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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 $8.99 \pm 1.01 \times 10^7$ cells/mL and $8.43 \pm 1.01 \times 10^7$ 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.

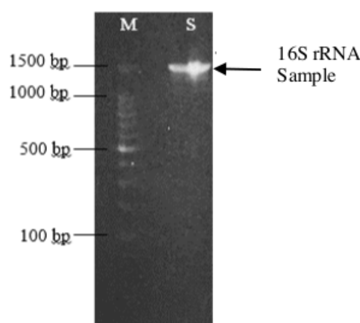

Figure 1. The electrophoresis of 16S rRNA gene amplification of indigenous *Bacillus* sp. EG6.4. M: 100bp DNA marker; S: sample of 16S rRNA gene

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

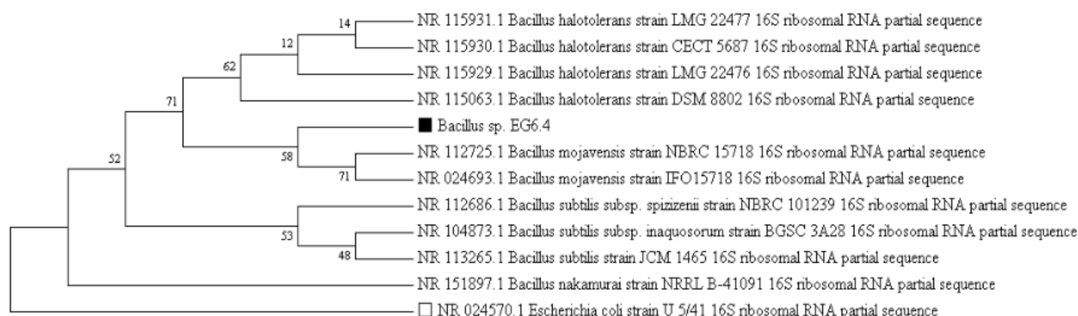

Figure 2. Phylogenetic tree of *Bacillus* sp. EG6.4 based on genetic similarity with other species in GenBank using Mega 7 application

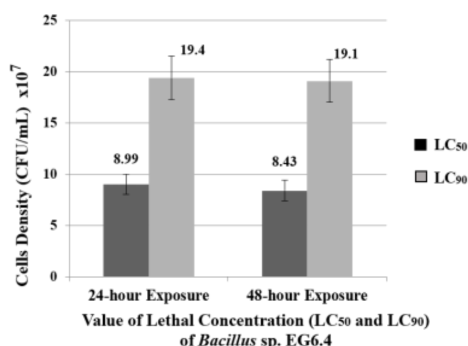

Figure 3. Values of LC_{50} and LC_{90} (CFU/mL) indigenous *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae after 24 and 48 hours of exposure

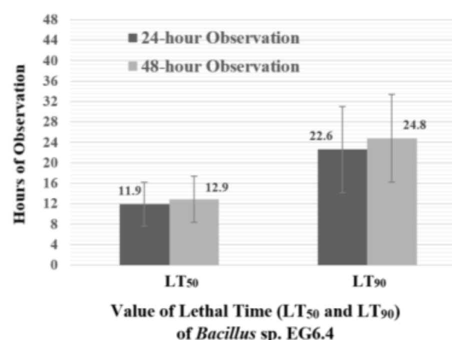

Figure 4. The lethal time 50% (c) and 90% (LT_{90}) of *Bacillus* sp. EG6.4 against *Aedes aegypti* third instar larvae on 24and 48hours exposure

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.

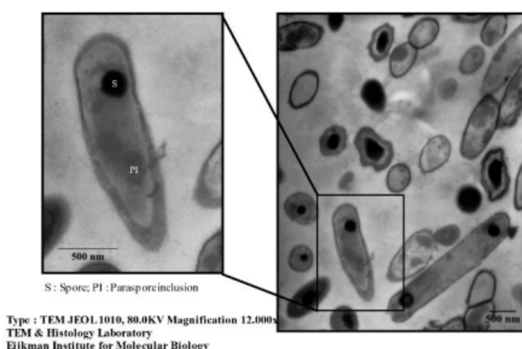

Figure 5. Organelles of indigenous *Bacillus* sp. EG6.4 endospore cells visible using transmission electron microscopy (TEM). S: Forespore, PI: Parasporal Inclusion

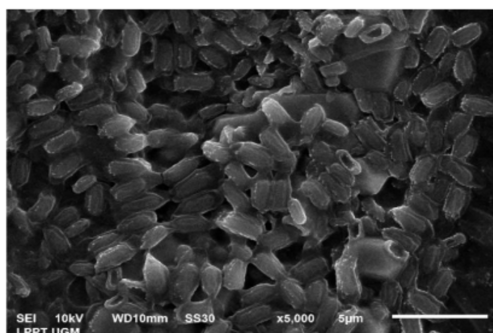

Figure 6. Visible spores of indigenous *Bacillus* sp. EG6.4 using Scanning Electron Microscopy (SEM)

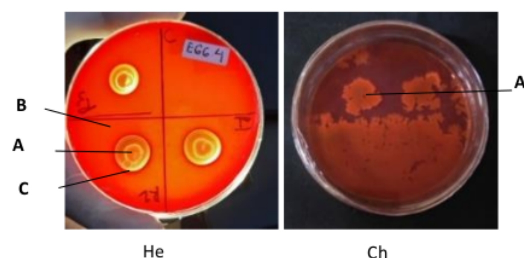

Figure 7. Hemolytic (He) and chitinolytic (Ch) activity of indigenous *Bacillus* sp. EG6.4 on blood agar plate media (He) and colloidal chitin plate media (Ch). Note: A: Colony of isolate; B: Blood agar plate, C: Clear zone around colony of isolate

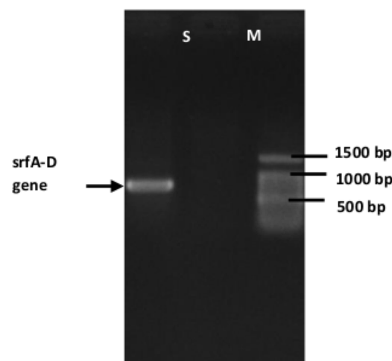

Figure 8. Electrophoresis results of srfA-D gene *Bacillus mojavensis* EG6.4. M: 500bp DNA marker; S: Sample of srfA-D gene

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 for 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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REFERENCES

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25 (17): 3389-3402. DOI: 10.1093/nar/25.17.3389.

Aramideh S, Shahram M, Abbas H, Ghassemi-Kahrizeh LE. 2016. Isolation, toxicity, and detection of *Cry* genes *Bacillus thuringiensis* isolates from West-Azerbaijan Province, Iran. J Entomol Zool Stud 4 (6): 111-116.

Arora N, Ahmad T, Rajagopal R, Bhatnagar RK. 2003. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1, Biochem Biophys Res Commun 307: 620-625. DOI: 10.1016/s0006-291x(03)01228-2.

Ausubel FM. 2003. Preparation of Genomic DNA from bacteria. In: Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds). Current Protocols in Molecular Biology. John Wiley & Sons, Inc., Cambridge.

Bacon CW, Hinton DM. 2002. Endophytic and biological control potential of *Bacillus mojavensis* and related species. BioControl 23**:** 274-284. DOI: 10.1006/bcon.2001.1016.

Bais HP, Fall R, Vivanco JM. 2004. Biocontrol of *Bacillus subtilis* against infection of rabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol 134: 307-319. DOI: 10.1104/pp.103.028712.

Benelli G, Jeffries CL, Walker, T. 2016. Biological control of mosquito vectors: past, present, and future. Insects 7 (52): 1-18. DOI: 10.3390/insects7040052.

Berekaa MM, Ezzeldin MF. 2018. Exopolysaccharide from *Bacillus mojavensis* DAS10-1: production and characterization mahmoud. J Pure Appl Microbiol 12 (2): 633-640. DOI: 10.22207/JPAM.12.2.21.

Blacutt AA, Mitchell TR, Bacon CW, Gold SE. 2016. *Bacillus mojavensis* RRC101 lipopeptides provoke physiological and metabolic changes during antagonism against *Fusarium verticillioides*. MPMI 29 (9): 713-723. DOI: 10.1094/MPMI-05-16-0093-R.

Boyce R, Lenhart A, Kroeger A, Velayudhan R, Roberts B, Horstick O. 2013. *Bacillus thuringiensis israelensis* for the control of dengue vectors: systematic literature review. Trop Med Intl Health 18 (5): 564-577. DOI: 10.1111/tmi.12087.

Brown T. 2016. A Gene cloning and DNA analysis: An introduction. Seventh Edition. John Wiley & Sons.

Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM. 1996. Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol 12 (1): 82-84. DOI: 10.1007/BF00327807.

Chimkhan N, Thammasittirong SNR, Roytrakul S, Krobthong S, Thammasittirong A. 2022. Proteomic response of *Aedes aegypti* larvae to Silver/Silver Chloride Nanoparticles synthesized using *Bacillus thuringiensis* subsp. israelensis metabolites. Insects 13: 641. DOI: 10.3390/insects13070641.

Colonna WJ, Mart ME, Nyman JA, Green C, Glatza CE. 2017. Hemolysis as a rapid screening technique for assessing the toxicity of native surfactin and a genetically engineered derivative. Environ Prog Sustain Energy 36 (2): 505-510. DOI: 10.1002/ep.12444.

Dahmana H, Mediannikov O. 2020. Mosquito-borne diseases emergence/resurgence and how to effectively control it biologically. 9 (310): 1-26. DOI: 10.3390/pathogens9040310.

Dahmana H, Raoult D, Fenollar F, Mediannikov O. 2020. Insecticidal activity of bacteria from larvae breeding site with natural larvae mortality: Screening of separated supernatant and pellet fractions. Pathogens 9 (486): 1-15. DOI:10.3390/pathogens9060486.

Das A, Mukherjee AK. 2006. Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. Acta Trop 97 (2): 168-173. DOI: 10.1016/j.actatropica.2005.10.002.

Falqueto SA, Pitaluga BF, Sousa JR, Targanski SK, Campos MG, Mendes TAO, Silva GF, Silva DHF, Soares MA. 2021. *Bacillus* spp. metabolites are effective in eradicating *Aedes aegypti* (Diptera: Culicidae) larvae with low toxicity to non-target species. J Invertebr Pathol 179 (107525). DOI: 10.1016/j.jip.2020.107525.

Fatchiyah, Arumingtyas EL, Widyarti S, Rahayu S. 2011. Biologi molekuler, prinsip dasar analisis (Molecular biology, basic principles of analysis). Erlangga, Jakarta. [Indonesian]

Gama ZP, Yanuwiadi B, Kurniati TH. 2010. Strategi pemberantasan nyamuk aman lingkungan: Potensi *Bacillus thuringiensis* isolat Madura sebagai musuh alami nyamuk *Aedesaegypti* (Environmentally safe mosquito eradication strategy: Potential of *Bacillus thuringiensis* Madura isolate as natural enemy of *Aedes aegypti* mosquito). Jurnal Pembangunan dan Alam Lestari 1 (1): 1-10. [Indonesian]

Geetha I, Manonmani AM. 2010. Surfactin: A novel mosquitocidal biosurfactant produced by *Bacillus subtilis* subsp. subtilis (VCRC B471) and influence of abiotic factors on its pupicidal efficacy. Lett Appl Microbiol 51: 406-412. DOI: 10.1111/j.1472-765X.2010.02912.x.

Geetha I, Paily KP, Manonmani AM. 2012. Mosquito adulticidal activity of a biosurfactant produced by *Bacillus subtilis* subsp. subtilis. Pest Manag Sci 68: 1447-1450. DOI: 10.1002/ps.3324.

Hmidet N, Ayed HB, Jacques P, Nasri M. 2017. Enhancement of surfactin and fengycin production by *Bacillus mojavensis* A21: application for diesel biodegradation. BioMed Res Intl Article ID 5893123: 1-8. DOI: 10.1155/2017/5893123.

Iftikhar S, Iqtedar M, Akhtar MS, Abdullah R, Kaleem A, Aihetasham A, Chaudhry A, Naz Sh, Sharif S. 2018. *Bacillus mojavensis* BTCB15-mediated synthesis of silver nanoparticles with mosquito larvicidal activity against vector-borne diseases. Nanosci Nanotechnol Lett 10 (7): 943-949. DOI: 10.1166/nnl.2018.2731.

Jasim B, Sreelakshmi S, Mathew J, Radhakrishnan EK. 2016. Identification of endophytic *Bacillus mojavensis* with highly specialised broad spectrum antibacterial activity. Biotech (6) 187: 1-10. DOI: 10.1007/s13205-016-0508-5.

Katak RM, Rocha EM, Oliveira JC, Muniz VA, Oliveira MR, Ferreira FAS, Silva WR, Roque RA, de Souza AQI, Souza-Neto JA, Terenius O, Marinotti O, Tadei WP. 2021. Larvicidal activities against *Aedes aegypti* of supernatant and pellet fractions from cultured *Bacillus* spp. isolated from Amazonian microenvironments. Trop Med Infect Dis 6 (104): 1-12. DOI: 10.3390/tropicalmed6020104.

Manonmani AM, Geetha I, Bhuvaneswari S. 2011. Enhanced production of mosquitocidal cyclic lipopeptide from *Bacillus subtilis* subsp. subtilis. Indian J Med Res 134: 476-482.

Melanie, Rustama MM, Sihotang IS, Kasmara H. 2018. Effectiveness of storage time formulation of *Bacillus thuringiensis* against *Aedes aegypti* larvae (Linnaeus). J Cropsaver 1 (1): 48-52. DOI: 10.24198/cropsaver.v1i1.16999.

Melo ALA, Soccrol VT, Soccrol CS. 2016. *Bacillus thuringiensis* : Mechanism of action, resitance, and new aplications : a Review. Crit Rev Biotechnol 36 2: 317-326. DOI: 10.3109/07388551.2014.960793.

Mounia YA, Chaouche NK, Dehimat L, Bataiche I, Ali KH, Cawoy H, Thonart P. 2014. Antifungal activity and bioactive compounds produced by *Bacillus mojavensis* and *Bacillus subtilis*. Afr J Microb Res 8 (6): 476-484. DOI: 10.5897/AJMR2013.6327.

Nafidiastri FA, Susetyo RD, Nurhariyati T, Supriyanto A, Geraldi A, Ni'matuzahroh, Fatimah, Salamun. 2021. Biosurfactant activity of indigenous *Bacillus* sp. ES4.3 isolated from endemic breeding sites of dengue hemorrhagic fever vector in Surabaya, East Java, Indonesia. Biodiversitas 2 (12): 5375-5381. DOI: 10.13057/biodiv/d221219.

Pearson WR. 2014. An introduction to sequence similarity (homology) searching. Curr Protoc Bioinformatics 27 (1): 3-1. DOI: 10.1002/0471250953.bi0301s42.

Poopathi S, Abidha S. 2013. Mosquitocidal bacterial toxins (*Bacillus sphaericus* and *Bacillus thuringiensis* serovar israelensis): mode of action, cytopathological effects and mechanism of resistance. J Physiol Pathophysiol 1 (3): 22-38.

Postelnicu T. 2011. *Probit Analysis*. Romanian Academy, Romania. DOI: 10.1007/978-3-642-04898-2_461.

Pratiwi EK, Samino S, Gama ZP, Nakagoshi N. 2013. Uji toksisitas *Bacillus thuringiensis* asal kota Nganjuk terhadap larva *Aedes aegypti* (Toxicity assay of *Bacillus thuringiensis* from Nganjuk City against *Aedes aegypti* larvae). Jurnal Biotropika 1 (4): 171-176. [Indonesian]

Promega Protocol. 2018. Wizard® Genomic DNA Purification Kit. Technical Manual. 1-19. Promega, USA.

Salamun, Fatimah, Fauzi A, Praduwana SN, Ni'matuzahroh. 2021. Larvicidal toxicity and parasporal inclusion of native *Bacillus thuringiensis* BK5.2 against *Aedes aegypti*. J Basic Clin Physiol Pharmacol 32 (4): 379-384. DOI: 10.1515/jbcpp-2020-0472.

Salamun, Ni'matuzahroh, Fatimah, Maswantari MIF, Rizka MU, Nurhariyati T, Supriyanto A. 2020. Diversity of indigenous entomopathogenic bacilli from domestics breeding sites of dengue haemorrhagic fever vector based on the toxicity against *Aedes aegypti* Larvae. Ecol Environ Cons 26: S21-S26.

Shahcheraghi SH, Ayatollahi J, Lotfi M. 2015. Applications of *Bacillus subtilis* as an important bacterium in medical sciences and human life. Trop J Med Res 18: 1-4. DOI: 10.4103/1119-0388.152530.

Suryadi BF, Yanuwiadi B, Ardyati T, Suharjono. 2016. Evaluation of entomopathogenic *Bacillus sphaericus* isolated from Lombok beach area against mosquito larvae. Asian Pac J Trop Biomed 6 (2): 148-154. DOI: 10.1016/J.APJTB.2015.10.013.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30 (12): 2725-2729. DOI: 10.1093/molbev/mst197.

Terra WR, Terra FC. 2005. Biochemistry of digestion. In: Gilbert LI, Iatrou K, Gill SS. (eds.). Comprehensive molecular insect science biochemand mol biology. Elsevier Press Oxford, UK. DOI: 10.1016/B0-44-451924-6/00053-3.

Thomas MB. 2017. Biological control of human disease vectors: A perspective on challenges and opportunities. BioControl 63: 61-69. DOI: 10.1007/s10526-017-9815-y.

Wang SL, Lin TY, Yen YH, Liao HF, Chen YJ. 2006. Bioconversion of shellfish chitin waste for the production of *Bacillus subtilis* W-188 chitinase. Carbohydr Res 34: 2507-2515. DOI: 10.1016/j.carres.2006.06.027.

Wang X-T, Liu B, Li X-Z, Lin W, Li D-A, Dong H, Wang L. 2022. Biosurfactants produced by novel facultative-halophilic *Bacillus* sp. XT-2 with biodegradation of long chain n-alkane and the application for enhancing waxy oil recovery. Energy 240. DOI: 10.1016/j.energy.2021.122802.

Wuryaningsih YNS. 2007. Detektion antigen virus den on monocyts by streptavidin biotin test as early diagnostic for dengue fever haemorhagic. Biodiversitas J Biol Divers 8 (3): 174-178. ISSN: 1412-033X.

Zhao P, Quan C, Wang Y, Wang J, Fan S. 2014. *Bacillus amyloliquefaciens* Q-426 as a potential biocontrol agent against *Fusarium oxysporum* f. sp. spinaciae. J Basic Microbiol 54: 448-456. DOI: 10.1002/jobm.201200414.

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