 1**) and is related to other
225 bacteria (**Figure 2**).
226 *Bacillus* sp. EG6.4 has a close relationship with *Bacillus mojavensis* and *Bacillus halotolerans*. *B. mojavensis* and *B.*
227 *halotolerans* are two species of bacteria that are closely related to *Bacillus subtilis* however it differs significantly in terms
228 of fatty acid content, DNA sequences, and transgenic species resistance (Bacon and Hinton 2002). *B. mojavensis* have
229 been known that produce secondary matabolites such as oxygenated monoterpenes and lipopeptides, group of
230 biosurfactants, that have promising applications in agricultural, food industry, and clinical fields. In agriculture, *B.*
231 *mojavensis* as antimicrobial agent against pathogenic bacteria and fungi (Camele *et al.,* 2019). *B. halotolerans* has been
232 known that produces biosurfactants. Based on research reported by Wang et al. (2002), biosurfactants from *B. halotolerans*
233 can be used as agent to remediation in polluted environment petroleum.
234 The values of LC_{50} and LC_{90} are the concentrations of microorganisms that cause 50% and 90% mortality of the target
235 organism population. The lower the LC value of microorganisms, the higher the toxicity. Toxicity of *Bacillus* sp.

236 influenced by the type of toxin or secondary metabolites produced by microorganisms, the species and age of the target
237 larvae, and the media used for bacterial growth (Suryadi et al. 2016). Determination of LC_{50} and LC_{90} as well as LT_{50} and
238 LT_{90} was carried out to determine the concentration and length of time required by *Bacillus* sp. EG6.4 to kill 50% and 90%
239 and the length of time to kill 50% and 90% of third instar *A. aegypti* larvae. Based on the mortality rate of *A. aegypti*
240 larvae in the treatment, *Bacillus* sp. EG6.4 in the category of high toxicity. Based on the mortality rate of *A. aegypt*i larvae
241 by *Bacillus* sp. EG6.4, indicates that the isolate has the potential to be developed as a biolarvacidal agent.
242 The value of LC_{50} and LC_{90} values of *Bacillus* sp. EG6.4 has high potential (Figure 3), when compared to previous
243 studies. Gamma et al. (2010) reported that *B. thuringiensis* PWR4 32 isolated from Malang, Indonesia had an LC_{50} value
244 of 22.79 x 10^7 cells/mL at 72-hour exposure. Pratiwi et al. (2013) have reported that *B. thuringiensis* W.Swh.S.K2 isolated
245 from Nganjuk Indonesia, had an LC_{50} value of 3.53x10^7 cells/mL at 48-hour exposure. The number of spores consumed by
246 the larvae affects the rate of larval death. Gamma et al. (2010) found that as *B. thuringiensis* spores developed, more Cry
247 was released to kill *A. aegypti* larvae. The two isolates have different strains or species, so there is a difference in toxicity
248 between them. One of the characteristics of *Bacillus* sp. can produce endospores and protein crystals (parasporal
249 inclusions) at the time of cell sporulation.
250 *Bacillus* sp. EG6.4 shows a complete endospore with sections including a spore layer, spore, and paraspore inclusions
251 (**Figure 5; Figure 6**). *Bacillus* sp. EG6.4 produces paraspore inclusions, but the shape is massive and has not been proven
252 as a protein toxin that has larvicidal activity against *A. aegypti* larvae, so it is necessary to further investigate whether the
253 components of paraspore inclusions cause larval death. Iftikhar et al (2018) reported that *B. mojavensis* BTCB15
254 synthesized silver nanoparticles as nanosides against the larvae of *Culex quinquefasciatus*, *Anopheles stephensi*, and *A.*
255 *aegypti*, important vectors of disease transmission. The LC_{50} and LC_{90} for third instar larvae of the three species were 0.80,
256 0.75, and 1.2 ppm, and 0.80, 0.67, and 0.86 ppm, respectively. Iftikhar et al (2018), recommended the development of
257 silver nanoparticles produced by *B, mojavensis* BTCB15, which may play a role in combating mosquito populations,
258 thereby controlling vector-borne diseases. The larvicidal activity of silver nanoparticles (Ag/AgCl NPs) synthesized using
259 *Bti* supernatant showed that LC_{50}=0.133 g/mL, higher toxicity than that synthesized using insecticidal protein,
260 LC_{50}=0.148–0.217 g/mL (Chimkhan et al. 2022). Possible mechanism of action of Ag/AgCl NPs against *A. aegypti* larvae
261 is through mitochondrial dysfunction, DNA and protein damage, inhibition of cell proliferation, and cell apoptosis, and
262 recommended that Ag/AgCl NPs are an alternative approach to control *A. aegypti* larvae, a mosquito-borne disease vector
263 (Chimkhan et al. 2022). Further research needs to be done, whether *Bacillus* sp. EG6.4 can produce silver nanoparticles
264 that have a larvicidal effect on *A. aegypti* larvae.
265 Hemolytic activity assay of *Bacillus* sp. EG6.4 on blood agar showed a positive result (**Figure 7**). Biosurfactants have
266 been utilized as an alternative to chemical pesticides in the past. Previous studies found that biosurfactant-producing
267 bacteria are suitable for controlling plant pathogenic and insecticidal (Bais et al. 2004; Zhao et al. 2014). *B. subtilis* has
268 been reported as bacterial agent have producting mosquitocidal toxin (Das and Mukherjee 2006; Manonmani et al. 2011).
269 The toxicity of biosurfactants or their potential as biocontrol agents has not been well investigated. Biosurfactants, which
270 are surfactin produced by *Bacillus* strains, have been offered as a potential alternative to mosquito-borne disease control.
271 Biosurfactants produced by *Bacillus* strains have been shown to have mosquitocidal activity in adult mosquitos, killing
272 both immature and adult mosquitos (Geetha et al. 2012). Mosquitoes in the larval and pupal stages, respiration depends on
273 the siphon. The decrease in the surface tension of the water induced by the surfactant will prevent the siphon from
274 remaining in position on the outer layer of water. As a result, larvae and pupae lose contact with air and death occurs due
275 to respiratory failure. The low oxygen concentration causes the insect spiracles to remain open and causes insect death.
276 Other studies have shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects
277 (Zhao et al. 2014). From these studies it has been shown that biosurfactants affect the cuticle of insects, because they are
278 amphiphilic in the presence of hydrophobic and hydrophilic molecules that will damage cell membranes, then damage
279 epithelial cells, and cause larval death (Zhao et al. 2014).
280 *B. mojavensis* has been shown to create lipopeptides, which have a unique structure and are important antibacterial and
281 antifungal substances derived from surfactin, iturin, and fengysin (Mounia et al. 2014; Blacutt et al. 2016). According to
282 Jasim et al. (2016), lipopeptide in *B. mojavensis* has antiviral, antitumoral, antifungal, and antimycoplasmatic properties.
283 The production of lipopeptide compounds by *B. mojavensis* is influenced by environmental factors, especially the carbon
284 source is an important parameter in the formation of lipopeptides. Based on the research of Hmidet et al. (2017), *B.*
285 *mojavensis* produced surfactin and fengysin on all carbon sources used and the best production occurs in media with
286 glucose as a carbon source and the least production occurs in media with starch carbon sources. *B. mojavensis* also showed
287 α-hemolytic activity during its growth in blood agar (Berekaa and Ezzeldin 2018). These investigations revealed that
288 bacteria-produced biosurfactant can be employed as a biocontrol for plant pathogens and as an effective bioinsecticide.
289 Insecticidal metabolites with an amphiphilic structure comprising hydrophobic and hydrophilic molecules were created by
290 biosurfactants.
291 Melo et al. (2016) discovered that the larvicidal paraspore toxin produced by *B. thuringiensis* also produces chitinase
292 enzymes for agroindustrial use. The chitinolytic activity assay showed negative result (**Figure 7**). This chitinolytic activity
293 assay is also a screening effort to determine the potentials of a bacterium to produce chitinase. Chitinolytic
294 microorganisms have a lot of potential to be applied as biocontrol agents (Wang et al. 2006). The growth and development
295 of insects is highly dependent on the construction and structure of chitin (Merzendorfer and Zimoch, 2003). Chitinase

296 induces peritrophic membrane damage in the insect intestine which causes a decrease in digestive activity and interferes
297 with insect growth (Tera and Tera 2005). In a study conducted by Arora et al. (2003), purified chitinase from *Bacillus* spp.
298 and has been analyzed as an insecticide. However, the results of this study did not show a clear zone formed around the
299 colony, thus indicating a negative result.
300 The results of nucleotide BLAST analysis and BLAST protein srfA-D gene *B. mojavensis* EG6.4 showed the highest
301 similarity to *B. mojavensis* strain PS17 with access number CP066516.1 and surfactin biosynthesis thioesterase srfA-D
302 from *B. mojavensis* bacteria with access number QQF62274.1. The srfA-D from *B. mojavensis* EG6.4 has been known that
303 play a role in biosynthesis thioesterase enzyme to produce biosurfactants.
304 The indigenous *Bacillus* sp. EG6.4 is similar to *Bacillus mojavensis* and produced massive shape parasporal inclusion.
305 Bioassay results in the category of high toxicity against *A. aegypti* larvae. *Bacillus* sp. EG6.4 showed hemolytic activity
306 and be detected the srfA-D gene to produce surfactin, indicating its potency to produce biosurfactants. *Bacillus* sp. EG6.4
307 showed potential to be developed as a biocontrol agent for disease vectors and plant pests and plant diseases.

308 ## ACKNOWLEDGEMENTS

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315 **Conflicts of Interest:** There is no conflict of interest stated by the authors.
316

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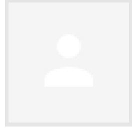

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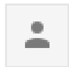

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Researchers found that the indigenous *Bacillus* species isolated from *Aedes aegypti* breeding sites, namely *Bacillus mojavensis* (EG6.4) had 97.89% similarity with *Bacillus mojavensis* strain NBRC 15718 and *Bacillus mojavensis* IFO 15718. The results of the larvicidal bioassay showed that the bacteria could kill larvae of *A. aegypti* in the category of high toxicity. Ultrastructural observations of bacterial cells did not Cry-toxin, but the hemolytic activity test showed positive results, so that the bacteria have produced biosurfactants with proven surfactin coding genes from these bacteria. The results of this study which are supported by the discussion of the literature review have shown that *Bacillus* sp. EG6.4 has the potential to be developed as a biocontrol agent for vector-borne diseases and plant pathogens or plant pests.

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Surabaya, Indonesia. August 11th 2022

Sincerely yours,

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Salamun

Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

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Abstract. This research was carried out for the molecular identification, as well as the determination and mechanism of action of the larvicidal toxicity of *Bacillus* sp. EG6.4 was isolated from the breeding sites of *Aedes aegypti* from Gresik City, East Java, Indonesia. Previous studies of *Bacillus* sp. EG6.4 is a Gram-positive endospore-forming bacteria. Molecular species identification using 16S rRNA gene sequencing showed that the isolate had 97.89% similarity with *Bacillus mojavensis*. The isolate showed larvicidal toxicity against *A. aegypti* larvae. The Lethal Concentration 50% (LC_{50}) values at 24- and 48-hours exposure were 8.99±1.01 x10^7 cells/mL and 8.43±1.01 x10^7 cells/mL, respectively. Meanwhile, the Lethal Time 50% (LT_{50}) value was 11.9±1.1 hours. To determine the larvicidal mechanisms, whether due to the production of chitinolytic enzymes or biosurfactants, chitinolytic and hemolytic assays were conducted, respectively. As a result, *Bacillus* sp. EG6.4 showed hemolytic but not chitinolytic activity, indicating its potency to produce biosurfactants. The detection using Transmission Electron Microscopy (TEM) showed that the isolate has oval-shaped endospores that were located subterminal with massive-shape parasporal inclusions. The detection of srfA-D genes showed that the isolate produced surfactin biosynthesis thioesterase. Thus, *Bacillus* sp. EG6.4 produced biosurfactant that potentially to be developed as a biocontrol agent for disease vectors and plant pathogens.

Key words: *Aedes aegypti*, *Bacillus mojavensis*, Biosurfactants, Larvicidal toxicity, parasporal inclusion, srfA-D gene

Running title: Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4

INTRODUCTION

Dengue fever (DF), a vector-borne disease and a serious public health problem in the world, this disease transmitted by the *Aedes aegypti* mosquito (Dahmana and Mediannikov, 2020; Falqueto et al., 2021). Vaccine development has been carried out, but the results have not been satisfactory. To suppress mosquito vector populations, including the use of chemical insecticides, it has had a negative impact on the environment and is toxic to non-target organisms (Dahmana and Mediannikov, 2020). So that one alternative to overcome this disease with integrated vector control (Melanie *et al*. 2018). Biological control experts suggest developing bioinsecticides as biocontrol agents for disease vectors (Thomas 2017)

The biodiversity of microorganisms is one of the abundant renewable biological resources in the world, including in Indonesia. The use of *Bacillus* in the biocontrol of vectors, pests, and plant diseases has grown rapidly (Benelli et al. 2016). *Bacillus thuringiensis* var. *israelensis* (Bti) used as a dengue vector biocontrol agent (BCAs) for the controlling of *A. aegypti* larvae (Boyce et al. 2013). During sporulation, *B. thuringiensis* produce parasporal inclusions and the resulting Cry toxin causes the death of several insect species (Aramideh et al. 2016).

The group of spore-forming bacteria will stop their growth under abnormal conditions and begin to increase their metabolic activity (Shahcheraghi et al. 2015). Sporulation is an effort to defend bacteria against an unfavorable environment. Several *Bacillus* species are reported to produce a protein toxin (Cry toxin) and also secondary metabolites that act as self-defense to survive. In the vegetative phase, bacteria grow and develop, but in unfavorable conditions will produce secondary metabolites such as biosurfactants, enzymes, or exotoxins to maintain their lives. Biosurfactant synthesis is associated with hemolytic activity, has been reported by Carrillo et al. (1996) and also applied by Colonna et al. (2017) as a rapid screening technique for assesing the toxicity of native surfactin. The hemolytic activity assay is a typical screening method for detecting biosurfactant activity. Biosurfactant generated by *Bacillus* sp. was found to be effective as a plant pathogenic and insecticidal agent in several tests (Bais et al. 2004). Nafidiastri et al. (2021) reported that indigenous *B. velezensis* ES4.3 resulted biosurfactant activity was indicated by the formation of clear zones,

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Commented [mm6]: We changed "*A. aegypti*" to "*Aedes aegypti*"

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48 emulsions, and a decrease in surface tension and the surfactin gene showed a 100% ID with the surfactin biosynthesis
49 thioesterase SrfA-D gene.
50 Isolation and characterization of indigenous *Bacillus* entomopathogenic species have been isolated from the breeding
51 sites of *A. aegypti* in Surabaya, Gresik, and Sidoarjo, East Java, Indonesia (Salamun et al. 2020). According to preliminary
52 test, about 133 *Bacillus* sp. are entomopathogenic against larvae of *A. aegypti*. The majority of *Bacillus* sp. isolates showed
53 mild to moderate toxicity, but 16 isolates were highly toxic, including *Bacillus* sp. EG6.4, which causes 100% larval
54 mortality. Salamun et al. (2020) identified microscopic, macroscopic, and biochemical phenotypic similarities indicating
55 that *Bacillus* sp. EG6.4 had a similarity level of 80.60% with *B. thuringiensis*. Mechanism of action of larvicidal toxicity
56 of *Bacillus* spp. against *A. aegypti* in two ways, namely in the sporulation phase the bacteria produce endotoxins which are
57 stored in the paraspore inclusions (pellet fraction) and produce secondary metabolites (supernatant fraction) which are
58 secreted out of the cells. (Dahmana et al. 2020; Falqueto et al., 2021; Katak et al. 2021).
59 Further research is needed for molecular identification to determine species name of *Bacillus* sp. EG6.4 and genetic
60 relationships with other bacilli, bioassays to determine larval mortality, and to detect the mechanism of action by detecting
61 the presence of paraspore inclusions, surfactin coding genes, and hemolytic and chitinolytic activities, so it is hoped that
62 the mechanism of action of *Bacillus* sp. EG6.4 as larvicidal can be identified.
63 This study aimed to molecular identification and larvicidal toxicity and larvicidal mechanism of the
64 indigenous *Bacillus* sp. EG6.4. The Identification of the species name is conducted with the 16S rRNA gene and traces
65 genetic affinity through a phylogenetic tree. The larvicidal activity to determine the LC_{50} and LC_{90}, as well as LT_{50} and
66 LT_{90} values. To determine the larvicidal mechanisms of action, with detection of endospore by Transmission Electron
67 Microscopy (TEM) and Scanning Electron Microsopy (SEM), surfactin coding genes (srfA-D) and detection of secondary
68 metabolite activity by hemolytic and chitinolytic activity assays.
69

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70 MATERIALS AND METHODS

71 **Molecular Identification**

72 **The DNA isolation**
73 The genomic DNA of *Bacillus* sp. EG6.4 was isolated using the Promega DNA Purification Kit (Promega, 2018). The
74 isolated DNA was visualized by electrophoresis. The results of genomic DNA electrophoresis of *Bacillus* sp. EG6.4 was
75 photographed under a UV Transilluminator. Ratio of absorbance at 260 nm and 280 nm is use assessed Purity of genomic
76 DNA by Thermo Scientific Multiskan GO Microdroplet Spectrophotometer.

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77 **Identification of 16S rRNA gene and phylogenetic tree**
78 Identification of 16S rRNA gene was initiated by culturing 2-3 ose isolates of *Bacillus* sp. EG6.4 into 20 mL of Luria
79 Bertani media, incubated at room temperature with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA,
80 extraction was carried out using the CTAB method (Ausubel et al. 2003). DNA purity and concentration values were
81 measured using Multiskan GO on λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene amplification was carried out using
82 Eppendorf Mastercycler. This process begins by adding GoTaq Green Master Mix and 16S rRNA primers, in the form of
83 primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was conditioned as follows: initial denaturation of 94°C
84 for 2 minutes, denaturation of 92°C for 30 seconds, annealing 55°C for 30 seconds, elongation of 72°C for 1 minute, final
85 elongation of 72°C for 5 minutes, 35 cycles. The PCR samples were sent to the 1st Base DNA Sequencing Service
86 Malaysia. Amplikon was sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al.
87 1997). The results of PCR were visualized through an electrophoresis process using 1% agarose gel followed by Ethidium
88 Bromide staining and observed in ultraviolet light. The data were also analyzed for their relation by building a
89 phylogenetic tree using MEGA 6.0 (Tamura et al. 2013).

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Commented [mm17]: We used MEGA 6 application to construct phylogeni tree

90 **The analysis of 16S rRNA gene**
91 The isolated DNA of *Bacillus* sp. EG6.4 was amplified using the Eppendors Mastercycler with the PCR method and
92 universal primers 27F and 1492R at 1st Base Sequencing Service in Singapore. The Bio Edit Sequence Alignment Editor
93 software version 7.2.5 was used to evaluate the data from the sequencing results. The 16S rRNA gene nucleotide sequence
94 from *Bacillus* sp. EG6.4 was matched with 16S rRNA gene sequences from other bacteria reported to GenBank. The Basic
95 Local Alignment Search Tool (BLAST) at The National Library of Medicine, National Center for Biotechnology
96 Information in Washington, DC (http://blast.ncbi.nml.nih.gov/) was used to find homology of the 16S rRNA gene.

97 **Analysis of phylogenetic tree**
98 The results of genomic DNA sequencing of *Bacillus* sp. EG6.4 which has been compared with other bacteria through
99 BLAST was then compiled to determine the relationship through a phylogenetic tree was created by Mega 7 program. A
100 phylogenetic tree was created by including FASTA from other species. All of these species were selected based on

101 nucleotide sequence of 16S rRNA gene, because relationships of a species with other species can be seen from the
102 sequence.
103
104 **Bioassay larvicidal toxicity**
105 Determination of the LC of *Bacillus* sp. EG6.4 against *A. aegypti* larvae was prepared by inoculating on Nutrient Yeast
106 Salt Medium (NYSM) broth were incubated on a rotary shaker (130 rpm) at 30°C for 72 hours. Then, bacterial density was
107 determined by spectrophotometer at A_{600nm}. Bioassays were conducted by Suryadi et al. (2016). The final culture
108 concentration was adjusted to variations concentration of (v/v) NYSM. Samples of 20 third-instar larvae of *A. aegypti*
109 were tested at six concentrations each treatment in triplicate. The larvae were seen to die after 24- and 48-hours exposure.
110 Lethal Time of *Bacillus* sp. EG6.4 of *A. aegypti* larvae was determined at a concentration of LC_{90}, as many as 20 larvae
111 were exposed, each treatment had three replications. Mortality of the larvae was scored after 0; 0.5; 1; 2; 4; 8; 10; 20; 24;
112 and 48-hours exposure along with NYSM (10% v/v) without inoculum as negative controls.
113 Bioassay of larval mortality (%) used to determine LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} of *Bacillus* sp. EG6.4
114 against *A. aegypti* larvae. Probit analysis using Minitab Version 17 was used to calculate the toxicity of *Bacillus* sp. EG6.4
115 against *A. aegypti* (Postelnicu, 2018).
116
117 **Larvicidal Mechanisms**

118 **Detection of parasporal inclusion**

119 The process of detecting *Bacillus* sp. EG6.4 parasporal inclusions included multiple phases, including rejuvenation,
120 purification, and growth on NYSM broth at 30 ^{0}C 72 hours, in a shaker incubator (130 rpm). After producing a pure liquid
121 culture of *Bacillus* sp. EG6.4, TEM examination was performed at the Eijkman Institute's TEM and Histology Laboratory in
122 Jakarta, while SEM study was performed at LPPT Gadjah Mada University in Yogyakarta using an SEI 10 kV WD10mm
123 SS30.

124 **Hemolytic and chitinolytic activity**

125 Hemolytic activity was tested using blood agar media acquired from the Surabaya Health Laboratory Center
126 and utilized to screen *Bacillus* sp. EG6.4 for haemolytic activity. Colony of isolates cultured for two days at room
127 temperature. The formation of distinct clear zones surrounding a colony. Chitinolytic activity was carried out by
128 growing *Bacillus* sp. EG6.4 on colloidal chitin agar media by the streak method. Observation of the clear zone
129 around the colonies was carried out after an incubation period of 4 days, by adding Congo red dye to Petri and
130 then washing it with NaOH.

131 **Detection of surfactin-coding gene**
132 Researchers used the DNA obtained from the identification of the 16S rRNA gene stage in this stage. The
133 approach for detecting the biosynthetic surfactin gene is similar to that for identifying the 16S rRNA gene, with
134 the exception of the primer employed. On the page ThermoFisher Scientific Oligo Perfect Primer Designer
135 cloning application, the srfA-D gene primers are self-designed. The surfactin gene primers that have been
136 designed are forward primer (5'- ATGAGCCAACTGTTCAAATCATTTG -3') and reverse primer (5'-
137 TCAGGAACTGGAAATCGGATGC -3').
138

139 **RESULTS AND DISCUSSION**

140 **Molecular identification**
141 Molecular identification (**Figure 1; Table 1**) showed that the *Bacillus* sp. EG6.4 had 97.89% similarity with *B.*
142 *mojavensis* strains NBRC 15718 and *B. mojavensis* IFO 15718 and was closely related to *B. halotolerans* strains DSM
143 8802 and *B. halotolerans* LMG 22476 (**Figure 2**).
144

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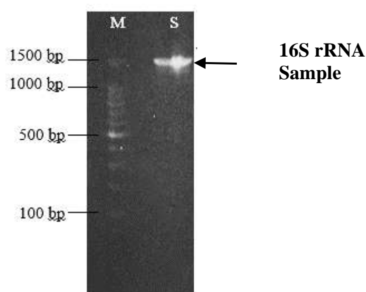


Figure 1. The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M=100bp DNA marker; S= sample of 16S rRNA gene.

Table 1. The name of species of indigenous *Bacillus* sp. EG6.4 based on 16S rRNA gene analysis by Basic Local Alignment Search Tool (BLAST)

Description	Accession	E value	Query Cover	% ID
Bacillus mojavensis strain NBRC 15718	NR 112725.1	0.0	100%	97.89
Bacillus mojavensis strain IFO 15718	NR 024693.1	0.0	100%	97.89
Bacillus halotolerans strain DSM 8802	NR 115063.1	0.0	100%	97.89

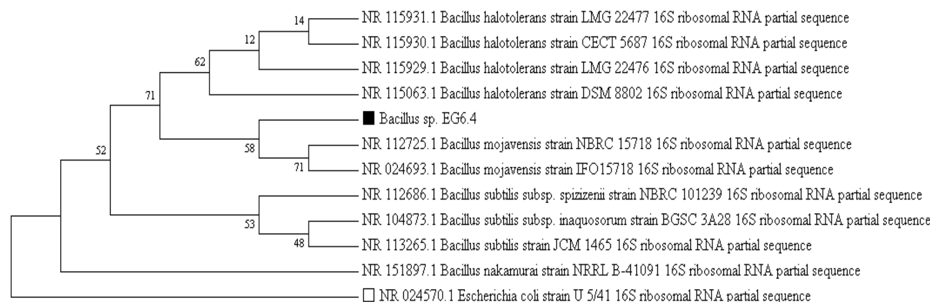


Figure 2. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application

Bioassay

The results of bioassay of *Bacillus* sp. EG6.4 against third instar larvae of *A. aegypti* in **Table 2**. The results of the Probit analysis, the LC_{50} values at 24- and 48-hours exposure were 8.99±1.01 x10^7 cells/mL and 8.43±1.01 x10^7 cells/mL, respectively (**Figure 3**). Meanwhile, the LT_{50} value was 11.9±1.1 hours (**Figure 4**).

Table 2. The effect of indigenous *Bacillus* sp. EG6.4 concentrations (OD_{600nm} and CFU/mL) on mortality of *Aedes aegypti* third instar larvae (%) after 24- and 48-hours exposure.

Treatments (Concentration Series)	Culture (per-10 mL NYSM)	OD_{600nm}	CFU/mL	24 h exposure, larval mortality = %	48 h exposure, larval mortality = %
C_1	0.5 mL	0.07	09.8 x 10^7	6.7 ± 5.8	6.7 ± 5.8
C_2	1.0 mL	0.15	2.29 x 10^7	20 ± 10	26.7 ± 5.8
C_3	2.5 mL	0.37	5.90 x 10^7	43.3 ± 15.3	43.3 ± 15.3
C_4	5.0 mL	0.75	12.1 x 10^7	73.3 ± 5.8	76.7 ± 5.8
C_5	10 mL	1.50	24.4 x 10^7	93.3 ± 5.8	93.3 ± 5.8

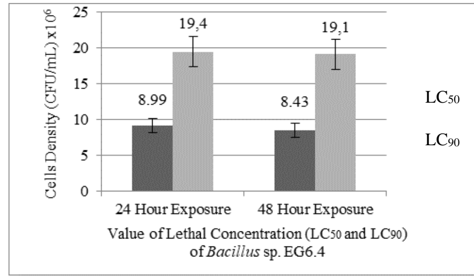


Figure 3. Value of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48 hours of exposure

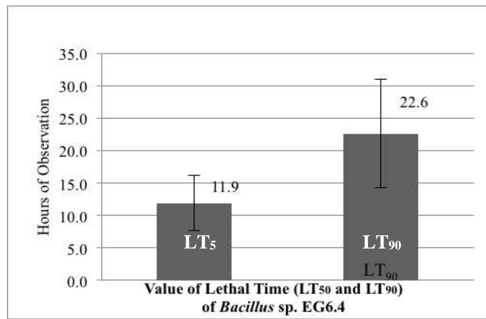


Figure 4. The Lethal Time 50% (LT_{50}) and Lethal Time 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae on observation: 0; 0.5; 1; 2; 4; 8; 10; 20; 24; 48-hours exposure

Larvicidal Toxicity Mechanism

Detection parasporal inclusion

Bacillus sp. EG6.4 is a Gram-positive and endospore-forming bacteria. Detection using TEM showed subterminal oval-shaped endospores (Salamun et al. 2020). Detection using TEM showed massive paraspore inclusions (**Figure 5**). Detection using SEM only showed spores and did not the Cry toxin (**Figure 6**).

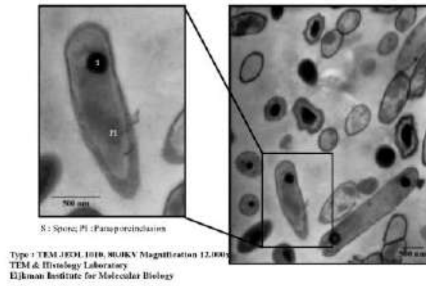


Figure 5. The organelles of indigenous *Bacillus* sp. EG6.4 endospore cells were visible using transmission electron microscopy (TEM). TEM JEOL1010 80.0 kV, 12.000x magnification, S=Forespore, PI=Parasporal Inclusion

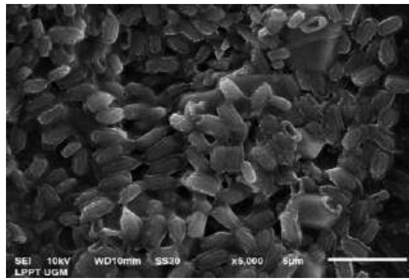
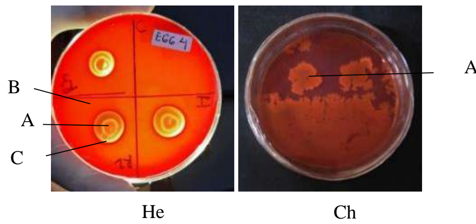


Figure 6. Scanning Electron Microscopy (SEM) showed only visible spore of indigenous *Bacillus* sp. EG6.4, in SEI 10kV WD10mm SS30 x5,000 magnification (5µm), by LPPT UGM, Yogyakarta.

Hemolytic and chitinolytic activity

The results of hemolytic and chitinolytic activity assays, showed that hemolytic but not chitinolytic activity (**Figure 7**), indicating its potential to produce biosurfactants.

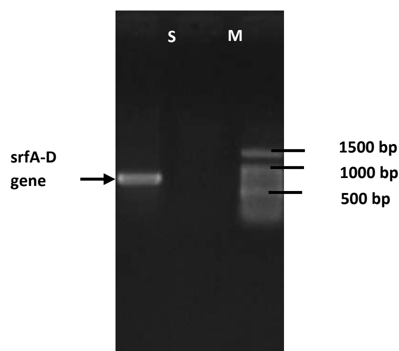


195 **Figure 7.** Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal
196 chitin plate media (Ch). Note: A=Colony of isolate; B=Blood agar plate, C=Clear zone around colony of isolate

Commented [SS23]: We added notes "Ä", "B", "C", "He", and "Ch" at Figure 7.

197 **Detection of surfactin-coding gene**

198 The electrophoresis results (**Figure 8**) showed a band of about 700 bp. Based on data in GenBank CP0514641, the
199 srfA-D gene of *B. mojavensis* was 729 bp. The srfA-D gene has encoded a thioesterase enzyme that is important in the
200 surfactin biosynthetic pathway. Band position on electrophoretic visualization showed that *B. mojavensis* EG6.4 had the
201 srfA-D gene.
202
203



204
205
206 **Figure 8.** Electrophoresis results of the srfA-D gene *Bacillus mojavensis* EG6.4 which was successfully amplified. Description
207 M=500bp DNA marker; S=Sample of srfA-D gene.
208
209 **Table 3**. Results of the Basic Local Alignment Search Tool (BLAST) nucleotide analysis the srfA-D gene of *Bacillus mojavensis* EG6.4

Species Name with srfA-D Gene	No. Accession	E. value	QC	% ID	Description
Bacillus mojavensis strain PS17	CP066516.1	0,0	99%	98,35%	Complete genome
Bacillus mojavensis strain UCMB5075	CP051464.1	0,0	100%	94,92%	Complete genome

210
211 The nucleotide BLAST results from the srfA-D gene of *B. mojavensis* EG6.4 showed a similarity value of 98.35% with
212 the gene in *B. mojavensis* strain PS17 and had a similarity value of 94.92% with *B. mojavensis* strain UCMB5075 (**Table**
213 **3**). Further studies, the results of protein BLAST against the amino acid gene srfA-D *B. mojavensis* EG 6.4 obtained the
214 highest similarity of 98.35% with surfactin biosynthesis thioesterase SrfA-D from the bacterium *B. mojavensis* strain
215 PS17.

216 **Discussion**
217 The results of the isolation of *Bacillus* sp. EG6.4 has DNA purity = 1.84 (A260/A280), DNA concentration 44.7
218 (ng/µl), has been confirmed by electrophoresis showing DNA size is well above 1500bp. The DNA migration during
219 electrophoresis is influenced by agarose concentration, DNA molecule size, voltage, and temperature (Fatchiyah et al.
220 2011; Brown, 2016). The results of the 16S rRNA gene amplification was visualized by electrophoresis (**Figure 1**),
221 showed that a band measuring about 1550bp and about 500 bases at the end of the sequence is a hypervariable region.
222 Pearson (2014) reported that the 16S rRNA gene can be used for the molecular identification of microorganisms. The
223 results of purification and sequencing of the 16S rRNA gene of *Bacillus* sp. EG6.4 showed a nucleotide sequence with a
224 size of 1424 bp. Based on BLAST analysis, *Bacillus* sp. EG6.4 is similar to *B. mojavensis* (**Table 1**) and is related to other
225 bacteria (**Figure 2**).

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226 *Bacillus* sp. EG6.4 has a close relationship with *Bacillus mojavensis* and *Bacillus halotolerans*. *B. mojavensis* and *B.*
227 *halotolerans* are two species of bacteria that are closely related to *Bacillus subtilis* however it differs significantly in terms
228 of fatty acid content, DNA sequences, and transgenic species resistance (Bacon and Hinton 2002). *B. mojavensis* have
229 been known that produce secondary matabolites such as oxygenated monoterpenes and lipopeptides, group of
230 biosurfactants, that have promising applications in agricultural, food industry, and clinical fields. In agriculture, *B.*
231 *mojavensis* as antimicrobial agent against pathogenic bacteria and fungi (Camele *et al.,* 2019). *B. halotolerans* has been
232 known that produces biosurfactants. Based on research reported by Wang et al. (2002), biosurfactants from *B. halotolerans*
233 can be used as agent to remediation in polluted environment petroleum.
234 The values of LC_{50} and LC_{90} are the concentrations of microorganisms that cause 50% and 90% mortality of the target
235 organism population. The lower the LC value of microorganisms, the higher the toxicity. Toxicity of *Bacillus* sp.

236 influenced by the type of toxin or secondary metabolites produced by microorganisms, the species and age of the target
237 larvae, and the media used for bacterial growth (Suryadi et al. 2016). Determination of LC_{50} and LC_{90} as well as LT_{50} and
238 LT_{90} was carried out to determine the concentration and length of time required by *Bacillus* sp. EG6.4 to kill 50% and 90%
239 and the length of time to kill 50% and 90% of third instar *A. aegypti* larvae. Based on the mortality rate of *A. aegypti*
240 larvae in the treatment, *Bacillus* sp. EG6.4 in the category of high toxicity. Based on the mortality rate of *A. aegypt*i larvae
241 by *Bacillus* sp. EG6.4, indicates that the isolate has the potential to be developed as a biolarvacidal agent.
242 The value of LC_{50} and LC_{90} values of *Bacillus* sp. EG6.4 has high potential (Figure 3), when compared to previous
243 studies. Gamma et al. (2010) reported that *B. thuringiensis* PWR4 32 isolated from Malang, Indonesia had an LC_{50} value
244 of 22.79 x 10^7 cells/mL at 72-hour exposure. Pratiwi et al. (2013) have reported that *B. thuringiensis* W.Swh.S.K2 isolated
245 from Nganjuk Indonesia, had an LC_{50} value of 3.53x10^7 cells/mL at 48-hour exposure. The number of spores consumed by
246 the larvae affects the rate of larval death. Gamma et al. (2010) found that as *B. thuringiensis* spores developed, more Cry
247 was released to kill *A. aegypti* larvae. The two isolates have different strains or species, so there is a difference in toxicity
248 between them. One of the characteristics of *Bacillus* sp. can produce endospores and protein crystals (parasporal
249 inclusions) at the time of cell sporulation.
250 *Bacillus* sp. EG6.4 shows a complete endospore with sections including a spore layer, spore, and paraspore inclusions
251 (**Figure 5; Figure 6**). *Bacillus* sp. EG6.4 produces paraspore inclusions, but the shape is massive and has not been proven
252 as a protein toxin that has larvicidal activity against *A. aegypti* larvae, so it is necessary to further investigate whether the
253 components of paraspore inclusions cause larval death. Iftikhar et al (2018) reported that *B. mojavensis* BTCB15
254 synthesized silver nanoparticles as nanosides against the larvae of *Culex quinquefasciatus*, *Anopheles stephensi*, and *A.*
255 *aegypti*, important vectors of disease transmission. The LC_{50} and LC_{90} for third instar larvae of the three species were 0.80,
256 0.75, and 1.2 ppm, and 0.80, 0.67, and 0.86 ppm, respectively. Iftikhar et al (2018), recommended the development of
257 silver nanoparticles produced by *B, mojavensis* BTCB15, which may play a role in combating mosquito populations,
258 thereby controlling vector-borne diseases. The larvicidal activity of silver nanoparticles (Ag/AgCl NPs) synthesized using
259 *Bti* supernatant showed that LC_{50}=0.133 g/mL, higher toxicity than that synthesized using insecticidal protein,
260 LC_{50}=0.148–0.217 g/mL (Chimkhan et al. 2022). Possible mechanism of action of Ag/AgCl NPs against *A. aegypti* larvae
261 is through mitochondrial dysfunction, DNA and protein damage, inhibition of cell proliferation, and cell apoptosis, and
262 recommended that Ag/AgCl NPs are an alternative approach to control *A. aegypti* larvae, a mosquito-borne disease vector
263 (Chimkhan et al. 2022). Further research needs to be done, whether *Bacillus* sp. EG6.4 can produce silver nanoparticles
264 that have a larvicidal effect on *A. aegypti* larvae.
265 Hemolytic activity assay of *Bacillus* sp. EG6.4 on blood agar showed a positive result (**Figure 7**). Biosurfactants have
266 been utilized as an alternative to chemical pesticides in the past. Previous studies found that biosurfactant-producing
267 bacteria are suitable for controlling plant pathogenic and insecticidal (Bais et al. 2004; Zhao et al. 2014). *B. subtilis* has
268 been reported as bacterial agent have producting mosquitocidal toxin (Das and Mukherjee 2006; Manonmani et al. 2011).
269 The toxicity of biosurfactants or their potential as biocontrol agents has not been well investigated. Biosurfactants, which
270 are surfactin produced by *Bacillus* strains, have been offered as a potential alternative to mosquito-borne disease control.
271 Biosurfactants produced by *Bacillus* strains have been shown to have mosquitocidal activity in adult mosquitos, killing
272 both immature and adult mosquitos (Geetha et al. 2012). Mosquitoes in the larval and pupal stages, respiration depends on
273 the siphon. The decrease in the surface tension of the water induced by the surfactant will prevent the siphon from
274 remaining in position on the outer layer of water. As a result, larvae and pupae lose contact with air and death occurs due
275 to respiratory failure. The low oxygen concentration causes the insect spiracles to remain open and causes insect death.
276 Other studies have shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects
277 (Zhao et al. 2014). From these studies it has been shown that biosurfactants affect the cuticle of insects, because they are
278 amphiphilic in the presence of hydrophobic and hydrophilic molecules that will damage cell membranes, then damage
279 epithelial cells, and cause larval death (Zhao et al. 2014).
280 *B. mojavensis* has been shown to create lipopeptides, which have a unique structure and are important antibacterial and
281 antifungal substances derived from surfactin, iturin, and fengysin (Mounia et al. 2014; Blacutt et al. 2016). According to
282 Jasim et al. (2016), lipopeptide in *B. mojavensis* has antiviral, antitumoral, antifungal, and antimycoplasmatic properties.
283 The production of lipopeptide compounds by *B. mojavensis* is influenced by environmental factors, especially the carbon
284 source is an important parameter in the formation of lipopeptides. Based on the research of Hmidet et al. (2017), *B.*
285 *mojavensis* produced surfactin and fengysin on all carbon sources used and the best production occurs in media with
286 glucose as a carbon source and the least production occurs in media with starch carbon sources. *B. mojavensis* also showed
287 α-hemolytic activity during its growth in blood agar (Berekaa and Ezzeldin 2018). These investigations revealed that
288 bacteria-produced biosurfactant can be employed as a biocontrol for plant pathogens and as an effective bioinsecticide.
289 Insecticidal metabolites with an amphiphilic structure comprising hydrophobic and hydrophilic molecules were created by
290 biosurfactants.
291 Melo et al. (2016) discovered that the larvicidal paraspore toxin produced by *B. thuringiensis* also produces chitinase
292 enzymes for agroindustrial use. The chitinolytic activity assay showed negative result (**Figure 7**). This chitinolytic activity
293 assay is also a screening effort to determine the potentials of a bacterium to produce chitinase. Chitinolytic
294 microorganisms have a lot of potential to be applied as biocontrol agents (Wang et al. 2006). The growth and development
295 of insects is highly dependent on the construction and structure of chitin (Merzendorfer and Zimoch, 2003). Chitinase

296 induces peritrophic membrane damage in the insect intestine which causes a decrease in digestive activity and interferes
297 with insect growth (Tera and Tera 2005). In a study conducted by Arora et al. (2003), purified chitinase from *Bacillus* spp.
298 and has been analyzed as an insecticide. However, the results of this study did not show a clear zone formed around the
299 colony, thus indicating a negative result.
300 The results of nucleotide BLAST analysis and BLAST protein srfA-D gene *B. mojavensis* EG6.4 showed the highest
301 similarity to *B. mojavensis* strain PS17 with access number CP066516.1 and surfactin biosynthesis thioesterase srfA-D
302 from *B. mojavensis* bacteria with access number QQF62274.1. The srfA-D from *B. mojavensis* EG6.4 has been known that
303 play a role in biosynthesis thioesterase enzyme to produce biosurfactants.
304 The indigenous *Bacillus* sp. EG6.4 is similar to *Bacillus mojavensis* and produced massive shape parasporal inclusion.
305 Bioassay results in the category of high toxicity against *A. aegypti* larvae. *Bacillus* sp. EG6.4 showed hemolytic activity
306 and be detected the srfA-D gene to produce surfactin, indicating its potency to produce biosurfactants. *Bacillus* sp. EG6.4
307 showed potential to be developed as a biocontrol agent for disease vectors and plant pests and plant diseases.

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316

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Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

Abstract. This research was carried out for the molecular identification, as well as the determination and mechanism of action of ~~the~~ larvicidal toxicity of *Bacillus* sp. EG6.4 ~~was~~ isolated from ~~the~~ breeding sites of *Aedes aegypti* from Gresik City, East Java, Indonesia. ~~Previous studies of~~ *Bacillus* sp. EG6.4 ~~is~~ was a Gram-positive endospore-forming bacteria. Molecular species identification using 16S rRNA gene sequencing showed that ~~the~~ isolate had 97.89% similarity with *Bacillus mojavensis*. The isolate showed larvicidal toxicity against *A. aegypti* larvae. The ~~Lethal~~ lethal ~~Concentration~~ concentration 50% (LC_{50}) values at 24~~-~~ and 48-hours exposure were 8.99±1.01 ×~~x~~10^7 cells/mL and 8.43±1.01 ×~~x~~10^7 cells/mL, respectively, while lethal time 50% (LT_{50}) value was 11.9±1.1 hours. ~~Meanwhile, the Lethal Time 50% (LT_{50}) value was 11.9±1.1 hours. To determine the larvicidal mechanisms~~, ~~whether due to the production~~ Production of chitinolytic enzymes or biosurfactants, chitinolytic and hemolytic assays were conducted to determine the larvicidal mechanism~~, respectively~~. As a result, *Bacillus* sp. EG6.4 showed hemolytic, but not chitinolytic activity, indicating its potency to produce biosurfactants. ~~The detection using~~ Transmission Electron Microscopy (TEM) result showed that ~~the~~ isolate ~~has~~ had oval-shaped endospores ~~that were~~ located subterminal with massive-shape parasporal inclusions. The detection of srfA-D gene~~s~~ showed that ~~the~~ isolate produced surfactin biosynthesis thioesterase. Thus, *Bacillus* sp. EG6.4 produced biosurfactant that potentially to be developed as a biocontrol agent for disease vectors and plant pathogens.

~~**Key words**~~ **Keywords**: *Aedes aegypti*, *Bacillus mojavensis*, ~~Biosurfactants~~ biosurfactants, ~~Larvicidal~~ larvicidal toxicity, parasporal inclusion, srfA-D gene

Running title: Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4

INTRODUCTION

Dengue fever (DF), a vector-borne disease and a serious public health problem in the world, ~~this disease~~ is transmitted by the *Aedes aegypti* mosquito (Dahmana and Mediannikov~~,~~ 2020; Falqueto et al.~~,~~ 2021). Vaccines ~~development has~~ have been ~~carried out~~ developed, but the results have not been satisfactory. ~~To suppress mosquito vector populations, including the use of chemical insecticides,~~ Chemical insecticides are used to suppress mosquito vector populations, ~~it has had~~ but have a negative impact on the environment and ~~is~~ are toxic to non-target organisms (Dahmana and Mediannikov~~,~~ 2020). So that one alternative to overcome this disease with integrated vector control (Melanie et al. 2018). Biological control experts suggest developing bioinsecticides as biocontrol agents for disease vectors (Thomas 2017)

The biodiversity of microorganisms is one of the abundant renewable biological resources in the world, including in Indonesia. The use of *Bacillus* in the biocontrol of vectors, pests, and plant diseases has grown rapidly (Benelli et al. 2016). *Bacillus thuringiensis* var. *israelensis* (Bti) is used as a dengue vector biocontrol agent (BCAs) for ~~the~~ controlling of *A. aegypti* larvae (Boyce et al. 2013). During sporulation, *B. thuringiensis* produce parasporal inclusions and the resulting ~~Cry~~ cry toxin causes ~~the~~ death of several insect species (Aramideh et al. 2016).

The group of spore-forming bacteria ~~will stop~~ inhibits their growth under abnormal conditions and begins to increase their metabolic activity (Shahcheraghi et al. 2015). Sporulation is an effort to defend bacteria against an unfavorable environment. Several *Bacillus* species are reported to produce a protein toxin (~~Cry~~ cry toxin) and also secondary metabolites that act as self-defense to survive. In the vegetative phase, bacteria grow and develop, but in unfavorable conditions ~~will~~ they produce secondary metabolites, such as biosurfactants, enzymes, or exotoxins to maintain their lives. Biosurfactant synthesis is associated with hemolytic activity~~, has been reported by~~ (Carrillo et al. ~~(~~1996) and has also applied by Colonna et al. (2017) as a rapid screening technique for assesing the toxicity of native surfactin. The hemolytic activity assay is a typical screening method for detecting biosurfactant activity. Biosurfactant generated by *Bacillus* sp.

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~~was~~ is found to be effective as a plant pathogenic and insecticidal agent in several tests (Bais et al. 2004). Nafidiastri et al. (2021) reported that indigenous *B. velezensis* ES4.3 resulted biosurfactant activity was indicated by the formation of clear zones, emulsions, and a decrease in surface tension and the surfactin gene showed a 100% ID with the surfactin biosynthesis thioesterase SrfA-D gene.

Isolation and characterization of indigenous *Bacillus* entomopathogenic species have been isolated from the breeding sites of *A. aegypti* in Surabaya, Gresik, and Sidoarjo, East Java, Indonesia (Salamun et al. 2020). According to preliminary test, about 133 *Bacillus* sp. are entomopathogenic against larvae of *A. aegypti*. The majority of *Bacillus* sp. isolates showed mild to moderate toxicity, but 16 isolates were highly toxic, including *Bacillus* sp. EG6.4, which causes 100% larval mortality. Salamun et al. (2020) identified microscopic, macroscopic, and biochemical phenotypic similarities indicating that *Bacillus* sp. EG6.4 had a similarity level of 80.60% with *B. thuringiensis*. Mechanism of action of larvicidal toxicity of *Bacillus* spp. against *A. aegypti* in two ways, namely in the sporulation phase the bacteria produce endotoxins which are stored in the paraspore inclusions (pellet fraction) and produce secondary metabolites (supernatant fraction) which are secreted out of the cells. (Dahmana et al. 2020; Falqueto et al.~~,~~ 2021; Katak et al. 2021).

Further research is needed for molecular identification to determine species name of *Bacillus* sp. EG6.4 and genetic relationships with other bacilli, bioassays to determine larval mortality, and to detect the mechanism of action by detecting the presence of paraspore inclusions, surfactin coding genes, and hemolytic and chitinolytic activities, so it is hoped that the mechanism of action of *Bacillus* sp. EG6.4 as larvicidal can be identified.

~~This study aimed~~ The aim of this study was to identify the isolated species at molecular ~~identification~~ level and to determine larvicidal toxicity and larvicidal mechanism of the indigenous *Bacillus* sp. EG6.4. ~~The Identification of the~~ Further, species name ~~is~~ was conducted with the 16S rRNA gene and traces genetic affinity through a phylogenetic tree. ~~The larvicidal~~ Larvicidal activity ~~to~~ was determined by ~~the~~ LC_{50} and LC_{90}~~, as well as LT_{50} and LT_{90}~~ values. To determine the larvicidal mechanisms of action, with detection of endospore by Transmission Electron Microscopy (TEM) and Scanning Electron Microsopy (SEM), surfactin coding genes (srfA-D) and detection of secondary metabolite activity by hemolytic and chitinolytic activity assays.

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MATERIALS AND METHODS

Molecular Identification

~~The~~ DNA isolation

The genomic DNA of *Bacillus* sp. EG6.4 was isolated using the Promega DNA Purification Kit (Promega~~,~~ 2018). ~~The isolated~~ Isolated DNA was visualized by electrophoresis. The results of genomic DNA electrophoresis of *Bacillus* sp. EG6.4 was photographed under a UV Transilluminator. The purity of genomic DNA was measured using the ratio ~~Ratio~~ of absorbance at 260 nm and 280 nm ~~is use assessed Purity of genomic DNA~~ by Thermo Scientific Multiskan GO Microdroplet Spectrophotometer.

Identification of 16S rRNA gene and phylogenetic tree

Identification of 16S rRNA gene was initiated by culturing 2-3 ose isolates of *Bacillus* sp. EG6.4 into 20 mL of Luria Bertani media, incubated at room temperature with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out using the CTAB method (Ausubel et al. 2003). DNA purity and concentration values were measured using Multiskan GO ~~on~~ at λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene amplification was carried out using Eppendorf Mastercycler. ~~This~~ The process ~~begins~~ started by adding GoTaq Green Master Mix and 16S rRNA primers, in the form of primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was conditioned as follows: initial denaturation of 94°C for 2 minutes, denaturation of 92°C for 30 seconds, annealing 55°C for 30 seconds, elongation of 72°C for 1 minute, final elongation of 72°C for 5 minutes, 35 cycles. The PCR samples were sent to ~~the~~ 1st Base DNA Sequencing Service Malaysia. ~~Amplikon~~ Amplicon was sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The results of PCR were visualized through an electrophoresis process using 1% agarose gel followed by ~~Ethidium~~ ethidium ~~Bromide~~ bromide staining and observed in ultraviolet light. The data were also analyzed for their relation by building a phylogenetic tree using MEGA 6.0 (Tamura et al. 2013).

~~The analysis~~ Analysis of 16S rRNA gene

~~The isolated~~ Isolated DNA of *Bacillus* sp. EG6.4 was amplified using the ~~Eppendors~~ eppendors ~~Mastercycler~~ mastercycler with the PCR method and universal primers 27F and 1492R at 1st Base Sequencing Service in Singapore. The Bio Edit Sequence Alignment Editor software version 7.2.5 was used to evaluate the data from the sequencing results. The 16S rRNA gene nucleotide sequence from *Bacillus* sp. EG6.4 was matched with 16S rRNA gene sequences from other bacteria reported to GenBank. The Basic Local Alignment Search Tool (BLAST) at The National Library of Medicine, National Center for Biotechnology Information in Washington, DC (http://blast.ncbi.nml.nih.gov/) was used to find homology of the 16S rRNA gene.

Analysis of phylogenetic tree

The results of genomic DNA sequencing of *Bacillus* sp. EG6.4, which has been compared with other bacteria through BLAST, ~~was~~ were then compiled to determine the relationship through a phylogenetic tree was created by Mega 7 program. A phylogenetic tree was created by including FASTA from other species. All of these species were selected based on nucleotide sequence of 16S rRNA gene, because relationships of a species with other species can be seen from the sequence.

Bioassay larvicidal toxicity

Determination of ~~the~~ LC of *Bacillus* sp. EG6.4 against *A. aegypti* larvae was prepared by inoculating on Nutrient Yeast Salt Medium (NYSM) broth ~~were~~ was incubated on a rotary shaker (130 rpm) at 30°C for 72 hours and then bacterial density was determined by spectrophotometer at 600nm. ~~Then, bacterial density was determined by spectrophotometer at A_{600nm}.~~ Bioassay~~s~~ ~~were~~ was conducted ~~by~~ according to Suryadi et al. (2016). The final culture concentration was adjusted to variations concentration of (v/v) NYSM. Samples of 20 third-instar larvae of *A. aegypti* were tested at six concentrations each treatment in triplicate. ~~The larvae~~ Larvae were ~~seen to~~ died after 24- and 48-hours exposure. The ~~Lethal~~ lethal ~~Time~~ time of *Bacillus* sp. EG6.4 of *A. aegypti* larvae was determined at a concentration of LC_{90}, as many as 20 larvae were exposed, and each treatment had three replications. Mortality of ~~the~~ larvae was scored after 0~~;~~, 0.5~~;~~, 1~~;~~, 2~~;~~, 4~~;~~, 8~~;~~, 10~~;~~, 20~~;~~, 24~~;~~ and 48-hours exposure along with NYSM (10% v/v) without inoculum served as negative controls.

Bioassay of larval mortality (%) used to determine LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} of *Bacillus* sp. EG6.4 against *A. aegypti* larvae. Probit analysis using Minitab ~~Version~~ version 17 was used to calculate the toxicity of *Bacillus* sp. EG6.4 against *A. aegypti* (Postelnicu~~,~~ 2018).

Larvicidal Mechanisms

Detection of parasporal inclusion

The process of detecting *Bacillus* sp. EG6.4 parasporal inclusions included multiple phases, including rejuvenation, purification, and growth on NYSM broth at 30 °C 72 hours, in a shaker incubator (130 rpm). After producing a pure liquid culture of *Bacillus* sp. EG6.4, TEM examination was performed at the Eijkman Institute's TEM and Histology Laboratory in Jakarta, while SEM study was performed at LPPT Gadjah Mada University in Yogyakarta using an SEI 10 kV WD10mm SS30.

Hemolytic and chitinolytic activity

Hemolytic activity was tested using blood agar media acquired from the Surabaya Health Laboratory Center and utilized to screen *Bacillus* sp. EG6.4 for h~~a~~emolytic activity. Colony of isolates was cultured for two days at room temperature. ~~The formation of distinct~~ Distinct clear zones were formed surrounding a colony. Chitinolytic activity was carried out by growing *Bacillus* sp. EG6.4 on colloidal chitin agar media by the streak method. Observation of the clear zone around the colonies was carried out after an incubation period of 4 days, by adding Congo red dye to ~~Petri~~ petri dish and then washing it with NaOH.

Detection of surfactin-coding gene

~~Researchers used the~~ The DNA obtained was used from the identification of the 16S rRNA gene stage in this stage. The approach for detecting the biosynthetic surfactin gene ~~is~~ was similar to that for identifying the 16S rRNA gene, with the exception of the primer employed. On the page Thermo Fisher Scientific Oligo Perfect Primer Designer cloning application, the srfA-D gene primers ~~are~~ were self-designed. The surfactin gene primers that ~~have been~~ were designed are forward primer (5'- ATGAGCCAACTGTTCAAATCATTTG -3') and reverse primer (5'- TCAGGAACTGGAAATCGGATGC -3').

RESULTS AND DISCUSSION

Molecular identification

Molecular identification results (Figure 1~~;~~ and Table 1) showed that ~~the~~ *Bacillus* sp. EG6.4 had 97.89% similarity with *B. mojavensis* strains NBRC 15718 and *B. mojavensis* IFO 15718 and was closely related to *B. halotolerans* strains DSM 8802 and *B. halotolerans* LMG 22476 (Figure 2).

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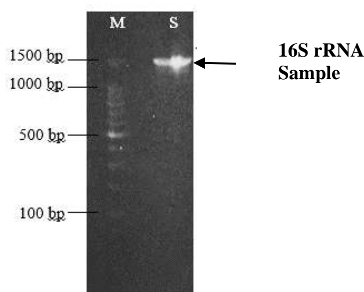

Figure 1. The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M=100bp DNA marker; S= sample of 16S rRNA gene.

Table 1. ~~The name of species of indigenous *Bacillus* sp. EG6.4 based on~~ 16S rRNA gene analysis of indigenous *Bacillus* sp. EG6.4 by Basic Local Alignment Search Tool (BLAST)

Description	Accession No	E value	Query Cover	% ID
Bacillus mojavensis strain NBRC 15718	NR 112725.1	0.0	100%	97.89
Bacillus mojavensis strain IFO 15718	NR 024693.1	0.0	100%	97.89
Bacillus halotolerans strain DSM 8802	NR 115063.1	0.0	100%	97.89

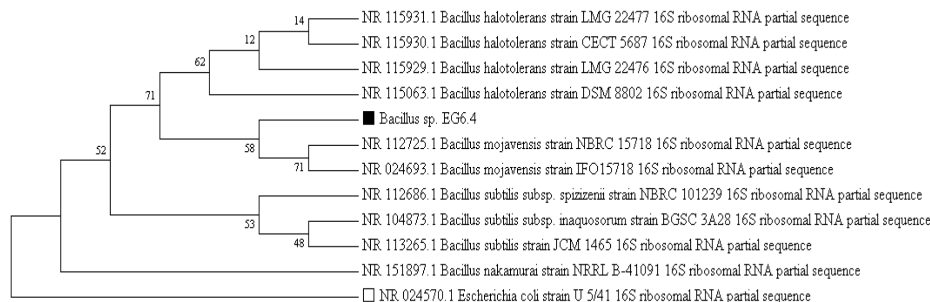

Figure 2. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application

Bioassay Results

The results of bioassay of *Bacillus* sp. EG6.4 against third instar larvae of *A. aegypti* are presented in Table 2. The results of ~~the Probit~~ probit analysis showed that~~, the~~ LC_{50} values at 24- and 48-hours exposure were 8.99±1.01 ~~×x~~10^7 cells/mL and 8.43±1.01 ~~×x~~10^7 cells/mL, respectively (Figure 3), whereas LT_{50} values were 11.9±1.1 and hours and 22.6 ……… (Figure 4). ~~Meanwhile, the LT_{50} value was 11.9±1.1 hours (Figure 4).~~

Table 2. The effect of indigenous *Bacillus* sp. EG6.4 concentrations ~~(OD_{600nm} and CFU/mL)~~ on mortality of *Aedes aegypti* third instar larvae (%) after 24~~-~~ and 48-hours exposure.

Treatments (Concentration Series)	Culture (per-10 mL NYSM)	$OD_{600\,nm}$	CFU/mL	24 h exposure, larval mortality = %	48 h exposure, larval mortality = %
C_1	0.5 mL	0.07	09.8 x 10^7	6.7 ± 5.8	6.7 ± 5.8
C_2	1.0 mL	0.15	2.29 x 10^7	20 ± 10	26.7 ± 5.8
C_3	2.5 mL	0.37	5.90 x 10^7	43.3 ± 15.3	43.3 ± 15.3
C_4	5.0 mL	0.75	12.1 x 10^7	73.3 ± 5.8	76.7 ± 5.8

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C_5	10 mL	1.50	24.4 x 10^7	93.3 ± 5.8	93.3 ± 5.8

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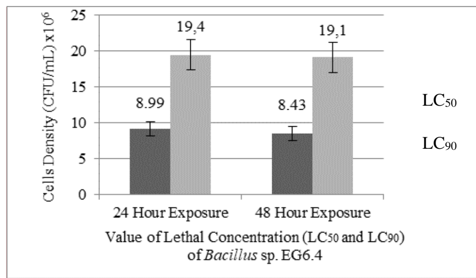

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177 **Figure 3.** Values of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48
178 hours of exposure

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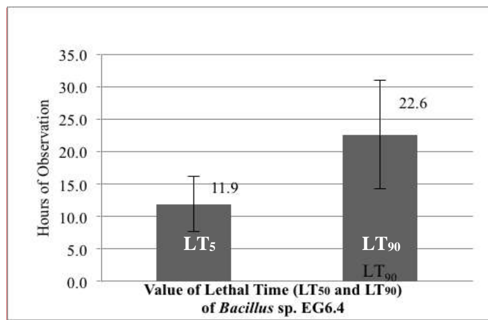

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182 **Figure 4**. The Lethal Lethal Time time 50% ($LT_{50}c$) and Lethal Time 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third
183 instar larvae on observation: 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48 hours exposure

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184 **Larvicidal Toxicity Mechanism**

185 **Detection parasporal inclusion**

186 Result revald that *Bacillus* sp. EG6.4 is was a Gram-positive and endospore-forming bacteria. Detection using TEM
187 showed subterminal oval-shaped endospores (Salamun et al. 2020). Detection using TEM result showed massive paraspore
188 inclusions (Figure 5), while SEM showed only spores but not cry toxin (Figure 6). Detection using SEM only showed
189 spores and did not the Cry toxin (**Figure 6**).

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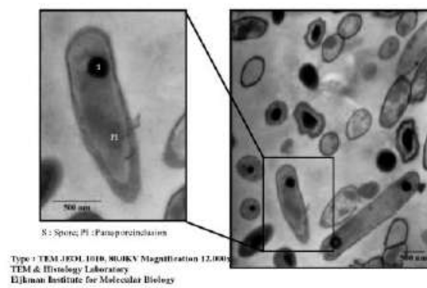

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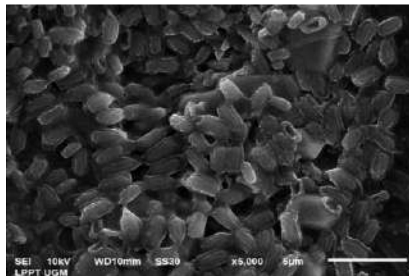
191 **Figure 5.** The organelles Organelles of indigenous *Bacillus* sp. EG6.4 endospore cells were visible using transmission electron
192 microscopy (TEM). TEM JEOL1010 80.0 kV, 12.000x magnification, S=Forespore, PI=Parasporal Inclusion



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194 **Figure 6.** Scanning Electron Microscopy (SEM) showed only visible Visible spores of indigenous *Bacillus* sp. EG6.4 using Scanning
195 Electron Microscopy (SEM), in SEI 10kV WD10mm SS30 x5,000 magnification (5μm), by LPPT UGM, Yogyakarta.

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197 **Hemolytic and chitinolytic activity**

198 The results of hemolytic and chitinolytic activity assays, showed that hemolytic activity but not chitinolytic activity
199 (Figure 7), indicating its potential to produce biosurfactants.

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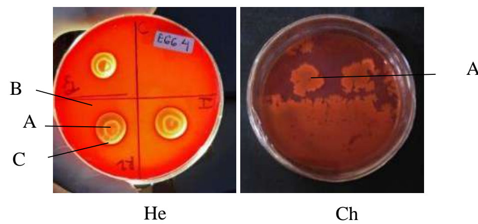

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Figure 7. Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal chitin plate media (Ch). Note: A=Colony of isolate; B=Blood agar plate, C=Clear zone around colony of isolate

Detection of surfactin-coding gene

The electrophoresis results ~~**(Figure 8)**~~ showed a band of about 700 bp. Based on data in GenBank CP0514641, ~~the~~ srfA-D gene of *B. mojavensis* was 729 bp (Figure 8). The srfA-D gene has encoded a thioesterase enzyme that is important in the surfactin biosynthetic pathway. Band position on electrophoretic visualization showed that *B. mojavensis* EG6.4 had ~~the~~ srfA-D gene.

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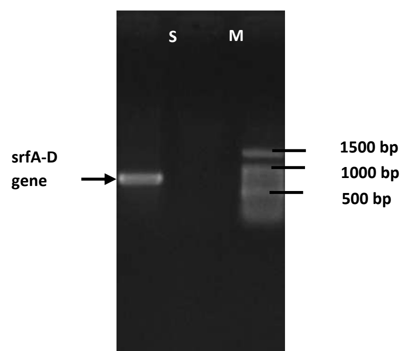

Figure 8. Electrophoresis results of ~~the~~ srfA-D gene *Bacillus mojavensis* EG6.4. ~~which was successfully amplified. Description~~ M=500bp DNA marker; S=Sample of srfA-D gene.

Table 3. Results of ~~the~~ Basic Local Alignment Search Tool (BLAST) nucleotide analysis ~~the~~ of srfA-D gene of *Bacillus mojavensis* EG6.4

Species Name with srfA-D Gene	~~No.~~ Accession No.	E. value	QC	% ID	Description
Bacillus mojavensis strain PS17	CP066516.1	0,.0	99%	98,.35%	Complete genome
Bacillus mojavensis strain UCMB5075	CP051464.1	0,.0	100%	94,.92%	Complete genome

The nucleotide BLAST results from ~~the~~ srfA-D gene of *B. mojavensis* EG6.4 showed a similarity value of 98.35% with the gene in *B. mojavensis* strain PS17 and had a similarity value of 94.92% with *B. mojavensis* strain UCMB5075 (Table 3). Further studies, the results of protein BLAST against the amino acid gene srfA-D *B. mojavensis* EG 6.4 obtained the highest similarity of 98.35% with surfactin biosynthesis thioesterase ~~SrfA~~srfA-D from the bacterium *B. mojavensis* strain PS17.

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Discussion

The results of ~~the~~ isolation of *Bacillus* sp. EG6.4 ~~has~~ showed DNA purity = 1.84 (A260/A280), DNA concentration 44.7 (ng/µl), ~~has been~~ which was confirmed by electrophoresis ~~showing~~ with DNA size ~~is well~~ above 1500bp. The DNA migration during electrophoresis ~~is~~ was influenced by agarose concentration, DNA molecule size, voltage, and temperature (Fatchiyah et al. 2011; Brown~~,~~ 2016). The result~~s~~ of ~~the~~ 16S rRNA gene amplification was visualized by electrophoresis ~~**(Figure 1)**~~, showed that a band measuring about 1550bp and ~~about~~ 500 bases at the end of the sequence ~~is~~ was a hypervariable region. Pearson (2014) reported that ~~the~~ 16S rRNA gene can be used for the molecular identification of microorganisms. The results of purification and sequencing of the 16S rRNA gene of *Bacillus* sp. EG6.4 showed a nucleotide sequence with a size of 1424 bp. Based on BLAST analysis, *Bacillus* sp. EG6.4 ~~is~~ was similar to *B. mojavensis* ~~**(Table 1)**~~ and ~~is~~ was related to other bacteria ~~**(Figure 2)**~~.

Bacillus sp. EG6.4 has a close relationship with *Bacillus mojavensis* and *Bacillus halotolerans*. *B. mojavensis* and *B. halotolerans* are two species of bacteria that are closely related to *Bacillus subtilis* however it differs significantly in terms of fatty acid content, DNA sequences, and transgenic species resistance (Bacon and Hinton 2002). *B. mojavensis* ~~have~~ has been known that produce secondary matabolites, such as oxygenated monoterpenes and lipopeptides, group of biosurfactants~~,~~ that have promising applications in agricultural, food industry, and clinical fields. In agriculture, *B. mojavensis* is used as antimicrobial agent against pathogenic bacteria and fungi (Camele et al.~~,~~ 2019). *B. halotolerans* has been known that produce~~s~~ biosurfactants. Based on research reported by Wang et al. (2002), biosurfactants from *B. halotolerans* can be used as agent to remediation in polluted environment petroleum.

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The values of LC_{50} and LC_{90} are the concentrations of microorganisms that cause 50% and 90% mortality of the target organism population. The lower the LC value of microorganisms, the higher the toxicity. Toxicity of *Bacillus* sp. influenced by the type of toxin or secondary metabolites produced by microorganisms, the species and age of the target larvae, and the media used for bacterial growth (Suryadi et al. 2016). Determination of LC_{50} and LC_{90} as well as LT_{50} and LT_{90} was carried out to determine the concentration and length of time required by *Bacillus* sp. EG6.4 to kill 50% and 90% and the length of time to kill 50% and 90% of third instar *A. aegypti* larvae. Based on the mortality rate of *A. aegypti* larvae in the treatment, *Bacillus* sp. EG6.4 ~~in the category of~~ showed high toxicity, which isolate has the potential to be developed as a biolarvacidal agent. ~~Based on the mortality rate of *A. aegypti* larvae by *Bacillus* sp. EG6.4, indicates that the isolate has the potential to be developed as a biolarvacidal agent.~~

The ~~value of~~ LC_{50} and LC_{90} values of *Bacillus* sp. EG6.4 ~~has~~ have high potential ~~(Figure 3)~~, when compared to previous studies. Gamma et al. (2010) reported that *B. thuringiensis* PWR4 32 isolated from Malang, Indonesia had an LC_{50} value of 22.79 × ~~x~~ 10^7 cells/mL at 72-hour exposure. Pratiwi et al. (2013) ~~have~~ reported that *B. thuringiensis* W.Swh.S.K2 isolated from Nganjuk Indonesia, had an LC_{50} value of $3.53x10^7$ cells/mL at 48-hour exposure. The number of spores consumed by the larvae affects the rate of larval death. Gamma et al. (2010) found that as *B. thuringiensis* spores developed, more Cry was released to kill *A. aegypti* larvae. The two isolates have different strains or species, so there is a difference in toxicity between them. One of the characteristics of *Bacillus* sp. is that it can produce endospores and protein crystals (parasporal inclusions) at the time of cell sporulation.

Bacillus sp. EG6.4 ~~shows~~ showed a complete endospore with sections, including a spore layer, spore, and paraspore inclusions ~~(**Figure 5; Figure 6**)~~. *Bacillus* sp. EG6.4 ~~produces~~ produced paraspore inclusions, but the shape ~~is~~ was massive and ~~has~~ was not ~~been~~ proven ~~as a~~to be a protein toxin that ~~has~~ had larvicidal activity against *A. aegypti* larvae., ~~so it is necessary to further investigate whether the components of paraspore inclusions cause larval death.~~ Iftikhar et al (2018) reported that *B. mojavensis* BTCB15 synthesized silver nanoparticles as nanosides against the larvae of *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, important vectors of disease transmission. The LC_{50} and LC_{90} for third instar larvae of the three species were 0.80, 0.75, and 1.2 ppm, and 0.80, 0.67, and 0.86 ppm, respectively. Iftikhar et al (2018), recommended the development of silver nanoparticles produced by *B*~~,~~. *mojavensis* BTCB15, which may play a role in combating mosquito populations, thereby controlling vector-borne diseases. The larvicidal activity of silver nanoparticles (Ag/AgCl NPs) synthesized using *Bti* supernatant showed that LC_{50}=0.133 g/mL, higher toxicity than that synthesized using insecticidal protein, LC_{50}=0.148–0.217 g/mL (Chimkhan et al. 2022). Possible mechanism of action of Ag/AgCl NPs against *A. aegypti* larvae is through mitochondrial dysfunction, DNA and protein damage, inhibition of cell proliferation, and cell apoptosis, and recommended that Ag/AgCl NPs are an alternative approach to control *A. aegypti* larvae, a mosquito-borne disease vector (Chimkhan et al. 2022). Further research needs to be done, whether *Bacillus* sp. EG6.4 can produce silver nanoparticles that ~~have a~~ can be used as antilarval ~~icidal effect on~~ against *A. aegypti* larvae.

Hemolytic activity assay of *Bacillus* sp. EG6.4 on blood agar showed a positive result ~~(**Figure 7**)~~. Biosurfactants have been utilized as an alternative to chemical pesticides in the past. Previous studies found that biosurfactant-producing bacteria are suitable for controlling plant pathogenic and insecticidal (Bais et al. 2004; Zhao et al. 2014). *B. subtilis* has been reported as a bacterial agent ~~have~~ produc~~t~~ing mosquitocidal toxin (Das and Mukherjee 2006; Manonmani et al. 2011). The toxicity of biosurfactants or their potential as biocontrol agents has not been well investigated. ~~Biosurfactants, which are surfactin produced by *Bacillus* strains, have been offered as a potential alternative to mosquito-borne disease control.~~ Biosurfactants produced by *Bacillus* strains have ~~been~~ shown ~~to have~~ mosquitocidal activity in adult mosquitos, killing both immature and adult mosquitos (Geetha et al. 2012). ~~Mosquitoes in the larval and pupal stages, respiration depends on the siphon. The decrease in the surface tension of the water induced by the surfactant will prevent the siphon from remaining in position on the outer layer of water. As a result, larvae and pupae lose contact with air and death occurs due to respiratory failure. The low oxygen concentration causes the insect spiracles to remain open and causes insect death.~~ Other studies have shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects (Zhao et al. 2014). From these studies it has been shown that biosurfactants affect the cuticle of insects, because they are amphiphilic in the presence of hydrophobic and hydrophilic molecules that ~~will~~ damage cell membranes, then damage epithelial cells, and cause larval death (Zhao et al. 2014).

B. mojavensis has been shown to create lipopeptides, which have a unique structure and are important antibacterial and antifungal substances derived from surfactin, iturin, and fengysin (Mounia et al. 2014; Blacutt et al. 2016). According to Jasim et al. (2016), lipopeptide in *B. mojavensis* has antiviral, antitumoral, antifungal, and antimycoplasmatic properties. ~~The production of lipopeptide compounds by *B. mojavensis* is influenced by environmental factors, especially the carbon source is an important parameter in the formation of lipopeptides.~~ Based on the research of Hmidet et al. (2017), *B. mojavensis* produced surfactin and fengysin on all carbon sources used and the best production occurs in media with glucose as a carbon source and the least production occurs in media with starch carbon sources. *B. mojavensis* also showed α-hemolytic activity during its growth in blood agar (Berekaa and Ezzeldin 2018). These investigations revealed that bacteria-produced biosurfactant can be employed as a biocontrol for plant pathogens and as an effective bioinsecticide. ~~Insecticidal metabolites with an amphiphilic structure comprising hydrophobic and hydrophilic molecules were created by biosurfactants.~~

The chitinolytic activity assay showed negative result. Melo et al. (2016) discovered that ~~the~~ larvicidal paraspore toxin produced by *B. thuringiensis* also produces chitinase enzymes for agroindustrial use. ~~The chitinolytic activity assay~~

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~~showed negative result (**Figure 7**)~~. ~~This chitinolytic activity assay is also a screening effort to determine the potentials of a bacterium to produce chitinase.~~ Chitinolytic microorganisms have a lot of potential to be applied as biocontrol agents (Wang et al. 2006). ~~The growth and development of insects is highly dependent on the construction and structure of chitin (Merzendorfer and Zimoch, 2003). Chitinase induces peritrophic membrane damage in the insect intestine which causes a decrease in digestive activity and interferes with insect growth (Tera and Tera 2005). In a study conducted by~~ Arora et al. (2003) reported that~~,~~ purified chitinase from *Bacillus* spp. ~~and~~ has been analyzed as an insecticide. ~~However, the results of this study did not show a clear zone formed around the colony, thus indicating a negative result.~~

The results of nucleotide BLAST analysis and BLAST protein srfA-D gene *B. mojavensis* EG6.4 showed ~~the~~ highest similarity to *B. mojavensis* strain PS17 with accession number CP066516.1 and surfactin biosynthesis thioesterase srfA-D from *B. mojavensis* bacteria with accession number QQF62274.1. The srfA-D from *B. mojavensis* EG6.4 ~~has been~~ is known ~~that~~ to play a role in biosynthesis thioesterase enzyme to produce biosurfactants.

The indigenous *Bacillus* sp. EG6.4 is similar to *Bacillus mojavensis* and ~~produced~~ produces massive shape of parasporal inclusion. Bioassay results ~~in the category of~~ showed high toxicity against *A. aegypti* larvae. *Bacillus* sp. EG6.4 showed hemolytic activity and ~~be detected~~ found that ~~the~~ srfA-D gene ~~to~~ produces surfactin, indicating its potency to produce biosurfactants. It is concluded from the present result that *Bacillus* sp. EG6.4 ~~showed potential to~~ can be developed as a biocontrol agent for disease vectors and plant pests and plant diseases.

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Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

Abstract. This research was carried out for the molecular identification, as well as the determination and mechanism of action of larvicidal toxicity of *Bacillus* sp. EG6.4 isolated from breeding sites of *Aedes aegypti* from Gresik City, East Java, Indonesia. *Bacillus* sp. EG6.4 was a Gram-positive endospore-forming bacteria. Molecular species identification using 16S rRNA gene sequencing showed that isolate had 97.89% similarity with *Bacillus mojavensis*. The isolate showed larvicidal toxicity against *A. aegypti* larvae. The lethal concentration 50% (LC_{50}) values at 24 and 48hours exposure were 8.99±1.01 $\times 10^7$ cells/mL and 8.43±1.01 $\times 10^7$ cells/mL, respectively, while lethal time 50% (LT_{50}) value was 11.9±1.1 hours. , Production of chitinolytic enzymes or biosurfactants, chitinolytic and hemolytic assays were conducted to determine the larvicidal mechanism. As a result, *Bacillus* sp. EG6.4 showed hemolytic, but not chitinolytic activity, indicating its potency to produce biosurfactants. Transmission Electron Microscopy (TEM) result showed that isolate had oval-shaped endospores located subterminal with massive-shape parasporal inclusions. The detection of srfA-D gene showed that isolate produced surfactin biosynthesis thioesterase. Thus, *Bacillus* sp. EG6.4 produced biosurfactant that potentially to be developed as a biocontrol agent for disease vectors and plant pathogens.

Keywords: *Aedes aegypti*, *Bacillus mojavensis*, biosurfactants, larvicidal toxicity, parasporal inclusion, srfA-D gene

Running title: Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4

INTRODUCTION

Dengue fever (DF), a vector-borne disease and a serious public health problem in the world, is transmitted by the *Aedes aegypti* mosquito (Dahmana and Mediannikov 2020; Falqueto et al. 2021). Vaccines have been developed, but the results have not been satisfactory. Chemical insecticides are used to suppress mosquito vector populations, but have a negative impact on the environment and are toxic to non-target organisms (Dahmana and Mediannikov 2020). ~~So that one alternative to overcome this disease with integrated vector control~~ The effectiveness of a storage time formulation of *Bacillus thuringiensis* has been investigated against an *A. aegypti* larvae, and it is recommended that this be an option to overcome the disease with integrated vector control (Melanie et al. 2018). Biological control experts suggest developing bioinsecticides as biocontrol agents for disease vectors (Thomas 2017).

The biodiversity of microorganisms is one of the abundant renewable biological resources in the world, including in Indonesia. The use of *Bacillus* in the biocontrol of vectors, pests, and plant diseases has grown rapidly (Benelli et al. 2016). *Bacillus thuringiensis* var. *israelensis* (Bti) is used as a dengue vector biocontrol agent (BCAs) for controlling of *A. aegypti* larvae (Boyce et al. 2013). During sporulation, *B. thuringiensis* produce parasporal inclusions and the resulting cry toxin causes death of several insect species (Aramideh et al. 2016).

The group of spore-forming bacteria inhibits their growth under abnormal conditions and begins to increase their metabolic activity (Shahcheraghi et al. 2015). Sporulation is an effort to defend bacteria against an unfavorable environment. Several *Bacillus* species are reported to produce a protein toxin (cry toxin) and also secondary metabolites that act as self-defense to survive. In the vegetative phase, bacteria grow and develop, but in unfavorable conditions they produce secondary metabolites, such as biosurfactants, enzymes, or exotoxins to maintain their live. Biosurfactant synthesis is associated with hemolytic activity (Carrillo et al. 1996) and has also applied by Colonna et al. (2017) as a rapid screening technique for assesing the toxicity of native surfactin. The hemolytic activity assay is a typical screening

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The effectiveness of the storage time formulation of *Bacillus thuringiensis* has been investigated against *A. aegypti* larvae, so recommended that is one alternative to overcome this disease with integrated vector control.

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method for detecting biosurfactant activity. Biosurfactant generated by *Bacillus* sp. is found to be effective as a plant pathogenic and insecticidal agent in several tests (Bais et al. 2004). Nafidiastri et al. (2021) reported that indigenous *B. velezensis* ES4.3 resulted biosurfactant activity was indicated by the formation of clear zones, emulsions, and a decrease in surface tension and the surfactin gene showed a 100% ID with the surfactin biosynthesis thioesterase SrfA-D gene.

Isolation and characterization of indigenous *Bacillus* entomopathogenic species have been isolated from the breeding sites of *A. aegypti* in Surabaya, Gresik, and Sidoarjo, East Java, Indonesia (Salamun et al. 2020). According to preliminary test, about 133 *Bacillus* sp. are entomopathogenic against larvae of *A. aegypti*. The majority of *Bacillus* sp. isolates showed mild to moderate toxicity, but 16 isolates were highly toxic, including *Bacillus* sp. EG6.4, which causes 100% larval mortality. Salamun et al. (2020) identified microscopic, macroscopic, and biochemical phenotypic similarities indicating that *Bacillus* sp. EG6.4 had a similarity level of 80.60% with *B. thuringiensis*. Mechanism of action of larvicidal toxicity of *Bacillus* spp. against *A. aegypti* in two ways, namely in the sporulation phase the bacteria produce endotoxins which are stored in the paraspore inclusions (pellet fraction) and produce secondary metabolites (supernatant fraction) which are secreted out of the cells. (Dahmana et al. 2020; Falqueto et al. 2021; Katak et al. 2021).

Further research is needed for molecular identification to determine species name of *Bacillus* sp. EG6.4 and genetic relationships with other bacilli, bioassays to determine larval mortality, and to detect the mechanism of action by detecting the presence of paraspore inclusions, surfactin coding genes, and hemolytic and chitinolytic activities, so it is hoped that the mechanism of action of *Bacillus* sp. EG6.4 as larvicidal can be identified.

The aim of this study was to identify the isolated species at molecular level and to determine larvicidal toxicity and larvicidal mechanism of the indigenous *Bacillus* sp. EG6.4. Further, species name was conducted with the 16S rRNA gene and traces genetic affinity through a phylogenetic tree. Larvicidal activity was determined by LC_{50} and LC_{90} values. To determine the larvicidal mechanisms of action, with detection of endospore by Transmission Electron Microscopy (TEM) and Scanning Electron Microsopy (SEM), surfactin coding genes (srfA-D) and detection of secondary metabolite activity by hemolytic and chitinolytic activity assays.

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MATERIALS AND METHODS

Molecular Identification

DNA isolation

The genomic DNA of *Bacillus* sp. EG6.4 was isolated using the Promega DNA Purification Kit (Promega 2018). Isolated DNA was visualized by electrophoresis. The results of genomic DNA electrophoresis of *Bacillus* sp. EG6.4 was photographed under a UV Transilluminator. The purity of genomic DNA was measured using the ratio of absorbance at 260 nm and 280 nm by Thermo Scientific Multiskan GO Microdroplet Spectrophotometer.

Identification of 16S rRNA gene and phylogenetic tree

Identification of 16S rRNA gene was initiated by culturing 2-3 ose isolates of *Bacillus* sp. EG6.4 into 20 mL of Luria Bertani media, incubated at room temperature with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out using the CTAB method (Ausubel et al. 2003). DNA purity and concentration values were measured using Multiskan GO at λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene amplification was carried out using Eppendorf Mastercycler. The process started by adding GoTaq Green Master Mix and 16S rRNA primers, in the form of primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was conditioned as follows: initial denaturation of 94°C for 2 minutes, denaturation of 92°C for 30 seconds, annealing 55°C for 30 seconds, elongation of 72°C for 1 minute, final elongation of 72°C for 5 minutes, 35 cycles. The PCR samples were sent to 1st Base DNA Sequencing Service Malaysia. Amplicon was sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The results of PCR were visualized through an electrophoresis process using 1% agarose gel followed by ethidium bromide staining and observed in ultraviolet light. The data were also analyzed for their relation by building a phylogenetic tree using MEGA 6.0 (Tamura et al. 2013).

Analysis of 16S rRNA gene

Isolated DNA of *Bacillus* sp. EG6.4 was amplified using the eppendors mastercycler with the PCR method and universal primers 27F and 1492R at 1st Base Sequencing Service in Singapore. The Bio Edit Sequence Alignment Editor software version 7.2.5 was used to evaluate the data from the sequencing results. The 16S rRNA gene nucleotide sequence from *Bacillus* sp. EG6.4 was matched with 16S rRNA gene sequences from other bacteria reported to GenBank. The Basic Local Alignment Search Tool (BLAST) at The National Library of Medicine, National Center for Biotechnology Information in Washington, DC (http://blast.ncbi.nml.nih.gov/) was used to find homology of the 16S rRNA gene.

Analysis of phylogenetic tree

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The results of genomic DNA sequencing of *Bacillus* sp. EG6.4, which has been compared with other bacteria through BLAST, were then compiled to determine the relationship through a phylogenetic tree was created by Mega 7 program. A

phylogenetic tree was created by including FASTA from other species. All of these species were selected based on nucleotide sequence of 16S rRNA gene, because relationships of a species with other species can be seen from the sequence.

Bioassay larvicidal toxicity

Determination of LC of *Bacillus* sp. EG6.4 against *A. aegypti* larvae was prepared by inoculating on Nutrient Yeast Salt Medium (NYSM) broth was incubated on a rotary shaker (130 rpm) at 30°C for 72 hours and then bacterial density was determined by spectrophotometer at 600nm. Bioassay was conducted according to Suryadi et al. (2016). The final culture concentration was adjusted to variations concentration of (v/v) NYSM. Samples of 20 third-instar larvae of *A. aegypti* were tested at six concentrations each treatment in triplicate. Larvae were died after 24- and 48-hours exposure. The lethal time of *Bacillus* sp. EG6.4 of *A. aegypti* larvae was determined at a concentration of LC_{90}, as many as 20 larvae were exposed, and each treatment had three replications. Mortality of larvae was scored after 0, 0.5, 1, 2, 4, 8, 10, 20, 24 and 48-hours exposure along with NYSM (10% v/v) without inoculum served as negative control.

Bioassay of larval mortality (%) used to determine LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} of *Bacillus* sp. EG6.4 against *A. aegypti* larvae. Probit analysis using Minitab version 17 was used to calculate the toxicity of *Bacillus* sp. EG6.4 against *A. aegypti* (Postelnicu 2018).

Larvicidal Mechanisms

Detection of parasporal inclusion

The process of detecting *Bacillus* sp. EG6.4 parasporal inclusions included multiple phases, including rejuvenation, purification, and growth on NYSM broth at 30 °C 72 hours, in a shaker incubator (130 rpm). After producing a pure liquid culture of *Bacillus* sp. EG6.4, TEM examination was performed at the Eijkman Institute's TEM and Histology Laboratory in Jakarta, while SEM study was performed at LPPT Gadjah Mada University in Yogyakarta using an SEI 10 kV WD10mm SS30.

Hemolytic and chitinolytic activity

Hemolytic activity was tested using blood agar media acquired from the Surabaya Health Laboratory Center and utilized to screen *Bacillus* sp. EG6.4 for hemolytic activity. Colony of isolates was cultured for two days at room temperature. Distinct clear zones were formed surrounding a colony. Chitinolytic activity was carried out by growing *Bacillus* sp. EG6.4 on colloidal chitin agar media by the streak method. Observation of the clear zone around the colonies was carried out after an incubation period of 4 days, by adding Congo red dye to petri dish and then washing it with NaOH.

Detection of surfactin-coding gene

The DNA obtained was used from the identification of the 16S rRNA gene stage in this stage. The approach for detecting the biosynthetic surfactin gene was similar to that for identifying the 16S rRNA gene, with the exception of the primer employed. On the page Thermo Fisher Scientific Oligo Perfect Primer Designer cloning application, the srfA-D gene primers were self-designed. The surfactin gene primers that were designed are forward primer (5'- ATGAGCCAACTGTTCAAATCATTTG -3') and reverse primer (5'- TCAGGAACTGGAAATCGGATGC -3').

RESULTS AND DISCUSSION

Molecular identification

Molecular identification results (Figure 1and Table 1) showed that *Bacillus* sp. EG6.4 had 97.89% similarity with *B. mojavensis* strains NBRC 15718 and *B. mojavensis* IFO 15718 and was closely related to *B. halotolerans* strains DSM 8802 and *B. halotolerans* LMG 22476 (Figure 2).

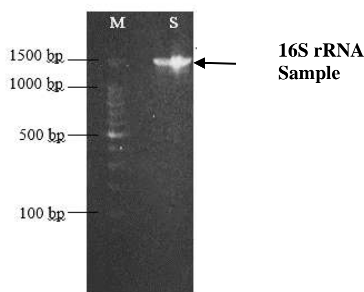

Figure 1. The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M=100bp DNA marker; S= sample of 16S rRNA gene.

Table 1. 16S rRNA gene analysis of indigenous *Bacillus* sp. EG6.4 by Basic Local Alignment Search Tool (BLAST)

Description	Accession No.	E value	Query Cover	% ID
Bacillus mojavensis strain NBRC 15718	NR 112725.1	0.0	100%	97.89
Bacillus mojavensis strain IFO 15718	NR 024693.1	0.0	100%	97.89
Bacillus halotolerans strain DSM 8802	NR 115063.1	0.0	100%	97.89

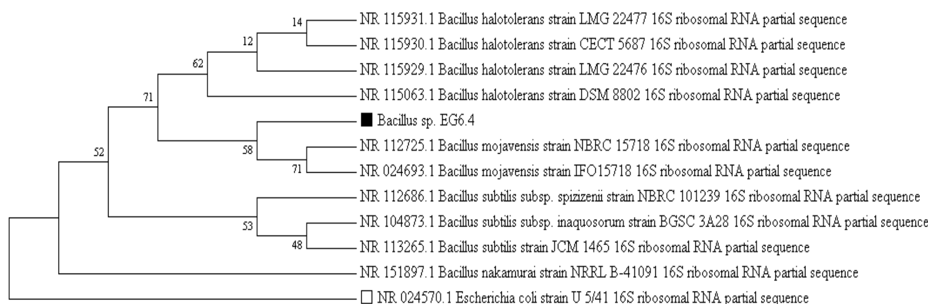

Figure 2. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application

Bioassay Results

The results of bioassay of *Bacillus* sp. EG6.4 against third instar larvae of *A. aegypti* are presented in Table 2. The results of probit analysis showed thatLC$_{50}$ values at 24- and 48-hours exposure were 8.99±1.01 ×10^{7} cells/mL and 8.43±1.01 ×10^{7} cells/mL, respectively (Figure 3), whereas LT$_{50}$ values were 11.9±1.1 hours and ~~hours and 22.6~~ 22.6±8.4 hours~~………~~ (Figure 4).

Table 2. The effect of indigenous *Bacillus* sp. EG6.4 concentrations on mortality of *Aedes aegypti* third instar larvae (%) after 24 and 48-hours exposure.

Treatments (Concentration Series)	Culture (per-10 mL NYSM)	OD$_{600\ nm}$	CFU/mL	24 h exposure, larval mortality = %	48 h exposure, larval mortality = %
C$_1$	0.5 mL	0.07	09.8 x 10^{7}	6.7 ± 5.8	6.7 ± 5.8
C$_2$	1.0 mL	0.15	2.29 x 10^{7}	20 ± 10	26.7 ± 5.8
C$_3$	2.5 mL	0.37	5.90 x 10^{7}	43.3 ± 15.3	43.3 ± 15.3

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at 24- and 48-hours observation were 11.9±1.1 hours and 22.6±8.4 hours, respectively.

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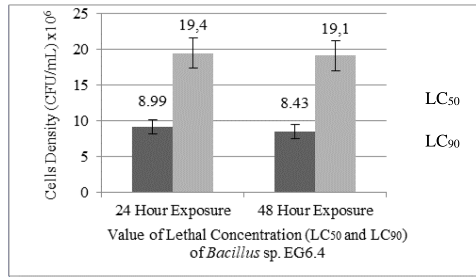
C_4	5.0 mL	0.75	12.1 x 10^7	73.3 ± 5.8	76.7 ± 5.8
C_5	10 mL	1.50	24.4 x 10^7	93.3 ± 5.8	93.3 ± 5.8



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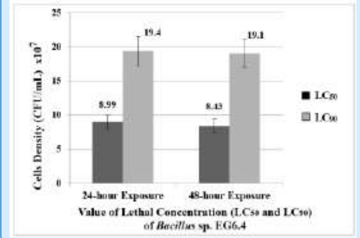

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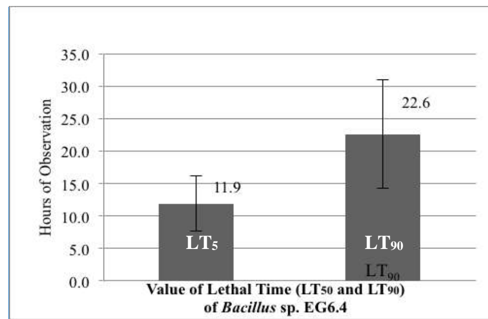
Figure 3. Values of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48 hours of exposure



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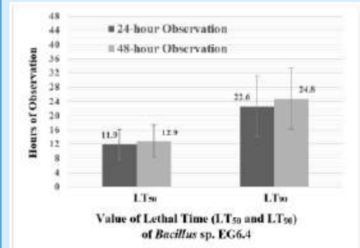

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Figure 4. The ~~Lethal~~ lethal time 50% (c) and 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae on 24and 48hours exposure

Larvicidal Toxicity Mechanism

Detection parasporal inclusion

Result revald that *Bacillus* sp. EG6.4 was a Gram-positive and endospore-forming bacteria. Detection using TEM showed subterminal oval-shaped endospores (Salamun et al. 2020). TEM result showed massive paraspore inclusions (Figure 5), while SEM showed only spores but not cry toxin (Figure 6).

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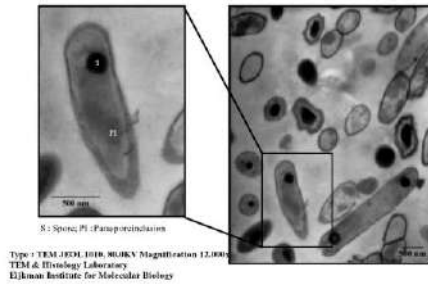

Figure 5. Organelles of indigenous *Bacillus* sp. EG6.4 endospore cells visible using transmission electron microscopy (TEM). S=Forespore, PI=Parasporal Inclusion

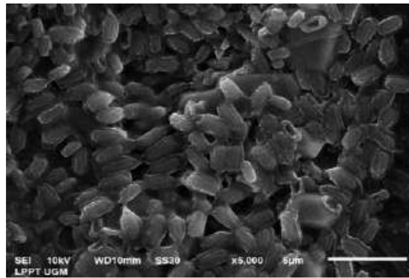

Figure 6. Visible spores of indigenous *Bacillus* sp. EG6.4 using Scanning Electron Microscopy (SEM).

Hemolytic and chitinolytic activity

The results showed hemolytic activity but not chitinolytic activity (Figure 7), indicating its potential to produce biosurfactants.

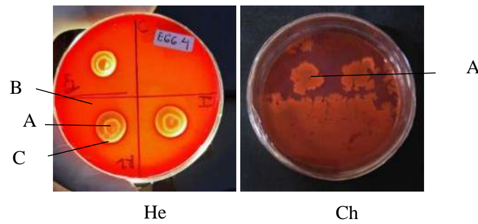

Figure 7. Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal chitin plate media (Ch). Note: A=Colony of isolate; B=Blood agar plate, C=Clear zone around colony of isolate

197 **Detection of surfactin-coding gene**

198 The electrophoresis results showed a band of about 700 bp. Based on data in GenBank CP0514641, srfA-D gene of *B.* 199 *mojavensis* was 729 bp (Figure 8). The srfA-D gene has encoded a thioesterase enzyme that is important in the surfactin 200 biosynthetic pathway. Band position on electrophoretic visualization showed that *B. mojavensis* EG6.4 had srfA-D gene.

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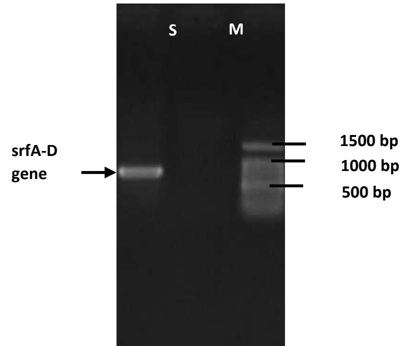

203
204
205 **Figure 8.** Electrophoresis results of srfA-D gene *Bacillus mojavensis* EG6.4. M=500bp DNA marker; S=Sample of srfA-D gene.
206
207 **Table 3**. Results of Basic Local Alignment Search Tool (BLAST) nucleotide analysis of srfA-D gene of *Bacillus mojavensis* EG6.4

Species Name with srfA-D Gene	Accession No.	E. value	QC	% ID	Description
Bacillus mojavensis strain PS17	CP066516.1	0.0	99%	98.35%	Complete genome
Bacillus mojavensis strain UCMB5075	CP051464.1	0.0	100%	94.92%	Complete genome

208
209 The nucleotide BLAST results from srfA-D gene of *B. mojavensis* EG6.4 showed a similarity value of 98.35% with the 210 gene in *B. mojavensis* strain PS17 and had a similarity value of 94.92% with *B. mojavensis* strain UCMB5075 (Table 3). 211 Further studies, the results of protein BLAST against the amino acid gene srfA-D *B. mojavensis* EG 6.4 obtained the 212 highest similarity of 98.35% with surfactin biosynthesis thioesterase srfA-D from the bacterium *B. mojavensis* strain PS17.

213 **Discussion**

214 The results of isolation of *Bacillus* sp. EG6.4 showed DNA purity = 1.84 (A260/A280), DNA concentration 44.7 215 (ng/µl), which was confirmed by electrophoresis with DNA size above 1500bp. The DNA migration during 216 electrophoresis was influenced by agarose concentration, DNA molecule size, voltage, and temperature (Fatchiyah et al. 217 2011; Brown 2016). The result of 16S rRNA gene amplification was visualized by electrophoresis, showed that a band 218 measuring about 1550bp and 500 bases at the end of the sequence was a hypervariable region. Pearson (2014) reported that 219 16S rRNA gene can be used for the molecular identification of microorganisms. The results of purification and sequencing 220 of the 16S rRNA gene of *Bacillus* sp. EG6.4 showed a nucleotide sequence with a size of 1424 bp. Based on BLAST 221 analysis, *Bacillus* sp. EG6.4 was similar to *B. mojavensis* and was related to other bacteria.

222 *Bacillus* sp. EG6.4 has a close relationship with *Bacillus mojavensis* and *Bacillus halotolerans*. *B. mojavensis* and *B.* 223 *halotolerans* are two species of bacteria that are closely related to *Bacillus subtilis* however it differs significantly in terms 224 of fatty acid content, DNA sequences, and transgenic species resistance (Bacon and Hinton 2002). *B. mojavensis* has been 225 known that produce secondary matabolites, such as oxygenated monoterpenes and lipopeptides, group of biosurfactants 226 that have promising applications in agricultural, food industry, and clinical fields. In agriculture, *B. mojavensis* is used as 227 antimicrobial agent against pathogenic bacteria and fungi (Camele et al. 2019). *B. halotolerans* has been known that 228 produce biosurfactants. Based on research reported by Wang et al. (2002), biosurfactants from *B. halotolerans* can be used 229 as agent to remediation in polluted environment petroleum.

230 The values of LC_{50} and LC_{90} are the concentrations of microorganisms that cause 50% and 90% mortality of the target 231 organism population. The lower the LC value of microorganisms, the higher the toxicity. Toxicity of *Bacillus* sp. 232 influenced by the type of toxin or secondary metabolites produced by microorganisms, the species and age of the target 233 larvae, and the media used for bacterial growth (Suryadi et al. 2016). Determination of LC_{50} and LC_{90} as well as LT_{50} and 234 LT_{90} was carried out to determine the concentration and length of time required by *Bacillus* sp. EG6.4 to kill 50% and 90% 235 and the length of time to kill 50% and 90% of third instar *A. aegypti* larvae. Based on the mortality rate of *A. aegypti* 236 larvae in the treatment, *Bacillus* sp. EG6.4 showed high toxicity, which isolate has the potential to be developed as a 237 biolarvacidal agent.

238 The LC_{50} and LC_{90} values of *Bacillus* sp. EG6.4 have high potential, when compared to previous studies. Gamma et al. 239 (2010) reported that *B. thuringiensis* PWR4 32 isolated from Malang, Indonesia had an LC_{50} value of 22.79×10^7 cells/mL

at 72-hour exposure. Pratiwi et al. (2013) reported that *B. thuringiensis* W.Swh.S.K2 isolated from Nganjuk Indonesia, had an LC_{50} value of $3.53x10^7$ cells/mL at 48-hour exposure. The number of spores consumed by the larvae affects the rate of larval death. Gamma et al. (2010) found that as *B. thuringiensis* spores developed, more Cry was released to kill *A. aegypti* larvae. The two isolates have different strains or species, so there is a difference in toxicity between them. One of the characteristics of *Bacillus* sp. is that it can produce endospores and protein crystals (parasporal inclusions) at the time of cell sporulation. *Bacillus* sp. EG6.4 showed a complete endospore with sections, including a spore layer, spore, and paraspore inclusions. *Bacillus* sp. EG6.4 produced paraspore inclusions, but the shape was massive and was not proven to be a protein toxin that had larvicidal activity against *A. aegypti* larvae. Iftikhar et al (2018) reported that *B. mojavensis* BTCB15 synthesized silver nanoparticles as nanosides against the larvae of *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, important vectors of disease transmission. The LC_{50} and LC_{90} for third instar larvae of the three species were 0.80, 0.75, and 1.2 ppm, and 0.80, 0.67, and 0.86 ppm, respectively. Iftikhar et al (2018), recommended the development of silver nanoparticles produced by *B. mojavensis* BTCB15, which may play a role in combating mosquito populations, thereby controlling vector-borne diseases. The larvicidal activity of silver nanoparticles (Ag/AgCl NPs) synthesized using *Bti* supernatant showed that LC_{50}=0.133 g/mL, higher toxicity than that synthesized using insecticidal protein, LC_{50}=0.148–0.217 g/mL (Chimkhan et al. 2022). Possible mechanism of action of Ag/AgCl NPs against *A. aegypti* larvae is through mitochondrial dysfunction, DNA and protein damage, inhibition of cell proliferation, and cell apoptosis, and recommended that Ag/AgCl NPs are an alternative approach to control *A. aegypti* larvae, a mosquito-borne disease vector (Chimkhan et al. 2022). Further research needs to be done, whether *Bacillus* sp. EG6.4 can produce silver nanoparticles that can be used as antilarval against *A. aegypti* larvae.

Hemolytic activity assay of *Bacillus* sp. EG6.4 on blood agar showed a positive result. Biosurfactants have been utilized as an alternative to chemical pesticides in the past. Previous studies found that biosurfactant-producing bacteria are suitable for controlling plant pathogenic and insecticidal (Bais et al. 2004; Zhao et al. 2014). *B. subtilis* has been reported as a bacterial agent producing mosquitocidal toxin (Das and Mukherjee 2006; Manonmani et al. 2011). The toxicity of biosurfactants or their potential as biocontrol agents has not been well investigated. Biosurfactants produced by *Bacillus* strains have shown mosquitocidal activity in adult mosquitos, killing both immature and adult mosquitos (Geetha et al. 2012). Other studies have shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects (Zhao et al. 2014). From these studies it has been shown that biosurfactants affect the cuticle of insects, because they are amphiphilic in the presence of hydrophobic and hydrophilic molecules that damage cell membranes, then damage epithelial cells, and cause larval death (Zhao et al. 2014). *B. mojavensis* was reported to synthesize lipopeptides ~~has been shown to create lipopeptides~~, which have a unique structure and are important antibacterial and antifungal substances derived from surfactin, iturin, and fengysin (Mounia et al. 2014; Blacutt et al. 2016). According to Jasim et al. (2016), lipopeptide in *B. mojavensis* has antiviral, antitumoral, antifungal, and antimycoplasmatic properties. Based on the research of Hmidet et al. (2017), *B. mojavensis* produced surfactin and fengysin on all carbon sources used and the best production occurs in media with glucose as a carbon source and the least production occurs in media with starch carbon sources. *B. mojavensis* also showed α-hemolytic activity during its growth in blood agar (Berekaa and Ezzeldin 2018). These investigations revealed that bacteria-produced biosurfactant can be employed as a biocontrol for plant pathogens and as an effective bioinsecticide.

The chitinolytic activity assay showed negative result. Melo et al. (2016) discovered that larvicidal paraspore toxin produced by *B. thuringiensis* also produces chitinase enzymes for agroindustrial use. . Chitinolytic microorganisms have a lot of potential to be applied as biocontrol agents (Wang et al. 2006). Arora et al. (2003) reported that purified chitinase from *Bacillus* spp. has been analyzed as an insecticide. The results of nucleotide BLAST analysis and BLAST protein srfA-D gene *B. mojavensis* EG6.4 showed highest similarity to *B. mojavensis* strain PS17 with accession number CP066516.1 and surfactin biosynthesis thioesterase srfA-D from *B. mojavensis* bacteria with accession number QQF62274.1. The srfA-D from *B. mojavensis* EG6.4 is known to play a role in biosynthesis thioesterase enzyme to produce biosurfactants.

The indigenous *Bacillus* sp. EG6.4 is similar to *Bacillus mojavensis* and produces massive shape of parasporal inclusion. Bioassay results showed high toxicity against *A. aegypti* larvae. *Bacillus* sp. EG6.4 showed hemolytic activity and found that srfA-D gene produces surfactin, indicating its potency to produce biosurfactants. It is concluded from the present result that *Bacillus* sp. EG6.4 can be developed as a biocontrol agent for disease vectors and plant pests and plant diseases.

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Author contributions: The research team was in charge of the full material of this publication, including the subsections on molecular identification, bioassays, ultra-structural cells, and hemolytic and chitinolytic assays, according to the theme. All authors appreciate constructive criticism on their work.

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has been shown to synthesize lipopeptides in its secondary metabolites

297 **Conflicts of Interest:** There is no conflict of interest stated by the authors.
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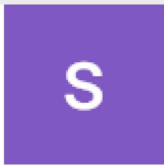

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Potential biocontrol agent of indigenous *Bacillus* sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions

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Abstract. *Salamun, Susetyo RD, Nafidiastri FA, Zain RA, Sari RP, Geraldi A, Fatimah, Ni'matuzahroh. 2022. Potential biocontrol agent of indigenous* Bacillus *sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions Biodiversitas 23: 5431-5438.* This research was carried out for molecular identification, as well as the determination and mechanism of action of larvicidal toxicity of *Bacillus* sp. EG6.4 isolated from breeding sites of *Aedes aegypti* from Gresik, East Java, Indonesia. *Bacillus* sp. EG6.4 was a Gram-positive endospore-forming bacteria. Molecular species identification using 16S rRNA gene sequencing showed that isolate had 97.89% similarity with *Bacillus mojavensis*. The isolate showed larvicidal toxicity against *A. aegypti* larvae. The lethal concentration 50% (LC_{50}) values at 24 and 48hours exposure were 8.99±1.01 $\times 10^7$ cells/mL and 8.43±1.01 $\times 10^7$ cells/mL, respectively, while lethal time 50% (LT_{50}) value was 11.9±1.1 hours. Production of chitinolytic enzymes or biosurfactants, chitinolytic and hemolytic assays were conducted to determine the larvicidal mechanism. As a result, *Bacillus* sp. EG6.4 showed hemolytic, but not chitinolytic activity, indicating its potency to produce biosurfactants. Transmission Electron Microscopy (TEM) result showed that isolate had oval-shaped endospores located subterminal with massive-shape parasporal inclusions. The detection of srfA-D gene showed that isolate produced surfactin biosynthesis thioesterase. Thus, *Bacillus* sp. EG6.4 produced biosurfactant that potentially to be developed as a biocontrol agent for disease vectors and plant pathogens.

Keywords: *Aedes aegypti*, *Bacillus mojavensis*, biosurfactants, larvicidal toxicity, parasporal inclusion, srfA-D gene

INTRODUCTION

Dengue fever (DF), a vector-borne disease and a serious public health problem in the world, is transmitted by the *Aedes aegypti* mosquito (Wuryaningsih 2007; Dahmana and Mediannikov 2020; Falqueto et al. 2021). Vaccines have been developed, but the results have not been satisfactory. Chemical insecticides are used to suppress mosquito vector populations but negatively impact the environment and are toxic to non-target organisms (Dahmana and Mediannikov 2020). The effectiveness of a storage time formulation of *Bacillus thuringiensis* has been investigated against an *A. aegypti* larvae, and it is recommended that this be an option to overcome the disease with integrated vector control (Melanie et al. 2018). Biological control experts suggest developing bioinsecticides as biocontrol agents for disease vectors (Thomas 2017). The biodiversity of microorganisms is one of the abundant renewable biological resources in the world, including in Indonesia. The use of *Bacillus* in the biocontrol of vectors, pests, and plant diseases has grown rapidly (Benelli et al. 2016). *Bacillus thuringiensis* var. *israelensis* (Bti) is used as a dengue vector biocontrol agent (BCAs) for controlling *A. aegypti* larvae (Boyce et al. 2013). During sporulation, *B. thuringiensis* produce parasporal inclusions and the resulting cry toxin causes death of several insect species (Aramideh et al. 2016).

The group of spore-forming bacteria inhibits their growth under abnormal conditions and begins to increase their metabolic activity (Shahcheraghi et al. 2015). Sporulation is an effort to defend bacteria against an unfavorable environment. Several *Bacillus* species are reported to produce a protein toxin (cry toxin) and also secondary metabolites that act as self-defense to survive. In the vegetative phase, bacteria grow and develop, but in unfavorable conditions they produce secondary metabolites, such as biosurfactants, enzymes, or exotoxins to maintain their live. Biosurfactant synthesis is associated with hemolytic activity (Carrillo et al. 1996) and has also applied by Colonna et al. (2017) as a rapid screening technique for assessing the toxicity of native surfactin. The hemolytic activity assay is a typical screening method for detecting biosurfactant activity. Biosurfactant generated by *Bacillus* sp. is found to be effective as a plant pathogenic and insecticidal agent in several tests (Bais et al. 2004). Nafidiastri et al. (2021) reported that indigenous *B. velezensis* ES4.3 resulted biosurfactant activity was

indicated by the formation of clear zones, emulsions, and a decrease in surface tension and the surfactin gene showed a 100% ID with the surfactin biosynthesis thioesterase SrfA-D gene.

Isolation and characterization of indigenous *Bacillus* entomopathogenic species have been isolated from the breeding sites of *A. aegypti* in Surabaya, Gresik, and Sidoarjo, East Java, Indonesia (Salamun et al. 2020). According to a preliminary test, about 133 *Bacillus* sp. are entomopathogenic against larvae of *A. aegypti*. The majority of *Bacillus* sp. isolates showed mild to moderate toxicity, but 16 isolates were highly toxic, including *Bacillus* sp. EG6.4, which causes 100% larval mortality. Salamun et al. (2020) identified microscopic, macroscopic, and biochemical phenotypic similarities indicating that *Bacillus* sp. EG6.4 had a similarity level of 80.60% with *B. thuringiensis*. Mechanism of action of larvicidal toxicity of *Bacillus* spp. against *A. aegypti* in two ways, namely in the sporulation phase the bacteria produce endotoxins which are stored in the paraspore inclusions (pellet fraction) and produce secondary metabolites (supernatant fraction) which are secreted out of the cells (Dahmana et al. 2020; Falqueto et al. 2021; Katak et al. 2021).

Further research is needed for molecular identification to determine species name of *Bacillus* sp. EG6.4 and genetic relationships with other bacilli, bioassays to determine larval mortality, and to detect the mechanism of action by detecting the presence of paraspore inclusions, surfactin coding genes, and hemolytic and chitinolytic activities, so it is hoped that the mechanism of action of *Bacillus* sp. EG6.4 as larvicidal can be identified.

The aim of this study was to identify the isolated species at molecular level and to determine larvicidal toxicity and larvicidal mechanism of the indigenous *Bacillus* sp. EG6.4. Further, species name was conducted with the 16S rRNA gene and traces genetic affinity through a phylogenetic tree. Larvicidal activity was determined by LC_{50} and LC_{90} values. To determine the larvicidal mechanisms of action, with detection of endospore by Transmission Electron Microscopy (TEM) and Scanning Electron Microsopy (SEM), surfactin coding genes (srfA-D) and detection of secondary metabolite activity by hemolytic and chitinolytic activity assays.

MATERIALS AND METHODS

Molecular identification

DNA isolation

The genomic DNA of *Bacillus* sp. EG6.4 was isolated using the Promega DNA Purification Kit (Promega 2018). Isolated DNA was visualized by electrophoresis. The results of genomic DNA electrophoresis of *Bacillus* sp. EG6.4 was photographed under a UV Transilluminator. The purity of genomic DNA was measured using the ratio of absorbance at 260 nm and 280 nm by Thermo Scientific Multiskan GO Microdroplet Spectrophotometer.

Identification of 16S rRNA gene and phylogenetic tree

Identification of 16S rRNA gene was initiated by culturing 2-3 ose isolates of *Bacillus* sp. EG6.4 into 20 mL of Luria Bertani media, incubated at room temperature with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out using the CTAB method (Ausubel et al. 2003). DNA purity and concentration values were measured using Multiskan GO at λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene amplification was carried out using Eppendorf Mastercycler. The process started by adding GoTaq Green Master Mix and 16S rRNA primers, in the form of primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was conditioned as follows: initial denaturation of 94°C for 2 minutes, denaturation of 92°C for 30 seconds, annealing 55°C for 30 seconds, elongation of 72°C for 1 minute, final elongation of 72°C for 5 minutes, 35 cycles. The PCR samples were sent to 1st Base DNA Sequencing Service Malaysia. Amplicon was sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The results of PCR were visualized through an electrophoresis process using 1% agarose gel followed by ethidium bromide staining and observed in ultraviolet light. The data were also analyzed for their relation by building a phylogenetic tree using MEGA 6.0 (Tamura et al. 2013).

Analysis of 16S rRNA gene

Isolated DNA of *Bacillus* sp. EG6.4 was amplified using the eppendorf mastercycler with the PCR method and universal primers 27F and 1492R at 1st Base Sequencing Service in Singapore. The Bio Edit Sequence Alignment Editor software version 7.2.5 was used to evaluate the data from the sequencing results. The 16S rRNA gene nucleotide sequence from *Bacillus* sp. EG6.4 was matched with 16S rRNA gene sequences from other bacteria reported to GenBank. The Basic Local Alignment Search Tool (BLAST) at The National Library of Medicine, National Center for Biotechnology Information in Washington, DC (http://blast.ncbi.nml.nih.gov/) was used to find homology of the 16S rRNA gene.

Analysis of phylogenetic tree

The results of genomic DNA sequencing of *Bacillus* sp. EG6.4, which has been compared with other bacteria through BLAST, was then compiled to determine the relationship through a phylogenetic tree created by Mega 7 program. A phylogenetic tree was created by including FASTA from other species. All of these species were selected based on nucleotide sequence of 16S rRNA gene, because relationships of a species with other species can be seen from the sequence.

Bioassay larvicidal toxicity

Determination of LC of *Bacillus* sp. EG6.4 against *A. aegypti* larvae was prepared by inoculating on Nutrient Yeast Salt Medium (NYSM) broth was incubated on a rotary shaker (130 rpm) at 30°C for 72 hours and then bacterial density was determined by spectrophotometer at 600nm. Bioassay was conducted according to Suryadi et al. (2016). The final culture concentration was adjusted to

variations concentration of (v/v) NYSM. Samples of 20 third-instar larvae of *A. aegypti* were tested at six concentrations each treatment in triplicate. Larvae were died after 24- and 48-hours exposure. The lethal time of *Bacillus* sp. EG6.4 of *A. aegypti* larvae was determined at a concentration of LC_{90}, as many as 20 larvae were exposed, and each treatment had three replications. Mortality of larvae was scored after 0, 0.5, 1, 2, 4, 8, 10, 20, 24 and 48-hours exposure along with NYSM (10% v/v) without inoculum served as negative control.

Bioassay of larval mortality (%) used to determine LC_{50} and LC_{90}, as well as LT_{50} and LT_{90} of *Bacillus* sp. EG6.4 against *A. aegypti* larvae. Probit analysis using Minitab version 17 was used to calculate the toxicity of *Bacillus* sp. EG6.4 against *A. aegypti* (Postelnicu 2018).

Larvicidal mechanisms

Detection of parasporal inclusion

The process of detecting *Bacillus* sp. EG6.4 parasporal inclusions included multiple phases, including rejuvenation, purification, and growth on NYSM broth at 30°C 72 hours, in a shaker incubator (130 rpm). After producing a pure liquid culture of *Bacillus* sp. EG6.4, TEM examination was performed at the Eijkman Institute's TEM and Histology Laboratory in Jakarta, while SEM study was performed at LPPT Gadjah Mada University in Yogyakarta using an SEI 10 kV WD10mm SS30.

Hemolytic and chitinolytic activity

Hemolytic activity was tested using blood agar media acquired from the Surabaya Health Laboratory Center and utilized to screen *Bacillus* sp. EG6.4 for hemolytic activity. Colony of isolates was cultured for two days at room temperature. Distinct clear zones were formed surrounding a colony. Chitinolytic activity was carried out by growing *Bacillus* sp. EG6.4 on colloidal chitin agar media by the streak method. Observation of the clear zone around the colonies was carried out after an incubation period of 4 days, by adding Congo red dye to petri dish and then washing it with NaOH.

Detection of surfactin-coding gene

The DNA obtained was used from the identification of the 16S rRNA gene stage in this stage. The approach for detecting the biosynthetic surfactin gene was similar to that for identifying the 16S rRNA gene, with the exception of the primer employed. On the page Thermo Fisher Scientific Oligo Perfect Primer Designer cloning application, the srfA-D gene primers were self-designed. The surfactin gene primers that were designed are forward primer (5'- ATGAGCCAACTGTTCAAATCATTTG -3') and reverse primer (5'- TCAGGAACTGGAAATCGGATGC -3').

RESULTS AND DISCUSSION

Molecular identification

Molecular identification results (Figure 1 and Table 1) showed that *Bacillus* sp. EG6.4 had 97.89% similarity with *B. mojavensis* strains NBRC 15718 and *B. mojavensis* IFO 15718 and was closely related to *B. halotolerans* strains DSM 8802 and *B. halotolerans* LMG 22476 (Figure 2).

Bioassay results

The results of bioassay of *Bacillus* sp. EG6.4 against third instar larvae of *A. aegypti* are presented in Table 2. The results of probit analysis showed thatLC_{50} values at 24- and 48-hours exposure was $8.99 \pm 1.01 \times 10^7$ cells/mL and $8.43 \pm 1.01 \times 10^7$ cells/mL, respectively (Figure 3), whereas LT_{50} values at 24- and 48-hours observation were 11.9 ± 1.1 hours and 22.6 ± 8.4 hours, respectively (Figure 4).

Larvicidal toxicity mechanism

Detection parasporal inclusion

Result revald that *Bacillus* sp. EG6.4 was a Gram-positive and endospore-forming bacteria. Detection using TEM showed subterminal oval-shaped endospores (Salamun et al. 2020). TEM result showed massive paraspore inclusions (Figure 5), while SEM showed only spores but not cry toxin (Figure 6).

Hemolytic and chitinolytic activity

The results showed hemolytic activity but not chitinolytic activity (Figure 7), indicating its potential to produce biosurfactants.

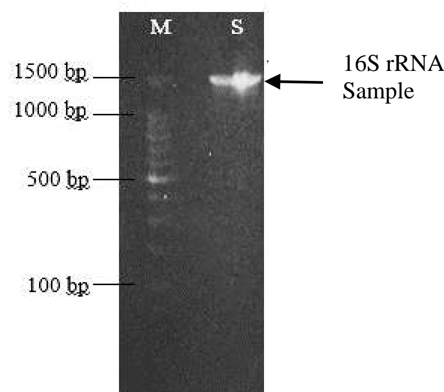

Figure 1. The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M=100bp DNA marker; S= sample of 16S rRNA gene

Table 1. 16S rRNA gene analysis of indigenous *Bacillus* sp. EG6.4 by Basic Local Alignment Search Tool (BLAST)

Description	Accession no.	E value	Query cover	% ID
Bacillus mojavensis strain NBRC 15718	NR 112725.1	0.0	100%	97.89
Bacillus mojavensis strain IFO 15718	NR 024693.1	0.0	100%	97.89

Bacillus halotolerans strain DSM 8802	NR 115063.1	0.0	100%	97.89

Table 2. The effect of indigenous *Bacillus* sp. EG6.4 concentrations on mortality of *Aedes aegypti* third instar larvae (%) after 24 and 48-hours exposure

Treatments (concentration series)	Culture (per-10 mL NYSM)	OD_{600nm}	CFU/mL	24 h exposure, larval mortality = %	48 h exposure, larval mortality = %
C_1	0.5 mL	0.07	09.8 x 10^7	6.7 ± 5.8	6.7 ± 5.8
C_2	1.0 mL	0.15	2.29 x 10^7	20 ± 10	26.7 ± 5.8
C_3	2.5 mL	0.37	5.90 x 10^7	43.3 ± 15.3	43.3 ± 15.3
C_4	5.0 mL	0.75	12.1 x 10^7	73.3 ± 5.8	76.7 ± 5.8
C_5	10 mL	1.50	24.4 x 10^7	93.3 ± 5.8	93.3 ± 5.8

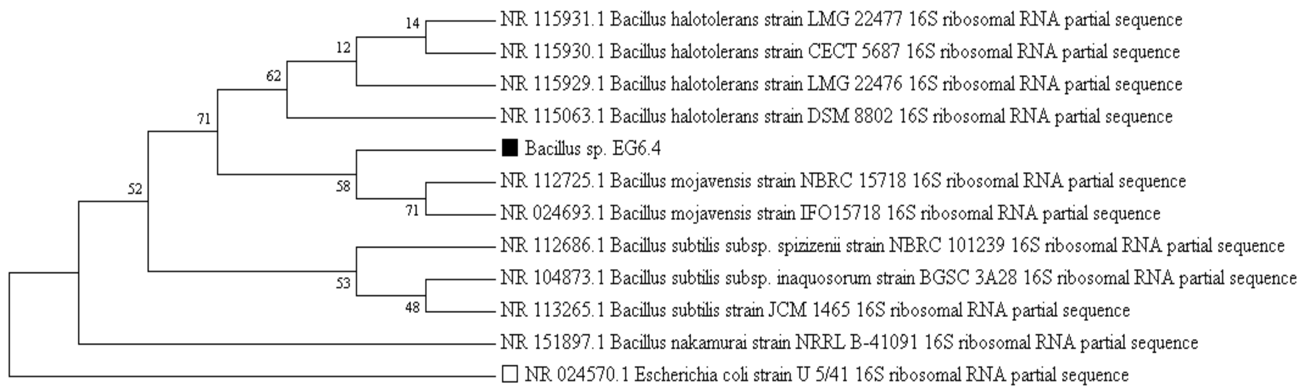

Figure 2. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application

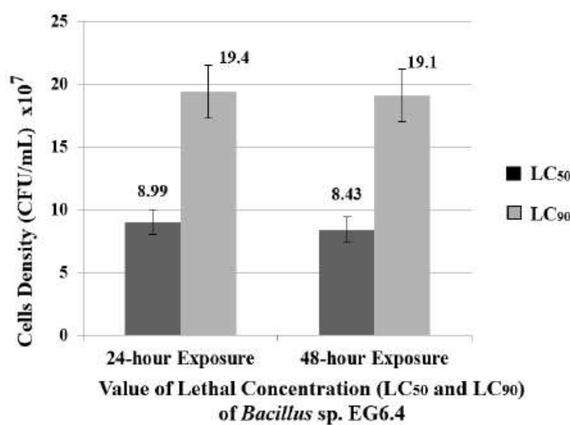

Figure 3. Values of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48 hours of exposure

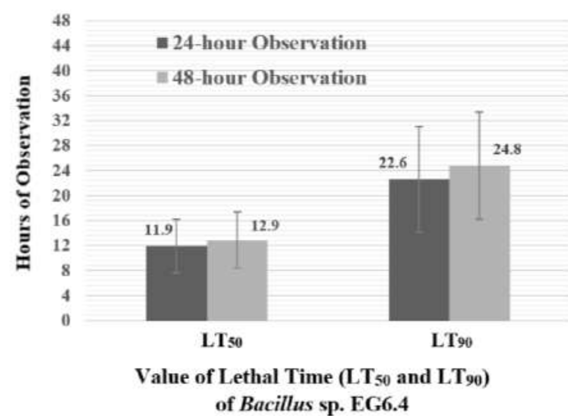

Figure 4. The lethal time 50% (c) and 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae on 24and 48hours exposure

Detection of surfactin-coding gene

The electrophoresis results showed a band of about 700 bp. Based on data in GenBank CP0514641, srfA-D gene of *B. mojavensis* was 729 bp (Figure 8). Band position on electrophoretic visualization showed that *B. mojavensis* EG6.4 had srfA-D gene. The nucleotide BLAST results from srfA-D gene of *B. mojavensis* EG6.4 showed a similarity value of 98.35% with the gene in *B. mojavensis* strain PS17 and had a similarity value of 94.92% with *B. mojavensis* strain UCMB5075 (Table 3). Further studies, the results of protein BLAST against the amino acid gene srfA-D *B. mojavensis* EG 6.4 obtained the highest similarity of 98.35% with surfactin biosynthesis thioesterase srfA-D from the bacterium *B. mojavensis* strain PS17.

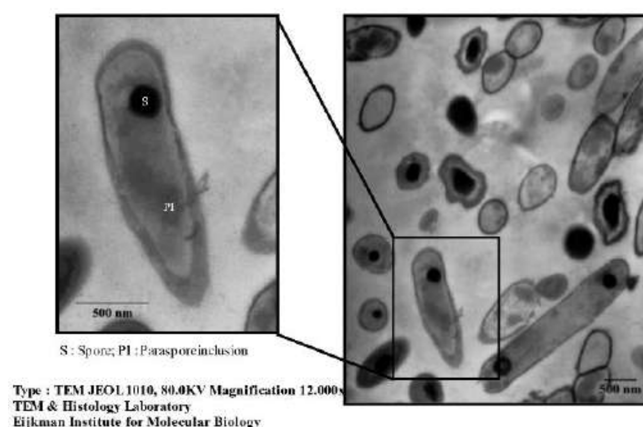

Figure 5. Organelles of indigenous *Bacillus* sp. EG6.4 endospore cells visible using transmission electron microscopy (TEM). S=Forespore, PI=Parasporal Inclusion

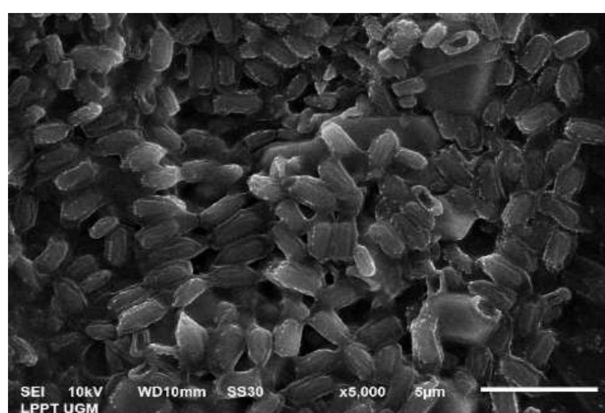

Figure 6. Visible spores of indigenous *Bacillus* sp. EG6.4 using Scanning Electron Microscopy (SEM)

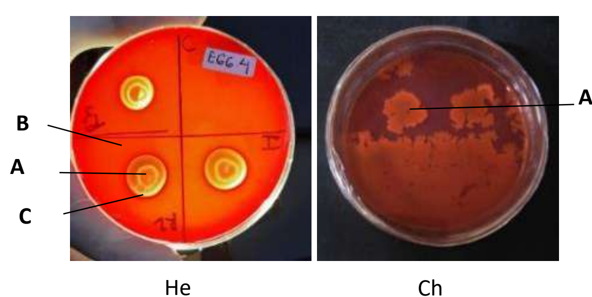

Figure 7. Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal chitin plate media (Ch). Note: A=Colony of isolate; B=Blood agar plate, C=Clear zone around colony of isolate

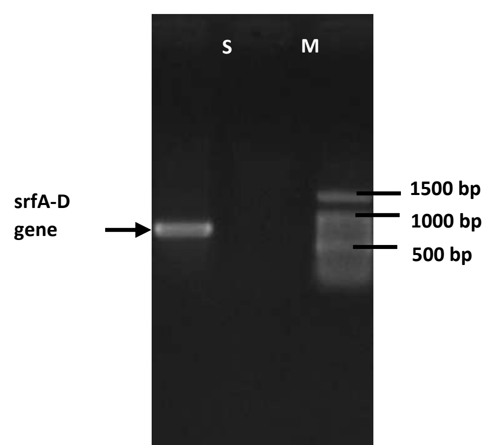

Figure 8. Electrophoresis results of srfA-D gene *Bacillus mojavensis* EG6.4. M=500bp DNA marker; S=Sample of srfA-D gene.

Discussion

The results of isolation of *Bacillus* sp. EG6.4 showed DNA purity = 1.84 (A260/A280), DNA concentration 44.7 (ng/µl), which was confirmed by electrophoresis with DNA size above 1500bp. The DNA migration during electrophoresis was influenced by agarose concentration, DNA molecule size, voltage, and temperature (Fatchiyah et al. 2011; Brown 2016). The result of 16S rRNA gene amplification was visualized by electrophoresis, showed that a band measuring about 1550bp and 500 bases at the end of the sequence was a hypervariable region. Pearson (2014) reported that 16S rRNA gene can be used for the molecular identification of microorganisms. The results of purification and sequencing of the 16S rRNA gene of *Bacillus* sp. EG6.4 showed a nucleotide sequence with a size of 1424 bp. Based on BLAST analysis, *Bacillus* sp. EG6.4 was similar to *B. mojavensis* and was related to other bacteria.

Bacillus sp. EG6.4 has a close relationship with *Bacillus mojavensis* and *Bacillus halotolerans*. *Bacillus mojavensis* and *B. halotolerans* are two species of bacteria that are closely related to *Bacillus subtilis* however it differs significantly in terms of fatty acid content, DNA sequences, and transgenic species resistance (Bacon and Hinton 2002). *Bacillus mojavensis* has been known that produce secondary metabolites, such as oxygenated monoterpenes and lipopeptides, group of biosurfactants that have promising applications in agricultural, food industry, and clinical fields. In agriculture, *B. mojavensis* is used as antimicrobial agent against pathogenic bacteria and fungi (Camele et al. 2019). *B. halotolerans* has been known that produce biosurfactants. Based on research reported by Wang et al. (2022), biosurfactants from *B. halotolerans* can be used as agent to remediation in polluted environment petroleum.

Table 3. Results of Basic Local Alignment Search Tool (BLAST) nucleotide analysis of srfA-D gene of *Bacillus mojavensis* EG6.4

Species name with srfA-D Gene	Accession no.	E. value	QC	% ID	Description
Bacillus mojavensis strain PS17	CP066516.1	0.0	99%	98.35%	Complete genome
Bacillus mojavensis strain UCMB5075	CP051464.1	0.0	100%	94.92%	Complete genome

The values of LC_{50} and LC_{90} are the concentrations of microorganisms that cause 50% and 90% mortality of the target organism population. The lower the LC value of microorganisms, the higher the toxicity. Toxicity of *Bacillus* sp. influenced by the type of toxin or secondary metabolites produced by microorganisms, the species and age of the target larvae, and the media used for bacterial growth (Suryadi et al. 2016). Determination of LC_{50} and LC_{90} as well as LT_{50} and LT_{90} was carried out to determine the concentration and length of time required by *Bacillus* sp. EG6.4 to kill 50% and 90% and the length of time to kill 50% and 90% of third instar *A. aegypti* larvae. Based on the mortality rate of *A. aegypti* larvae in the treatment, *Bacillus* sp. EG6.4 showed high toxicity, which isolate has the potential to be developed as a biolarvacidal agent.

The LC_{50} and LC_{90} values of *Bacillus* sp. EG6.4 have high potential, when compared to previous studies. Gama et al. (2010) reported that *B. thuringiensis* PWR4 32 isolated from Malang, Indonesia had an LC_{50} value of 22.79×10^7 cells/mL at 72-hour exposure. Pratiwi et al. (2013) reported that *B. thuringiensis* W.Swh.S.K2 isolated from Nganjuk Indonesia, had an LC_{50} value of 3.53×10^7 cells/mL at 48-hour exposure. The number of spores consumed by the larvae affects the rate of larval death. Gama et al. (2010) found that as *B. thuringiensis* spores developed, presence of cry toxin was released to kill *A. aegypti* larvae. The two isolates have different strains or species, so there is a difference in toxicity between them. One of the characteristics of *Bacillus* sp. is that it can produce endospores and protein crystals (parasporal inclusions) at the time of cell sporulation. *Bacillus* sp. EG6.4 showed a complete endospore with sections, including a spore layer, spore, and paraspore inclusions. *Bacillus* sp. EG6.4 produced paraspore inclusions, but the shape was massive and was not proven to be a protein toxin that had larvicidal activity against *A. aegypti* larvae. Iftikhar et al. (2018) reported that *B. mojavensis* BTCB15 synthesized silver nanoparticles as nanosides against the larvae of *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, important vectors of disease transmission. The LC_{50} and LC_{90} for third instar larvae of the three species were 0.80, 0.75, and 1.2 ppm, and 0.80, 0.67, and 0.86 ppm, respectively. Iftikhar et al. (2018), recommended the development of silver nanoparticles produced by *B. mojavensis* BTCB15, which may play a role in combating mosquito populations, thereby controlling vector-borne diseases. The larvicidal activity of silver nanoparticles (Ag/AgCl NPs) synthesized using *Bti* supernatant showed that LC_{50}=0.133 g/mL, higher toxicity than that synthesized using insecticidal protein, LC_{50}=0.148-0.217 g/mL (Chimkhan et al. 2022). Possible mechanism of action of Ag/AgCl NPs against *A. aegypti* larvae is through mitochondrial dysfunction, DNA and protein damage, inhibition of cell proliferation, and cell apoptosis, and recommended that Ag/AgCl NPs are an alternative approach to control *A. aegypti* larvae, a mosquito-borne disease vector (Chimkhan et al. 2022). Further research needs to be done, whether *Bacillus* sp. EG6.4 can produce silver nanoparticles that can be used as antilarval against *A. aegypti* larvae.

Hemolytic activity assay of *Bacillus* sp. EG6.4 on blood agar showed a positive result. Biosurfactants have been utilized as an alternative to chemical pesticides in the past. Previous studies found that biosurfactant-producing bacteria are suitable for controlling plant pathogenic and insecticidal (Bais et al. 2004; Zhao et al. 2014). *B. subtilis* has been reported as a bacterial agent producing mosquitocidal toxin (Das and Mukherjee 2006; Manonmani et al. 2011). The toxicity of biosurfactants or their potential as biocontrol agents has not been well investigated. Biosurfactants produced by *Bacillus* strains have shown mosquitocidal activity in adult mosquitos, killing both immature and adult mosquitos (Geetha et al. 2012). Other studies have shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects (Zhao et al. 2014). From these studies it has been shown that biosurfactants affect the cuticle of insects, because they are amphiphilic in the presence of hydrophobic and hydrophilic molecules that damage cell membranes, then damage epithelial cells, and cause larval death (Zhao et al. 2014). *Bacillus mojavensis* was reported to synthesize lipopeptides, which have a unique structure and are important antibacterial and antifungal substances derived from surfactin, iturin, and fengycin (Mounia et al. 2014; Blacutt et al. 2016). According to Jasim et al. (2016), lipopeptide in *B. mojavensis* has antiviral, antitumoral, antifungal, and antimycoplasmatic properties. Based on the research of Hmidet et al. (2017), *B. mojavensis* produced surfactin and fengycin on all carbon sources used and the best production occurs in media with glucose as a carbon source and the least production occurs in media with starch carbon sources. *B. mojavensis* also showed α-hemolytic activity during its growth in blood agar (Berekaa and Ezzeldin 2018). These investigations revealed that bacteria-produced biosurfactant can be employed as a biocontrol for plant pathogens and as an effective bioinsecticide.

The chitinolytic activity assay showed negative result. Melo et al. (2016) discovered that larvicidal paraspore toxin produced by *B. thuringiensis* also produces chitinase enzymes for agroindustrial use. Chitinolytic microorganisms have a lot of potential to be applied as biocontrol agents (Wang et al. 2006). Arora et al. (2003) reported that purified chitinase from *Bacillus* spp. has been analyzed as an insecticide. The results of nucleotide BLAST analysis and BLAST protein srfA-D gene *B. mojavensis* EG6.4 showed highest similarity to *B. mojavensis* strain PS17 with accession number CP066516.1 and surfactin biosynthesis thioesterase srfA-D from *B. mojavensis* bacteria with accession number QQF62274.1. The srfA-D from *B. mojavensis* EG6.4 is known to play a role in biosynthesis thioesterase enzyme to produce biosurfactants.

The indigenous *Bacillus* sp. EG6.4 is similar to *Bacillus mojavensis* and produces massive shape of parasporal inclusion. Bioassay results showed high toxicity against *A. aegypti* larvae. *Bacillus* sp. EG6.4 showed hemolytic activity and found that srfA-D gene produces surfactin, indicating its potency to produce biosurfactants. It is concluded from the present result that *Bacillus* sp. EG6.4

can be developed as a biocontrol agent for disease vectors and plant pests and plant diseases.

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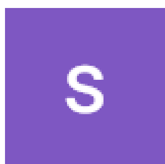

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