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Biosurfactant activity of indigenous *Bacillus* sp. ES4.3 isolated from endemic breeding sites of dengue hemorrhagic fever vector in Surabaya, East Java, Indonesia

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Abstract. *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 22: 5375-5381.* *Bacillus* spp. have shown the ability to results a variety of commercial bioactive compounds such as proteins, peptides, and lipopeptides (LPs). Some of the LPs produced by *Bacillus* spp. are surfactin, iturin, and fengicin. This study aimed to determine the name of the indigenous *Bacillus* sp. ES4.3, the biosynthesis surfactin gene, and the potential activity for biosurfactant produced by entomopathogenic *Bacillus* sp. ES4.3 isolated from endemic breeding sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia. Genomic DNA of *Bacillus* sp. ES4.3 was detected by isolating the DNA and visualizing it by electrophoresis. Furthermore, the 16S rRNA gene was amplified by the Polymerase Chain Reaction (PCR) method. The resulting nucleotide sequences were analyzed to find the relationship between *Bacillus* sp ES4.3 with another bacteria using MEGA version 6 software. Detection of biosynthesis surfactin gene was carried out by PCR method using *srf*AD primers. Analysis of the homology level of the surfactin gene was performed using the NCBI BLASTn and BLASTp genetic analysis program. The indigenous *Bacillus* sp. ES4.3 had 97.66% closeness to the species *Bacillus velezensis* FZB42 and the surfactin gene showed a 100% ID with the surfactin biosynthesis thioesterase SrfA-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity was indicated by the formation of clear zones, emulsions, and a decrease in surface tension in the values of 21.38 mN/m from the NB medium control and 33.74 mN/m from the distilled water control. The ability of *B. velezensis* ES4.3 to hemolyzed and reduce surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti.* Thus, *B. velezensis* ES4.3 has the potential to be developed as a biocontrol in disease vectors.

Keywords: 16S rRNA, *Bacillus* sp., emulsification activity, hemolytic activity, surface tension, surfactin

INTRODUCTION

Biosurfactants are surface active chemical compounds that can be synthesized by several microbial groups. Biosurfactants are an alternative to synthetic surfactants that are not biodegradable and harmful to the environment (Moro et al. 2018). Biosurfactants can be applied in various fields, such as in the food, pharmaceutical, cosmetic, and petroleum industries. They can be used for remediation in locations contaminated with oil and heavy metals (Nwaguma et al. 2016; Gomaa and El-Meihy 2019; Pele et al. 2019). Biosurfactants have several advantages over chemical surfactants, such as lower toxicity, higher biodegradability, not polluting the environment, nonharmful, greater and more specific selectivity (De Almeida et al. 2016; Chaves and Guimaraes 2018). Furthermore, biosurfactants are stable and efficient under adverse temperature, pH, and salinity, typically encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can also effectively reduce surface tension (ST) and interface stress (IT), as well as excellent foaming, emulsifying, and dispersing agents that are widely used in many industrial sectors (Jacques 2011; Pacwa-Plociniczak et al. 2011; Mulligan et al. 2014).

One of the microbial genus that can produce biosurfactants is Genus *Bacillus*. *Bacillus* known to be capable of synthesizing lipopeptide biosurfactants, such as surfactin, iturin, and fengicin (Mongkolthanaruk 2012). Surfactin consists of 7 amino acids ($_L$-leucine, $_D$-leucine, $_L$-aspartate acid, $_L$-valine, $_D$-leucine, $_L$-leucine, and $_L$-glutamic acid) bound to carboxyl and hydroxyl groups of fatty acid carbon atom number 12-16. Surfactin is synthesized by a complex mechanism, catalyzed by the Nonribosomal Peptide Synthetase (NRPS), encoded by the *srf*A operon. Surfactin is one of the three most important lipopeptides detected around the rhizosphere (Henry et al. 2011; Nihorimbere et al. 2012). Surfactin have a strong biosurfactant activity, it can suppress plant diseases (Cawoy et al. 2014) by the inhibition of bacterial growth, it breaks down the membranes or disintegrates it through physicochemical interactions (Deleu et al. 2013), suppressing fungi by encouraging the colonization of beneficial bacteria (Jia et al. 2015), and triggers a systemic resistance (Cawoy et al. 2014).

Detections the activity of biosurfactants can be used by (i) hemolytic activity tests, (ii) methods to analyze the surface activity, emulsifying activity, and surface tension/interface tension (Plaza 2014). In addition, identification of bacterial strains was also carried out, including 16S rRNA gene analysis and detection of biosynthesis surfactin gene, namely *srf*A-D. This methodology ensures that phenotypic and genotypic features are considered (Das et al. 2013), provides an overview of the role and importance of molecular genetics and the gene regulatory mechanisms behind surfactin biosynthesis of various microbes that have commercial importance.

The method is commonly used for screening and genetic identification of bacterial isolates that can rapidly produce biosurfactants through PCR. PCR was used to identify the 16S rRNA gene and gene involved in surfactin biosynthesis, called *srf*A-D (Mulligan et al. 2014). This study was to determine the name of the indigenous *Bacillus* sp. ES4.3, the surfactin biosynthesis gene, and the potential activity for biosurfactant produced by *Bacillus* sp. ES4.3 isolated from the breeding sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia.

MATERIALS AND METHODS

Isolate and media preparation

Bacillus sp. ES4.3 is a bacterial isolate that has been isolated from endemic breeding sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia in the previous research (Salamun et al. 2020). We used three media in this research. Nutrient Agar (NA) medium used for purification of *Bacillus* sp. ES4.3, Luria Bertani (LB) medium used for isolation of DNA *Bacillus* sp. ES4.3, and Nutrien Broth (NB) medium used for culturing *Bacillus* sp. ES4.3 for biosurfactant activity. The three media were prepared with distilled water and sterilized using an autoclave at 121ºC 1 atm. *Bacillus* sp. ES4.3 was cultured on the different three mediums and incubated at 35ºC for 24 hours.

Identification of 16S rRNA gene

Identification of 16S rRNA gene was initiated by culturing isolates of *Bacillus* sp. ES4.3 into 20 mL of Luria Bertani medium, incubated at 35ºC with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out using the CTAB method (Ausubel et al. 2003) with DNA Wizard Genomic DNA Purification Kit (Promega). 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 equipment. This process begins by adding GoTaq Green Master Mix and 16S rRNA primers, in 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 product was visualized through an electrophoresis process using 1% agarose gel followed by Ethidium Bromide staining and observed in ultraviolet light. The PCR samples were sent to the 1st Base DNA Sequencing Service Malaysia. Amplicon was sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The data were also analyzed for their relation by building a phylogenetic tree using MEGA version 6 software (Tamura et al. 2013).

Detection of biosynthesis surfactin gene

In this stage, to identify surfactine gene, the same procedures for 16S rRNA identification were performed using *srf*A-D gene primers. The *srf*A-D gene primers are self-designed on the page ThermoFisher Scientific Oligo Perfect Primer Designer cloning application.

Biosurfactant screening activity

Hemolytic test

Hemolytic test was carried out by culturing the bacterial isolate *Bacillus* sp. ES4.3 in sterile Blood Agar medium obtained from the Surabaya Laboratory. Isolate *Bacillus* sp. ES4.3 was inoculated into the Blood Agar medium by spot method. After that, it was incubated at 37ºC for 24 to 48 hours. The positive results of this test can be observed in the hemolysis zone and the color changes that occur around the bacterial colony.

Emulsification activity

Emulsification activity was carried out to determine the ability of *Bacillus* sp. ES4.3 in emulsifying liquid hydrocarbons, i.e. kerosene and diesel fuel. The bacteria were cultured in liquid NB medium and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied with kerosene, and used diesel fuel in different test tubes, and homogenized using vortex at high speed for 2 minutes. Stability was measured after 1 hour. Emulsification activity is determined by index calculation (E24). This calculation is done through the formula by Ozdal et al. (2017).

$$E24 = \frac{HE}{HS} \ x \quad 100\%$$

Where:
E24 : emulsification activity on 24 hours
HE : high of the emulsion layer
HS : high of total solution

Surface tension

The surface tension of the cell-free culture supernatant obtained by centrifugation was measured using the Kruss 100 tensiometer (Kruss GmbH, Hamburg, Germany) by the Du Nouy ring method. Measurements were replicated 3x to improve accuracy and average retrieval. This calculation is done through the formula by Chauhan et al. (2013).

$$\gamma = \gamma o \frac{\theta}{\theta o}$$

Where:

γ : the surface tension of the sample

γ_o: surface tension standard value of distilled water at t°C

θ : the indicated sample value according to the instrument scale

θ_o: distilled water value shown according to the instrument scale

RESULTS AND DISCUSSION

Analysis of 16S rRNA gene

Isolate of *Bacillus* sp. ES4.3 was identified by amplification and sequencing of the 16S rRNA gene using PCR. The sequences of *Bacillus* sp. ES4.3 DNA was analyzed using BioEdit Sequence Alignment Editor software version 7.2.5. and nucleotide Basic Local Alignment Search Tool (BLASTn) that followed by the website of the National Center for Biotechnology Information (NCBI) “http://www.ncbi.nlm.nih.gov”. Figure 1. showed the band of DNA from PCR result on agarose gel 1%.

The PCR results in Figure 1. showed the band of 16S rRNA gene from *Bacillus* sp. ES4.3 isolate. When it matched with size order of DNA marker, the size of the band measuring 1500bp.

The result of nucleotide Basic Local Alignment Search Tools (BLASTn) analysis in Table 1. indicates that this isolate is the *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with *Bacillus velezensis* strain FZB42 (GenBank access number NR_075005.2). This is because the results in Table 1. show the highest % ID is *B. velezensis* strain FZB42 compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

Analysis of phylogenetic tree

Figure 2. showed the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *B. velezensis.*These bacteria are used to calculate evolutionary distances and construct phylogenetic trees. The bootstrap test (1000 replications) is shown next to the branch. The consensus procedure was used to produce a bootstrap phenogram in the phylogenetic tree, analyzed by the Neighbor-Join Method.

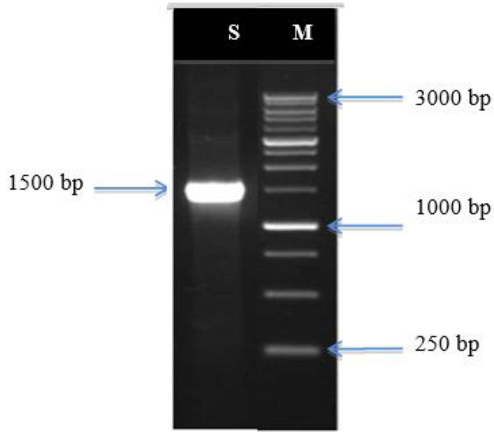

Figure 1. Electrophoresis result of DNA *Bacillus* sp. ES4.3 isolate marked with a band measuring 1500 bp. (S: Sample; M: Marker)

Table 1. The results of Basic Local Alignment Search Tools (BLAST) of *Bacillus* sp. ES4.3

Description	Scientific name	Query cover	% ID
Bacillus velezensis strain FZB42 16S ribosomal RNA gene, complete sequence	*Bacillus velezensis*	99%	97.66%
Bacillus atrophaeus strain NBRC 15539 16S ribosomal RNA gene, partial sequence	*Bacillus velezensis*	99%	97.52%
Bacillus atrophaeus strain JCM 9070 16S ribosomal RNA gene, partial sequence	*Bacillus velezensis*	99%	97.52%

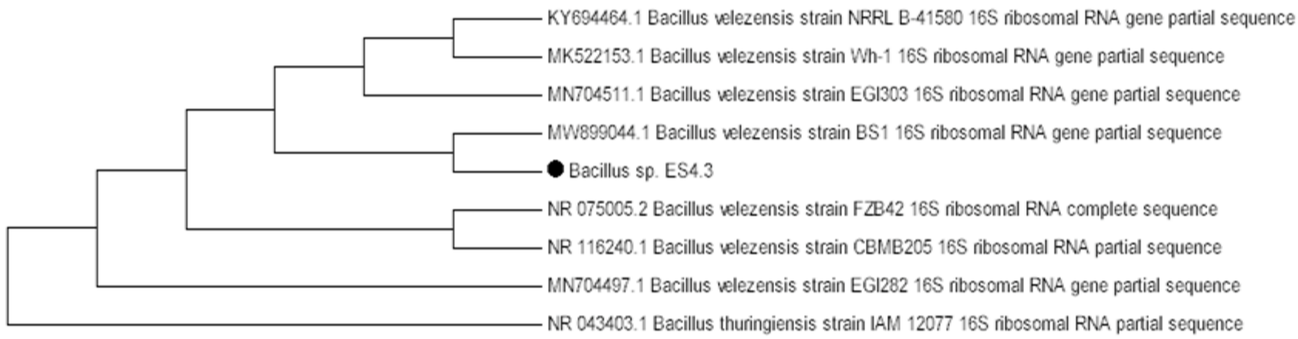

Figure 2. Phylogenetic tree of *Bacillus* sp. ES4.3 and another bacteria of *Bacillus velezensis* strains

Analysis of biosynthesis surfactin gene

The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *srf*A-D gene *B. velezensis* ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the *srf*A-D gene *B. velezensis* ES4.3 and another protein of the *srf*A-D gene *Bacillus* in GenBank. Figure 3. showed the band of *srf*A-D gene on agarose gel 1% with a successfully amplified size of 722 bp. Based on the BLASTp results, the *srf*A-D gene of *B. velezensis* ES4.3 protein has the highest similarity with the surfactin biosynthesis thioesterase SrfA-D from *Bacillus amyloliquefaciens* group bacteria in Genbank by 100% (GenBank access number WP_003156383.1). On the research of Rabbee et al. (2019), the research said that based on phylogenomic analysis, *B. velezensis* belong to the same clade as a *B. amyloliquefaciens*.

Screening of biosurfactant activity

Hemolytic activity

Hemolytic activity can be identified on Blood Agar medium with 24 hours observation by looking at the clear zone around the microbial colonies (Carillo et al. 1996). Hemolytic activity of *B. velezensis* ES4.3 can be seen in Figure 4.

Emulsification activity

Table 2. showed that the emulsification activity of the cell-free supernatant of *B. velezensis* ES4.3 used kerosene and diesel fuel at 1 hour and 24 hours observation. The emulsification activity of *B. velezensis* ES4.3 in kerosene showed an increase, while in diesel fuel, it decreased.

Surface tension

Table 3. showed that the surface tension value of the culture supernatant *B. velezensis* ES4.3, when it compared with the surface tension values of the distilled water control, NB medium control, and Tween control, the value of the culture supernatant of this isolate decreased to 21.38 mN/m from the NB medium control, 33.74 mN/m from the distilled water control, and 3.91 mN/m from the Tween control.

Discussion

The results of DNA isolation from *Bacillus* sp. ES4.3 showed the presence of a DNA band with a size of 1500 bp when it matched with the DNA marker (Figure 1). From these results, it can be said that there is a 16S rRNA gene, which is a DNA barcode for bacterial species. 16S rRNA gene sequencing serves as an approximation method for rapid and accurate bacterial identification. A bacterium represents the same genus if it has a similarity index above 95% and represents the same species if it is above 97% (Srinivasan et al. 2015; Johnson et al. 2019).

The *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with the *B. velezensis* strain FZB42 (GenBank access number NR_075005.2). These results are different from conventional identification results through observations of macroscopic, microscopic, and physiological characters carried out by Salamun et al. (2020), which stated that *Bacillus* sp. ES4.3 is *Bacillus sphaericus* with a comparable coefficient calculation (Ss) of 76.12%.

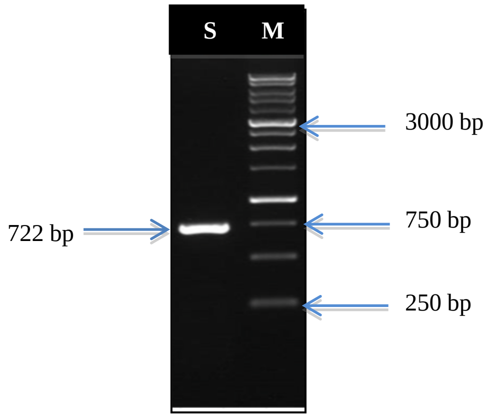

Figure 3. Electrophoresis results of the *srf*A-D gene in *Bacillus velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 722 bp. (S = Sample; M = Marker)

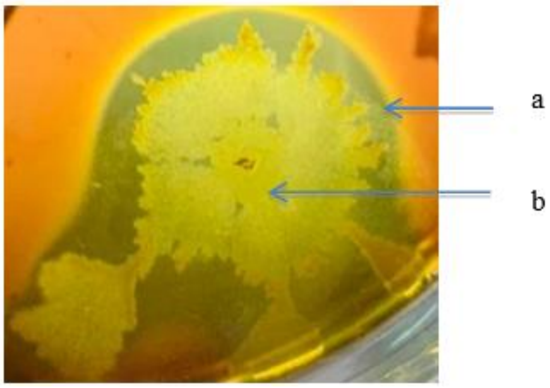

Figure 4. The clear zone is formed from the hemolytic activity of the *Bacillus velezensis* ES4.3 isolate on Blood Agar medium. Note: a. halo zone, b. Colony of *B. velezensis* ES4.3

Table 3. The surface tension value of the culture supernatant isolate *Bacillus velezensis* ES4.3 with an incubation time of 2 days

Treatment	Surface tension (mN/m)
Distilled water control	72.00 ± 0.00
NB medium control	59.64 ± 0.12
Supernatant *B. velezensis* ES4.3	38.26 ± 0.25
Tween control	34.35 ± 0.07

Table 2. Results of emulsification activity of supernatant of *Bacillus velezensis* ES4.3 on kerosene and diesel fuel

Treatment	Emulsification Activity (%)			
	Kerosene		Diesel fuel	
	1 hour	24 hours	1 hour	24 hourr
Supernatant *B. velezensis* ES4.3	31.08±0.22	14.52 ± 1.21	46.32±1.22	45.65 ± 0.46

Figure 2. showed the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *B. velezensis*. This shows that *Bacillus* sp. ES4.3 has a close relationship with *B. velezensis* BS1 and *B. velezensis* FZB42. It can be seen from the location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei* Damm, P.F.Cannon & Crous, a pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Lib.) de Bary isolated from lettuce. In addition, according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic tree, *Bacillus thuringiensis* is an outgroup.

The sequencing results were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *srf*A-D gene in *B. velezensis* ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the *srf*A-D gene *B. velezensis* ES4.3 and another protein of the gene *srf*A-D *Bacillus* in GenBank. Based on the results of BLASTp, the protein in the *srf*A-D gene from *B. velezensis* ES4.3 has the highest similarity of 100% with surfactin biosynthesis thioesterase *Srf*A-D from the *B. amyloliquefaciens* group bacteria in Genbank. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *B. velezensis* ES4.3. This sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B. velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *B. velezensis* ES4.3, identified as *B. velezensis* Htq6, with a successfully amplified size of 722 bp. The *srf*A-D gene is known to produce thioesterase, which is presumed to be involved in the lactonization process (Satpute et al. 2010).

The hemolytic activity can be identified by looking at the clear zone around the microbial colonies (Carillo et al. 1996). Hemolytic activity of *B. velezensis* ES4.3 can be seen in Figure 4. Carillo et al. (1996) stated that there is a relationship between hemolytic activity and biosurfactant production. In the Blood Agar medium, *B. velezensis* ES4.3 was inoculated. The presence of hemolytic activity, which is indicated by the formation of a halo zone around the colony, means the biosurfactants were produced.

From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis, which is indicated by the clear visible zone as a result of the lysis of all red blood cells and the release of hemoglobin from the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant concentration is low causing osmotic lysis (Zaragoza et al. 2010).

The clear zone in the Blood Agar medium corresponded to changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result, biosurfactants can directly interact with membrane lipids that enter the membrane, form pores in the larvae midgut, and destroy the membrane through detergent-like interactions (Butko 2003).

The results of the emulsification activity can be seen in Table 2. These results indicate differences in the results of the emulsification activity of the supernatant *B. velezensis* ES4.3 on kerosene and diesel fuel substrates. Better properties are characterized by a greater emulsion index value, which means that the surfactant has large emulsion stability. The results also showed a decrease in the value of emulsification activity after 24 hours, which indicates a relatively stable emulsification activity. This research proves that the biosurfactant product can be categorized as a bioemulsifier. The occurrence of emulsification activity in *B. velezensis* ES4.3 is indicated by the formation of foam, which creates a layer in the tube. The foam layer was then measured to calculate the emulsification activity value (Ni'matuzahroh et al. 2017). The emulsification index value indicates the stability of the emulsion, and the line above 50% showed good biosurfactant producers (Willumsen and Karlson 1997).

The mechanism by which surfactants work as emulgators is by reducing the tension between the surface of water and oil, so that a film layer is formed on the surface of the dispersed globules phase. Hence, emulsification activity is related to the parameters of biosurfactant production because it is a very good emulsifier. The indicators of biosurfactants that are produced by microorganisms can be seen in the emulsification activity of the media. Emulsification activity can be seen by the formation of an emulsion that looks like a bubble between the substrate and the media (Arifiyanto et al. 2020).

The surface tension value of the culture supernatant *B. velezensis* ES4.3 can be seen in Table 3. When compared with the surface tension values of the distilled water control, NB medium control, and Tween control, the value of the culture supernatant of this isolate decreased to 21.38 mN/m from the NB medium control, 33.74 mN/m from the distilled water control, and 3.91 mN/m from the Tween control. Bacteria can produce biosurfactants if they can reduce the surface tension value ≥ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant produced by bacterial isolates during the growth process (Arifiyanto et al. 2020). The decrease in surface tension can affect the entomopathogenic activity of *Aedes aegypti* larvae which causes low oxygen underwater, so that the larvae spiracles continue to open and make it death (Geetha 2010).

This study concluded that *Bacillus* sp. ES4.3 which was identified as *Bacillus velezensis* ES4.3, has a biosynthesis surfactin gene. The biosurfactant activity was indicated by the formation of clear zones, the formation of emulsions, and a decrease in surface tension in the values of 21.38 mN/m from the NB medium control and 33.74 mN/m from the distilled water control. The presence of these genes and the biosurfactant activity indicates that the *B. velezensis* ES4.3 can act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *B. velezensis* ES4.3 can be developed as a biocontrol in disease vectors and other fields of medicine, agriculture, pharmaceuticals, and waste treatments. Further research that can be done to produce surfactin include the production and optimization of biosurfactant production, as well as characterization of the resulting biosurfactant product.

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