Prospects of Indigenous *Bacillus subtilis* Strain LS9.1 as a Potential Biocontrol Agent against *Aedes aegypti* Larvae

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ABSTRACT

Biocontrol is one way that can be developed to overcome public health and agricultural problems. Biocontrol agents could be a microorganism, for example, from the *Bacillus* group. This study aims to determine the larvicidal toxicity of the *Bacillus subtilis* strain LS9.1 against *Aedes aegypti* larvae. The mode of action of the isolate caused larval death, which was detected with parasporal inclusions and secondary metabolites. Larvicidal toxicity with the bioassay method. Parasporal inclusion proteins were detected with the SDS-PAGE method. The Cry toxin coding gene with the PCR method and cell ultrastructure with transmission and scanning electron microscopy (TEM and SEM). The action of secondary metabolites was detected with hemolytic activity test. The isolate high toxicity to *A. aegypti* larvae. Parasporal inclusion proteins with molecular weights ranging from 72-7 kDa and the gene encoding a protein toxin in size 300 bp. The TEM and SEM results did not Cry-IV toxin. The hemolytic activity test showed a positive reaction correlated with biosurfactant production. Based on this study, the death of larvae is probably caused by secondary metabolites produced by *Bacillus subtilis* strain LS9.1. Thus, this isolate has the prospect of being an environmentally friendly biocontrol agent to disease vectors and insect pests.

1. Introduction

Disease vectors or plant pathogen and pests control using natural enemies are more perspective compared to insecticides with active chemical ingredients. Entomopathogens from microorganisms act as natural enemies producing toxic metabolites against insects (Dahmana and Mediannikov 2020). The advantages of this natural entomopathogen are target specific, does not kill non-targets, and is environmentally friendly (Thomas 2018; He *et al.* 2021).

The use of *Bacillus* in vector control or plant pathogen and pests had been developed. Boyce *et al.* (2013) reported that, *Bacillus thuringiensis* israelensis (Bti) has been shown as a biocontrol agent to be effective in controlling Dengue Hemorrhagic Fever vector. *B. thuringiensis* produces Cry toxin during the sporulation stage, and this toxin causes the death of some insect species (Soares-da-Silva *et al.* 2015). *B. thuringiensis* has been reported to successfully kill mosquito larvae of species of the genera *Aedes, Culex* and *Anopheles*. Studies on the isolation and identification of *Bacillus* are still being carried out, because of the numerous of disease vectors are transmitted by mosquitoes (Katak *et al.* 2021; Salamun *et al.* 2023).

Manonmani *et al.* (2011) recommended to increase mosquito cyclic lipopeptide production from *B. subtilis* subtilis. Geetha *et al.* (2012) reported the need to improve the mosquito adulticidal activity of biosurfactants produced by *B. subtilis* subtilis. *B. subtilis* also has been developed as a microbial agent for the green synthesis of silver nanoparticles, a perspective for the control of *A. aegypti* larvae (Wilson *et al.* 2022; Chimkhan *et al.* 2022). The other studies reported by

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Bais *et al.* (2004), Fira *et al.* (2018) and Kumbar *et al.* (2019) showed that *B. subtilis* could be developed for plant pathogens biocontrol agent. Shahcheraghi *et al.* (2015), also reported that *B. subtilis* as an important role in medical sciences and human lives.

Bacillus subtilis strain LS9.1 was one of the indigenous bacteria isolated from larvae of *A. aegypti* in the breeding sites of Surabaya city, East Java, Indonesia (Salamun *et al.* 2023). In the screening and confirmation test whole culture, a mixture of pellets and supernatant against larvae of *A. aegypti* with a larval mortality percentage of 90% at 24 hours exposure and 96.7% at 48 hours exposure (Salamun *et al.* 2023). This research the larvicidal toxicity test of whole culture, ultrastructure cells and molecular weight of parasporal inclusion protein, the coding gene of protein toxins and hemolytic activity test to detect mechanisms of actions the toxicity of *B. subtilis* strain LS9.1 against *A. aegypti* larvae. Researchers hope that the *B. subtilis* strain LS9.1 can be developed as an environmentally friendly biocontrol agent against disease vectors in public health or plant pathogens and insect pests in agriculture.

2. Materials and Methods

2.1. Larvicidal Toxicity Test

Larvicidal toxicity test was initiated by isolating of *B. subtilis* strain LS9.1 rejuvenated and cultured in Nutrient Yeast Salts Medium (NYSM) broth incubated at 35°C for 72 h at agitation 170 rpm (Suryadi *et al.* 2016). The number of cells (CFU/ml) of *B. subtilis* LS9.1 was calculated by serial dilution of bacterial culture and continued by counting the number of cells using the Total Plate Count (TPC) method. The larvicidal toxicity test used five concentrations with three replicates of twenty 3rd instar larvae of *A. aegypti*, and a 10% v/v culture media without bacteria as negative controls. The larvicidal toxicity test aimed to determine the lethal concentration (LC) and lethal time (LT) of *B. subtilis* strain LS9.1 against *A. aegypti* larvae. Determine of the LC_{50}, LC_{90}, LT_{50}, and LT_{90} using the MINITAB 17 software.

2.2. Cell Ultrastructure

The cells ultrastructural character of *B. subtilis* strain LS9.1 was determined by culturing pure isolate on NYSM broth, incubated at 35°C with agitation at 170 rpm for 72 h. The culture was analyzed by Transmission Electron Microscopy (TEM) using a TEM JEOL1010, 80.0 KV. The culture was also sonicated to break up the cells of *B. subtilis* strain LS9.1 which analyzed by Scanning Electron Microscopy (SEM) using SEI 10kV WD10mm SSO80.

2.3. Parasporal Inclusion Protein

Isolate of the *B. subtilis* strain LS9.1 was done by rejuvenating the Luria Bertani (LB) agar medium twice reinoculation and the isolate was inoculated on 2×SG medium. Protein samples were analyzed using SDS-PAGE method for protein molecules weight estimation. Characterization of protein molecular weights using 10% separating gel and 4% stacking gel. The results of SDS-PAGE visualization showed protein bands on the marker column with various sizes of molecular weights. The protein sample's molecular weight (bands) was calculated using the standard protein curve regression equation, $y = -1.7218x + 2.4421$ (y = log of molecular weight; x = Rf of protein sample). Relative mobility (Rf) markers are measured from bands per-distance migration (Altschul *et al.* 1997).

2.4. Toxin Coding Gene

Genomic DNA of *B. subtilis* strain LS9.1 was isolated using DNA Purification by Promega Kit (Tamura *et al.* 2013). The process of gene amplification, isolation of the Cry-IV DNA gene, and preparation of samples for electrophoresis was carried out as for the 16S rRNA gene (Ausubel *et al.* 2003; Salamun *et al.* 2023). The Cry-IV gene primers are self-designed, which were forward (5'-GCATATGATGTAGCGAAACAAG-3') and reverse (5'-GCGTGACATACCCATTTCC-3') (Aramideh *et al.* 2016).

2.5. Hemolytic Activity Test

Hemolytic activity test was carried out with Blood Agar media. *B. subtilis* strain LS9.1 is inoculated on Blood Agar media with the spot method. Then incubated for two days at room temperature. After incubation, were observed the clear zone around the colony. Based on the clear zone formed from the hemolytic activity test, it is classified into α, β, and γ hemolysis. The β hemolytic type shows the highest, α is intermediate, and γ is no hemolytic activity (Carrillo *et al.* 1996; Colonna *et al.* 2017).

3. Results

3.1. Larvicidal Toxicity

The larvicidal toxicity (LC and LT) of *B. subtilis* strain LS9.1 to 3rd instar larvae of *A. aegypti* is shown in Table 1 and 2. Overview of *A. aegypti* larvae exposed to *B.*

Table 1. The value of 50% lethal concentration (LC_{50}) of *Bacillus subtilis* strain LS9.1 against 3rd instar of *Aedes aegypti* larvae at 24- and 48-hour exposure

Isolate	Cells total (Cell/ml)	
	LC_{50} 24-hour (lower–upper)	LC_{50} 48-hour (lower–upper)
Bacillus subtilis strain LS9.1	$3.6 \times 10^7 \pm 0.2 \times 10^7$ $(3.2 \times 10^7 – 0.4 \times 10^7)$	$2.8 \times 10^7 \pm 0.2 \times 10^7$ $(2.4 \times 10^7 – 0.3 \times 10^7)$

Table 2. The lethal time (LT_{50} and LT_{90}) of *Bacillus subtilis* strain LS9.1 against 3rd instar larvae of *Aedes aegypti* at 24-hour exposure

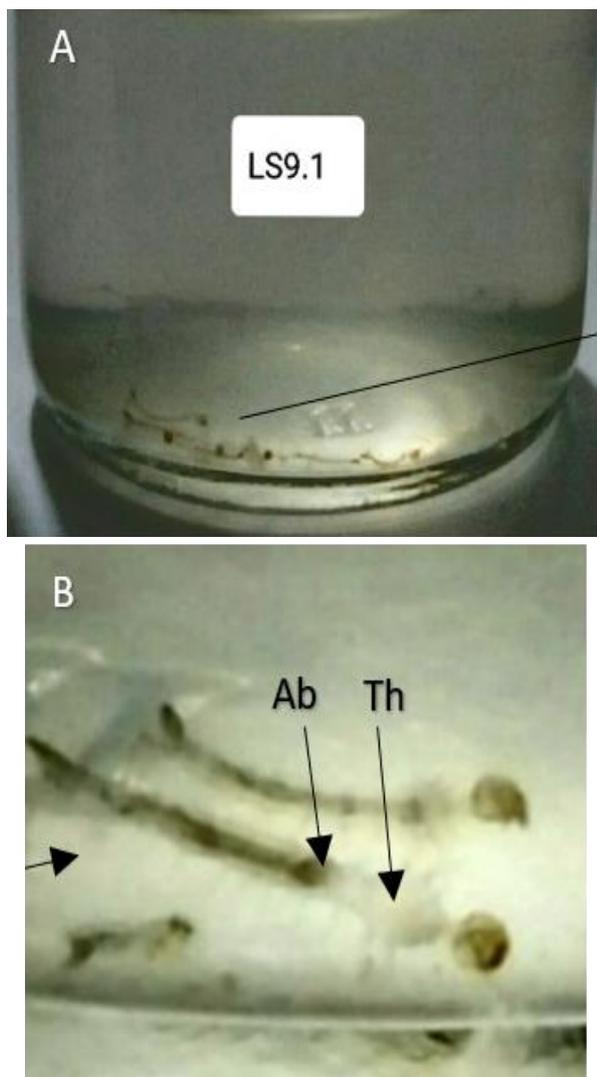| Isolate	LT_{50} (hour) (lower–upper)	LT_{90} (hour) (lower–upper)
Bacillus subtilis strain LS9.1	9.1 ± 0.5 hour (8.2-10.3 hour)	12.0 ± 1.1 hour (10.7–15.8 hour)

Figure 1. General description of *Aedes aegypti* larvae due to exposure to *Bacillus subtilis* strain LS9.1. Descriptions: larval death of bioassay results (A) has shown damage (B) to the abdominal (Ab) and thoracic (Th) regions (→)

subtilis strain LS9.1, has shown damage of abdomen and thorax regions (Figure 1).

3.2. Cells Ultrastructure

The observations by TEM (Figure 2) showed a cross section and a longitudinal section of both vegetative and endospore cells. The results of the detection of the sonicated endospore ultrastructure of *B. subtilis* strain LS9.1 by SEM observations showed in Figure 3. The observations by SEM showed that cell wall was lysed by the sonication method of *B. subtilis* strain LS9.1, has oval to cylindrical spores and has shown did no Cry-IV toxin structure.

3.3. Parasporal Inclusion Protein

The visualization of molecule weight of protein samples from *B. subtilis* strain LS9.1 in Figure 4. The molecular weight of the parasporal inclusion protein visualized on bands calculated by the standard equation in the method has sizes of 72 kDa, 53 kDa, 39 kDa, 21 kDa and 7 kDa.

3.4. Toxin Coding Gene

Detection of the Cry gene of *B. subtilis* strain LS9.1 using a PCR technique with Cry-IV specific primers (Aramideh *et al.* 2016), showed the presence of a single band measuring around 300 bp (Figure 5).

LS 9.1

Type : TEM JEOL 1010, 80.0KV
Magnification 10.000x
TEM & Histology Laboratory
Eijkman Institute for Molecular Biology

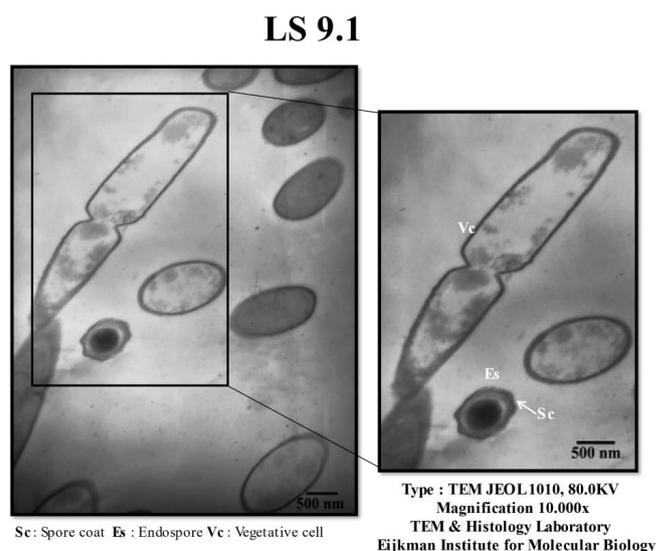Sc : Spore coat   Es : Endospore   Vc : Vegetative cell

Figure 2. The transmission electron microscopy (TEM) vegetative cells of *Bacillus subtilis* strain LS9.1. Descriptions: vegetative cell (Vc), Endospore (Es), Spore coat (Sc)
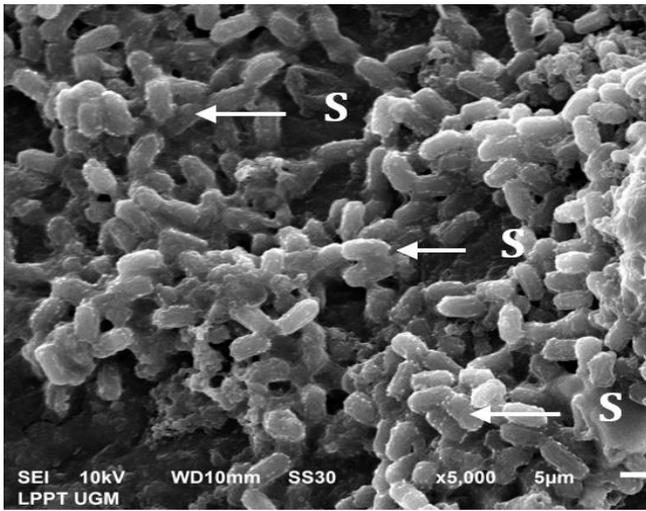
Figure 3. The scanning electron microscopy (SEM) of endospore from *Bacillus subtilis* strain LS9.1, whose cell walls were lysed by sonication method. Description: shows only Spore (S) and no cry-IV toxin

Figure 5. The band of the Cry gene from *Bacillus subtilis* strain LS9.1 was based on electrophoresis of the cry gene amplification using Cry-IV primers on 0.8% agarose gel. (Description: M = DNA marker, 100-1,500 bp; S = sample of *Bacillus subtilis* strain LS9.1, band at 300 bp)
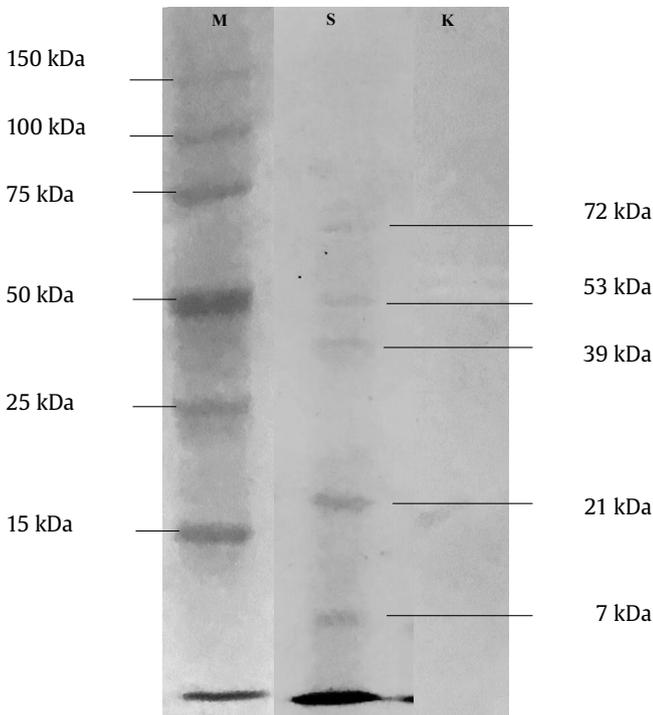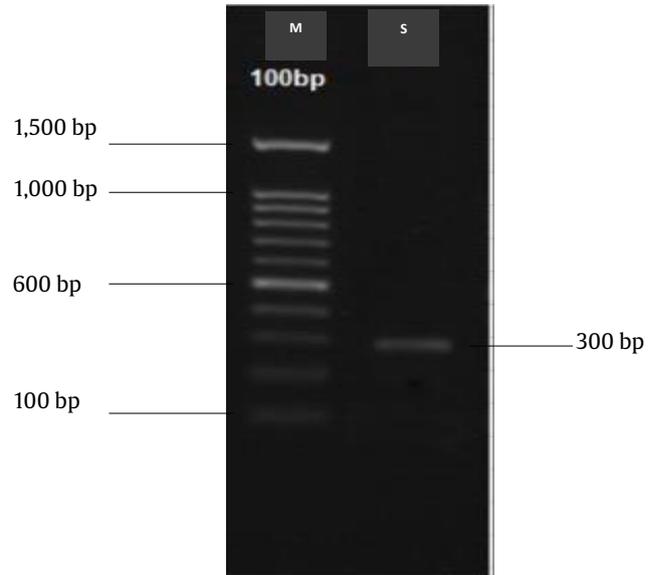

Figure 4. Parasporal inclusion proteins visualization from a sample of *Bacillus subtilis* strain LS9.1 by SDS-PAGE method. (Description: M = protein marker, 15-150 kDa; S = protein sample, 7-72 kDa of *Bacillus subtilis* strain LS9.1; K = control)

3.5. Hemolytic Activity

The results of the blood agar hemolytic activity test are shown in Figure 6. The results showed that there was a clear zone formed around the bacterial colonies, which indicated a positive reaction.
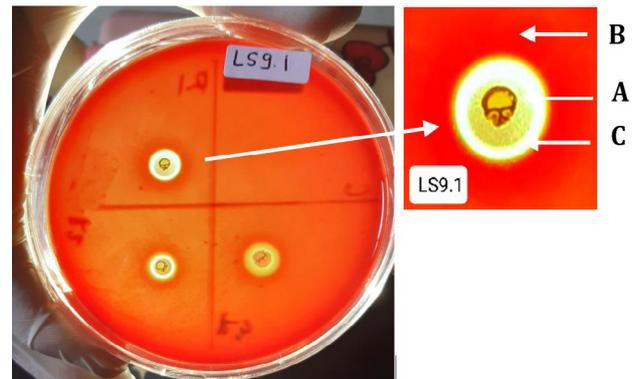

Figure 6. The hemolytic activity (C) of colony of *Bacillus subtilis* strain LS9.1 (A) on Blood Agar media (B) showed a β hemolytic activity or positive result

4. Discussion

Based on the studies (Table 1 and 2), the LC_{50} and LC_{90} values of *B. subtilis* strain LS9.1 have high potential as a biocontrol agent. Morphological images of larval samples exposed to *B. subtilis* strain LS9.1 shows damage to the abdominal and thoracic region (Figure 1). Salamun *et al.* (2021) reported that *B. thuringiensis* BK5.2 isolated from the Baluran National Park East Java, Indonesia had an LC_{50} value of 1.54×10^7 cells/ml at 48 h exposure. Pratiwi *et al.* (2013) reported that *B. thuringiensis* W.Swh.S.K2 from Nganjuk city, Indonesia, had an LC_{50} value of 3.53×10^7 cells/ml at

48 h of exposure. The results of this study have shown that the LC_{50} value of *B. subtilis* strain LS9.1 was $2.8 \times 10^7 \pm 0.2 \times 10^7$ cells/ml at 48 h exposure; the LC_{50} value was in between the results reported by Pratiwi *et al.* (2013) and Salamun *et al.* (2021). The number of endospore cells and secondary metabolites from the bacterial culture preparations affected on the mortality rate of the larvae. Gama *et al.* (2010) reported that the growth and development of *B. thuringiensis* cells in growth media affected the quantity of Cry toxin production, and the more Cry toxin, the more death of *A. aegypti* larvae. Suryadi *et al.* (2016), reported that the toxicity of indigenous *B. sphaericus* isolated from Lombok Island was influenced by the toxin protein, the age of the target larvae, and the bacterial growth media. Dahmana *et al.* (2020) and Katak *et al.* (2021) reported that there were differences in insecticidal activity between the supernatant and pellet fractions from cultures of *Bacillus strains* and suggested the characterization of insecticidal compounds and their mode of action.

The molecular identification of the 16S rRNA gene from *Bacillus* sp. strain LS9.1 by PCR method which was visualized by electrophoresis showing a DNA band of 1440 bp. and the BLAST analysis has a 99.98% similarity with *B. subtilis* subsp. inaquosorum (Salamun *et al.* 2023). Dunlap *et al.* (2020) have promoted that *B. subtilis* currently includes four subspecies, *B. subtilis* subsp. inaquosorum, *B. subtilis* subsp. subtilis, and *B. subtilis* subsp. stercoris and *B. subtilis* subsp. spizenii. Comparisons of the genomes of these bacilli suggests that the four subspecies should be promoted to species status, one of the main considerations being the lack of a particular phenotype. *B. subtilis* has antagonistic activity to various plant diseases, so it has the potential to be a biocontrol agent in the natural environment and is non-toxic and harmless. These bacteria are also reported to produce antimicrobial and antifungal compounds, and protection against plant root pathogens (Kumbar *et al.* 2019). Another study reported that cyclic lipopeptide compounds had inhibitory activity against plant pathogens (He *et al.* 2021). The genes for the biosynthesis of these antimicrobial peptides differ in each *Bacillus* species, an example of has been reported between strains of *B. amyloliquefaciens* and *B. subtilis* (Hossain *et al.* 2015). Manonmani *et al.* (2011) have reported that *B. subtilis* the ability to produce moquitocide toxins. However, research exploring the potential of *B. subtilis* as a biocontrol in vector-borne diseases, still needs to be developed until the applicable product.

This study has detected that the protein molecular weight, ranges from 72 kDa to 7 kDa (Figure 4). The difference in molecular weight was due to the isolates being able to produce one or more protein molecules during the sporulation phase. Thus, an *in vitro* test (bioassay) is needed to identify the larvicidal activity of each protein weight produced by *B. subtilis* strain LS9.1. Under unfavorable conditions, *B. subtilis* will stop growing and begin to increase its metabolic activity. Sporulation in the bacteria is necessary to survive in an unfavorable environment. Green synthesis of silver nanoparticles (Ag-NPs) mediated by the microbe *B. subtilis* (P3) showed strong larvicidal activity (LC_{50} = 22.65 mg/ml and LC_{90} = 55.10 mg/ml) against *A. aegypti* larvae (Wilson *et al.* 2022). The possible mechanism of action of Ag/AgCl NPs against larvae of *A. aegypti* is through mitochondrial dysfunction, DNA and protein damage, inhibited cell proliferation, and cell apoptosis. Thus, Ag/AgCl NPs are recommended as an alternative method to control *A. aegypti*, a mosquito-borne disease vector (Chimkhan *et al.* 2022). So that *B. subtilis* strain LS9.1 results from this study can also be used for the synthesis of green Ag-NPs and prospectively as a biocontrol agent to reduce the *A. aegypti* mosquito population.

Based on the visualization of the Cry gene from *B. subtilis* strain LS9.1 with Cry IV primer, a single band around 300 bp (Figure 5). These results do not match the report by Ben-Dov (2014) which is the primary reference for the research, based on the report the size of the Cry gene is 439 bp with amplification using the Cry-IV primer. The amplified gene of *B. subtilis* strain LS9.1 in this study has a size of around 300 bp. Ben-Dov (2014) reported that the gene with 305 bp is the Cry11 gene. This is also supported by TEM and SEM images on *B. subtilis* strain LS9.1, which it has not shown any Cry-IV toxin in the parasporal inclusions (Figure 2 and 3). The results of alignment analysis with the cry gene sequence from Ben-Dov *et al.* (1997), which has been registered in GenBank with Accession Number D00248, only show a similarity of 3.02%, which the Cry gene was not detected in *B. subtilis* strain LS9.1. Thus, the death of the larvae in this study was not caused by the Cry-IV toxin present in the parasporal inclusions.

In this research, the hemolytic activity test of *B. subtilis* strain LS9.1 on blood agar showed that around the bacterial colony, there was a clear zone of β hemolysis, indicating a positive reaction (Figure 6). Carrillo *et al.* (1996) found a correlation between biosurfactant production by bacteria and hemolytic

activity, and the blood hemolytic activity test recommended as a method to determine the presence of biosurfactant activity. Colonna *et al.* (2017) reported the presence of hemolytic activity as a rapid screening technique to assess the toxicity of native surfactin, one of the group of biosurfactants. Several studies have shown that biosurfactant-producing bacteria are suitable for controlling plant pathogens and insect pests (Bais *et al.* 2004; Zhao *et al.* 2014). *B. subtilis* has also been reported the ability to produce mosquito toxins (Das and Mukherjee 2006; Manonmani *et al.* 2011). Biosurfactants have been introduced as an alternative for controlling insects. The biosurfactants activity produced by *Bacillus strain* has been reported as mosquitoside activity against adult mosquitoes (Geetha *et al.* 2012).

Mode of action of *Bacillus* spp. against *A. aegypti* larvae through two mechanisms, during sporulation the bacteria produce endotoxins is stored in the parasporal inclusions (pellets) and produce secondary metabolites (supernatant) which are released from the cells into the culture media (Dahmana *et al.* 2020; Katak *et al.* 2021; Falqueto *et al.* 2021).

The action of a secondary metabolite of biosurfactants as a mosquitoside toxin that kill mosquito larvae and pupa. Biosurfactants also trigger a decrease of oxygen below the water surface (Mani *et al.* 2017). A decrease in oxygen concentrations causes the insect's spiracles to continue to open and can cause these insect's death. Research has shown that biosurfactant can control plant's pathogens and insect's pests (Zhao *et al.* 2014). From these studies, biosurfactants able to influence the cuticle of insects, because of their amphiphilic nature in the presence of hydrophobic and hydrophilic molecules (Mani *et al.* 2017). The death of *A. aegypti* larvae can be caused by biosurfactants produced by *B. subtilis* strain LS9.1 (Figure 6). Further research is recommended regarding the content of biosurfactant compounds in the supernatant cultured by *B. subtilis* strain LS9.1, and its relationship to abdominal and thoracic damage that causes larval death (Figure 1).

Indigenous entomopathogenic *B. subtilis* strain LS9.1 isolated from *A. aegypti* larvae was high toxicity against 3rd instar larvae of *A. aegypti*. Detection of ultrastructure cells did not show the presence of Cry-IV toxin in the parasporal inclusions, but from the detection of the coding gene using Cry-IV primer, a gene with 300 bp was found that was similar the Cry11 gene (305 bp). Also detected protein molecular weights ranged in size from 72 kDa, 53 kDa, 39 kDa, 21 kDa and 7 kDa. The hemolytic activity of *B. subtilis* strain LS9.1 showed positive results. It was suspected of producing biosurfactants. The indigenous *B. subtilis* strain LS9.1 has the potential as a biocontrol agent against disease vectors or plant pathogens and insect pests.

Acknowledgements

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