[biodiv] Editor Decision

2022-08-13 06:14 AM

Farah Aisyah Nafidiastri:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Potential biocontrol agent of indigenous Bacillus sp. EG6.4: Molecular identification, larvicidal toxicity, and mechanism of actions ".

Our decision is: Revisions Required

Reviewer A:

Dear Author,

Introduction is about 600-800 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. Please add some references that support your research background

Thank you

Recommendation: Revisions Required

[biodiv] Editor Decision External Inbox

Smujo Editors <smujo.id@gmail.com> Oct 28, 2021, 8:52 PM
to Farah, Rizky, Tri, Agus, Almando, Ni'matuzahroh, Fatimah, me

Farah Aisyah Nafidiastri; Rizky Danang Susetyo; Tri Nurhariyati, Agus Supriyanto, Almando Geraldi, Ni'matuzahroh, Fatimah, Salamun:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Biosurfactant Activity of Indigenous Bacillus sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever in Surabaya, East Java, Indonesia : Biosurfactant Activity Bacillus sp. ES4.3 Indonesia".

Our decision is: Revisions Required

Reviewer F:

Dear Editor,

The manuscript entitle "Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever in Surabaya, East Java, Indonesia" suggested for publication in Bidiversitas j. of biological diversity, the concept of the article was good but authors did not explain or clarify their points of view in the aim of the introductry part. Also, many parts in the manuscript requried carefully revision especially in the discussion part.

Authors should find the revised file form and carefully correct.

Regards

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

Revisi artikel biodiv farah

Inbox

farah aisyah

to me

Nov 12, 2021, 11:16 AM (18 hours ago)

Assalamualaikum Pak Salamun..
Mohon ijin..
Berikut saya kirimkan dua naskah revisi artikel biodiv..
Terkait judul berarti ditambahi kata-kata "Vector" nggih pak?
jika iya, mohon ijin setelah ini akan saya submit kembali pak..
Maturnuwun..

2 Attachments

Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

REV-revisiF-9557-...

Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

REVTrack-revisiF...

Salamun Salamy

to farah

5:46 AM (9 minutes ago)

Farah
Ok, silahkan hasil revisi kita di submit kembali ke Jurnal Biodiversitas, sesuai revisi terakhir. Semoga dapat diterima dan akhirnya bisa published.
Salamun

[biodiv] Editor Decision

External

Inbox

Anisa Septiasari <smujo.id@gmail.com> 10:19 AM (9 hours ago)

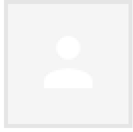
to FARAH, RIZKY, TRI, AGUS, ALMANDO, NI'MATUZAHROH, FATIMAH, me

FARAH AISYAH NAFIDIASTRI; RIZKY DANANG SUSETYO; TRI NURHARIYATI, AGUS SUPRIYANTO, ALMANDO GERALDI, NI'MATUZAHROH, FATIMAH, SALAMUN:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Biosurfactant activity of indigenous Bacillus sp. ES4.3 isolated from endemic breeding sites of dengue hemorrhagic fever in Surabaya, East Java, Indonesia : Biosurfactant Activity Bacillus sp. ES4.3 Indonesia".

Our decision is to: Accept Submission

Biodiversitas Journal of Biological Diversity



salamun
salamun <salamun@fst.unair.ac.id>

8:00 PM (13 minutes ago)

to Anisa

Thank you very much for your email about accepted decision. We are all authors very happy this information.

Dr. Salamun, M.Kes
Universitas Airlangga
Faculty of Science and Technology
Phone: +6281332198122
associates mailbox: salamun@fst.unair.ac.id

Smujo Editors <smujo.id@gmail.com> Tue, Nov 23, 2021, 8:26 PM
to FARAH, RIZKY, TRI, AGUS, ALMANDO, NI’MATUZAHROH, FATIMAH, me

FARAH AISYAH NAFIDIASTRI; RIZKY DANANG SUSETYO; TRI NURHARIYATI, AGUS SUPRIYANTO, ALMANDO GERALDI, NI’MATUZAHROH, FATIMAH, SALAMUN:

The editing of your submission, "Biosurfactant activity of indigenous Bacillus sp. ES4.3 isolated from endemic breeding sites of dengue hemorrhagic fever in Surabaya, East Java, Indonesia: Biosurfactant Activity Bacillus sp. ES4.3 Indonesia," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/9557

Biodiversitas Journal of Biological Diversity

Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

Manuscript received: DD MM 2021 (Date of abstract/manuscript submission). Revision accepted: ....

Abstract. *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 detected by isolating the DNA and visualizing 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 99.86 for % ID with the *srf*A-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 media control and 33.74 mN/m from the distilled water control. The ability of *Bacillus* sp. ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti*. Thus, *Bacillus* sp. ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

Running title: Biosurfactant Activity of *Bacillus* sp. ES4.3

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, petroleum industries, and 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, which is 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) which are 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), which is 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

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Commented [U7]: Unknown action of this bacterium for production of biosurfactant

Commented [mm8]: This bacterium has known activity in producing various kinds of biosurfactants, one of which is surfactin

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Commented [U11]: what is the relation between production of surfactant applications and using this bacterium as biocontrol for infectious diseases? So, what is the main target of this article????

Commented [mm12]: We deleted "pest, and plant diseases or waste treatment" and added previous sentences

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bacterial growth, it breaks down the membranes or disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 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 in Surabaya, East Java, Indonesia in the previous research (Salamun et al. 2020). We used three medium 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 on biosurfactant activity. The three medium were prepared with distilled water and sterilized using an autoclave at 121°C 1 atm. *Bacillus* sp. ES4.3 was cultured on the different three medium and incubated at 35°C 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 35°C 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 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 product were 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. Amplikon 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, we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is the primer used. 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 that 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 37°C 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 media and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by 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 or EI). This calculation is done through the formula by Ozdal et al. (2017).

Commented [mm14]: We added these statement and deleted "The media used for purification of *Bacillus* sp. ES4.3 is a slant Nutrient Agar medium, which used for the isolation of DNA is Luria Bertani medium and used for biosurfactant activity is Nutrien Broth medium. "

Commented [U15]: Authors should specify the media used for isolation and purification. Nutrient agar is a medium and LB another medium, please revise

Commented [mm16]: We deleted "aquadest" and replaced with this words

Commented [mm17]: We deleted "room temperature" and replaced with "35°C"

Commented [mm18]: We added the kit used for DNA isolation

Commented [U19]: Authors should mention the kit used for DNA isoaltion

Commented [mm20]: We added "equipment" for amplification proses

Commented [mm21]: We replaced "The result of PCR" with this words

Commented [mm22]: We have put this sentences after the visualized PCR product

Commented [mm23]: We added these words

Commented [mm24]: We deleted "This stage uses isolated DNA in the identification of 16S rRNA gene. This stage is the same as the procedure for the detection of the 16S rRNA gene, but the primers that has been used are different. " and replaced with this sentences

Commented [mm25]: We deleted "planting" and replaced with "culturing"

Commented [mm26]: We added these words and deleted "Sterile Blood Agar media was obtained from the Surabaya Laboratory"

Commented [mm27]: We deleted previous words "means of the"

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Commented [mm29]: We deleted "added" and replaced with "supplied by"

Commented [U30]: Mention the equation

102 $$E24 = \frac{HE}{HS} \; x \quad 100\%$$

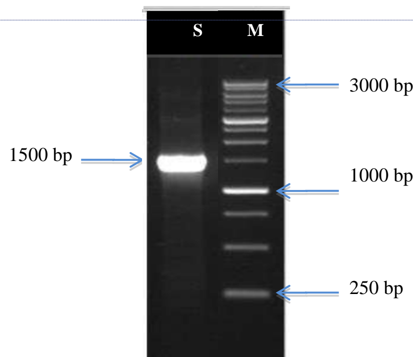
103
104 E24 : emulsification activity on 24 hours
105 HE : high of the emultion layer
106 HS : high of total solution
107
108 **Surface tension**
109 The surface tension of the cell-free culture supernatant obtained by centrifugation was measured using the Kruss 100
110 tensiometer (Kruss GmbH, Hamburg, Germany) by the Du Nouy ring method. Measurements were replicated 3x to
111 improve accuracy and average retrieval. This calculation is done through the formula by Chauhan et al. (2013).
112

113 $$g = go \frac{q}{qo}$$

114
115 γ : the surface tension of the sample
116 γ_o : surface tension standard value of distilled water at t°C
117 θ : the indicated sample value according to the instrument scale
118 θ_o : distilled water value shown according to the instrument scale

119 ## RESULTS AND DISCUSSION

120 **Analysis of 16S rRNA gene**
121 Isolate of *Bacillus* sp. ES4.3 was identified by amplification and sequencing of the 16S rRNA gene using PCR. The
122 sequences of *Bacillus* sp. ES4.3 DNA was analyzed using BioEdit Sequence Alignment Editor software version 7.2.5. and
123 nucleotide Basic Local Alignment Search Tool (BLASTn) that followed by the website of the National Center for
124 Biotechnology Information (NCBI) "http://www.ncbi.nlm.nih.gov". In Figure 1. showed the band of DNA from PCR
125 result on agarose gel 1%.
126–142



143 **Figure 1.** Electrophoresis result of DNA *Bacillus velezensis* ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M =
144 Marker)
145
146 The PCR result in Figure 1. showed the band of 16S rRNA gene from *Bacillus* sp. ES4.3 isolate. When it matched with
147 size order of DNA marker, the size of the band measuring 1500bp.
148 The result of nucleotide Basic Local Alignment Search Tools (BLASTn) analysis in Table 1. indicate that this isolate
149 as the *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with *Bacillus velezensis* strain FZB42 (GenBank access number
150 NR_075005.2). This is because the results in Table 1. show the highest % ID is *Bacillus velezensis* strain FZB42 when it
151 compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.
152
153
154
155

Commented [mm31]: We added the equation

Commented [U32]: Mention the formula

Commented [mm33]: We added the formula

Commented [mm34]: We deleted "Genes that have been isolation and amplified that confirmed by electrophoresis are shown in Figure 1. with a size of 1500 bp. " and replaced with this sentence

Commented [mm35]: We added label on the figure

Commented [U36]: No label or markers on the figure (Unnominated figure)

Commented [U37]: Please precise your concept from this figure

Commented [mm38]: We added these sentences to support the Figure 1.

Commented [U39]: So why authors do not mention this species in your work (Why Bacillus sp. only???)

Commented [mm40]: because in the some parts of manuscript (introduction and method) we don't yet know the species name of our isolate, so we have to use *Bacillus* sp. ES4.3

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%

Analysis of Phylogeni Tree

Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus 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 phylogeny tree, which is analyzed by Neighbor-Join Method.

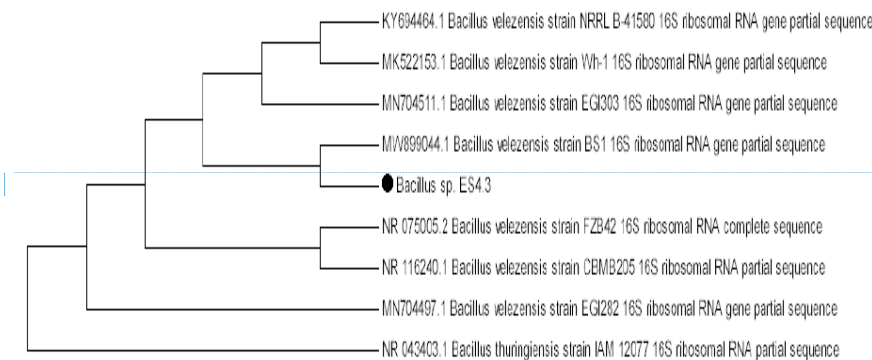


Figure 2. Phylogeni tree of *Bacillus velezensis* ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

Figure 3. shows that the sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *Srf*A-D gene *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 with another nucleotide of the *srf*A-D gene *Bacillus* in GenBank. Based on the BLASTp results, the amino acids in the *srf*A-D gene of *Bacillus* sp. ES4.3 have the highest similarity with the surfactin thioesterase *srf*A-D biosynthesis from *Bacillus amyloliquifaciens* group bacteria in Genbank by 99.86%. On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B. velezensis* belong to the same clade as a *B. amyloliquifaciens.*

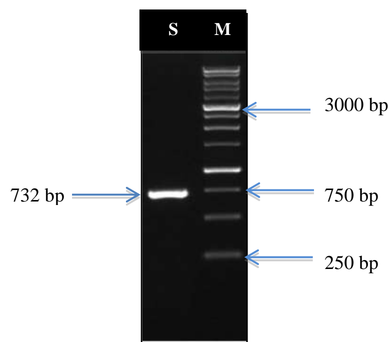


Figure 3. Electrophoresis results of the *srf*A-D gene in *Bacillus velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 732 bp. (S = Sample; M = Marker)

Commented [U41]: Not clear, authors should use good resolution

Commented [mm42]: We added these words

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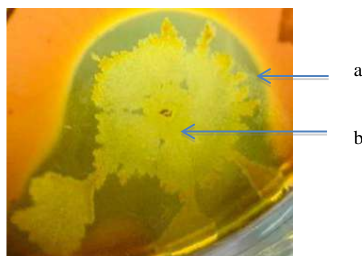
Commented [mm44]: We deleted "and" and replace with "with another", we also deleted "*B. velezensis*" and replace with "*Bacillus"*

Commented [mm45]: In this sentences, we matched our nucleotides of the *srf*A-D gene bacteria with another nucleotides of the *srf*A-D gene bacteria in GenBank data, (did not compare)

Commented [mm46]: We added these sentence for supporting our result

Commented [U47]: Authors made their comparison according *Bacillus amyloliquifaciens or B. velezensis*

210 **Screening of Biosurfactant Activity**
211 **Hemolytic Activity**
212 Hemolytic activity can be identified on Blood Agar media with 24 hours observation by looking at the clear zone
213 around the microbial colonies (Carillo et al. 1996). Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4.
214–226



227 **Figure 4.** The clear zone is formed from the hemolytic activity of the *Bacillus* sp. ES4.3 isolate on Blood Agar media. Notes: a) halo
228 zone, b) Colony of *Bacillus* sp. ES4.3.

229 **Emulsification Activity**
230 Table 2. shows that the emulsification activity of the cell-free supernatant of *Bacillus* sp. ES4.3 used kerosene and
231 diesel fuel at 1 hour and 24 hours observation. The emulsification activity of *Bacillus* sp. ES4.3 in kerosene showed an
232 increase, while in diesel fuel it showed a decrease.
233
234 **Table 2.** Results of emulsication activity of supernatant of *Bacillus* sp. ES4.3 on kerosene and diesel fuel.
235

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

236 **Surface Tension**
237 Table 3. shows that the surface tension value of the culture supernatant *Bacillus* sp. ES4.3, when it compared with the
238 surface tension values of the distilled water control, media control, and Tween control, the value of the culture supernatant
239 of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water control, and 3.91
240 mN/m from the Tween control.
241
242 **Table 3.** The surface tension value of the culture supernatant isolate *Bacillus* sp. ES4.3 with an incubation time of 2 days.
243

Treatment	Surface Tension (mN/m)
Distilled Water Control	72.00 ± 0.00
NB Control	59.64 ± 0.12
Supernatant *Bacillus* sp. ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

244

245 **Discussion**
246 The results of DNA isolation from *Bacillus* sp. ES4.3 showed the presence of a DNA band with a size of 1500 bp when
247 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
248 DNA barcode for bacterial species. 16S rRNA gene sequencing serves as an approximation method for rapid and accurate
249 bacterial identification. A bacterium is said to represent the same genus if it has a similarity index above 95% and
250 represents the same species if it is above 97% (Srinivasan et al. 2015; Johnson et al. 2019).
251 The *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with the *Bacillus velezensis* strain FZB42 (GenBank access
252 number NR_075005.2). These results are different from conventional identification results through observations of
253 macroscopic, microscopic, and physiological characters that have been carried out by Salamun et al. (2020), which stated
254 that *Bacillus* sp. ES4.3 is *Bacillus sphaericus* with a comparable coefficient calculation (Ss) of 76.12%.
255 Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *Bacillus velezensis.* This shows
256 that *Bacillus* sp. ES4.3 has a close relationship with *B. velezensis* BS1 and *B. velezensis* FZB42. It can be seen from the

Commented [U48]: Why authors did not extract surfactin from bacteria free cell supernatant for analysis?

Commented [mm49]: In this research we already used cell-free supernatant of *Bacillus* sp. ES4.3 for emulsification activity and surface tension analysis. The surfactin extract process will be carried out in future research to determine the type of surfactin produced.

Commented [U50]: The unit please or %???

Commented [mm51]: We added the unit of emulsification activity

Commented [mm52]: We deleted "aquadest" and replaced with this words

Commented [U53]: If authors use % is more logic

Commented [mm54]: We still use mN/m because it is the unit (international standard) of surface tension

Commented [U55]: Unit of measurement

Commented [mm56]: we've added the unit of measurement

Commented [U57]: Discussion should be revised grammatically

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257 location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that
258 could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis*
259 BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the
260 research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and
261 siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei*, a
262 pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis*
263 *cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* isolated from lettuce. In addition,
264 according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as
265 *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study
266 of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic
267 tree, *Bacillus thuringiensis* is an outgroup.

268 The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the
269 similarity of the *srf*A-D gene in *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine
270 the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 and the nucleotide gene *srf*A-D *B. velezensis*
271 in GenBank. Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin
272 biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity
273 with 99.86%. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *Bacillus* sp. ES4.3. This
274 sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B.*
275 *velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *Bacillus* sp.
276 ES4.3, identified as *B. velezensis* FZB42. The *srf*A-D gene is known to produce thioesterase, which is presumed to be
277 involved in the lactonization process (Satpute et al. 2010).

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

283 From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis,
284 which is indicated by the visible clear zone as a result of the lysis of all red blood cells and the release of hemoglobin from
285 the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because
286 biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane
287 antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different
288 mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and
289 the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant
290 concentration is low causing osmotic lysis (Zaragoza et al. 2010). The clear zone in the Blood Agar media corresponded to
291 changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of
292 cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result,
293 biosurfactants can directly interact with membrane lipids that enter the membrane and form pores in the larvae midgut and
294 destroy the membrane through detergent-like interactions (Butko 2003).

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

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

309 The surface tension value of the culture supernatant *Bacillus* sp. ES4.3 can be seen in Table 3. When compared with
310 the surface tension values of the distilled water control, NB media control, and tween control, the value of the culture
311 supernatant of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water
312 control, and 3.91 mN/m from the tween control. Bacteria have the potential to produce biosurfactants if they can reduce
313 the surface tension value ≥ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause
314 these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension
315 (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant
316 produced by bacterial isolates during the growth process (Arifiyanto et al. 2020). The decrease in surface tension can affect

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Commented [mm61]: We added "*Colletotrichum*" and deleted "*C.*"

Commented [U62]: Full name specie

Commented [mm63]: We added full name species

Commented [mm64]: We added full name species

Commented [mm65]: We support our discussion about the mechanism of vector death caused by surfactin (Comment 10)

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 media control and 33.74 mN/m from the distilled water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus velezensis* ES4.3 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus velezensis* ES4.3 has the potential to 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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Commented [U67]: What is the name of your strain???

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Commented [U69]: ????? *Bacillus* sp. ES4.3????

Commented [mm70]: Yes, it's true. Because we conclude that our indigenous species name have the potential to be developed as a biocontrol in disease vector

Commented [mm71]: We added these sentences to support Comment number 45

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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

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *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 detected by isolating the DNA and visualizing 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 99.86 for % ID with the *srf*A-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 media control and 33.74 mN/m from the distilled water control. The ability of *Bacillus* sp. ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti.* Thus, *Bacillus* sp. ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

Running title: Biosurfactant Activity of *Bacillus* sp. ES4.3

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, petroleum industries, and 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, which is 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) which are 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), which is 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

49 bacterial growth, it breaks down the membranes or disintegrate it through physicochemical interactions (Deleu et al. 2013),
50 suppressing fungi by encouraging the colonization of beneficial bacteria (Jia et al. 2015), and triggers a systemic resistance
51 (Cawoy et al. 2014).
52 Detections the activity of biosurfactants can be used by (i) hemolytic activity tests, (ii) methods to analyze the surface
53 activity, emulsifying activity, and surface tension/interface tension (Plaza 2014). In addition, identification of bacterial
54 strains was also carried out, including 16S rRNA gene analysis and detection of biosynthesis surfactin gene, namely *srf*A-
55 D. This methodology ensures that phenotypic and genotypic features are considered (Das et al. 2013), provided an
56 overview of the role and importance of molecular genetics and the gene regulatory mechanisms behind surfactin
57 biosynthesis of various microbes that have commercial importance.
58 The method that commonly used for screening and genetic identification of bacterial isolates that have the potential to
59 rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in
60 surfactin biosynthesis, which called *srf*A-D (Mulligan et al. 2014). This study was to determine the name of the indigenous
61 *Bacillus* sp. ES4.3, the surfactin biosynthesis gene, and the potential activity for biosurfactant produced by *Bacillus* sp.
62 ES4.3 isolated from the breeding sites of Dengue Hemorrhagic Fever in Surabaya, East Java, Indonesia.

63 ## MATERIALS AND METHODS

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

71 **Identification of 16S rRNA gene**
72 Identification of 16S rRNA gene was initiated by culturing isolates of *Bacillus* sp. ES4.3 into 20 mL of Luria Bertani
73 medium, incubated at 35ºC with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out
74 using the CTAB method (Ausubel et al. 2003) with DNA Wizard Genomic DNA Purification Kit (Promega). DNA purity
75 and concentration values were measured using Multiskan GO on λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene
76 amplification was carried out using Eppendorf Mastercycler equipment. This process begins by adding GoTaq Green
77 Master Mix and 16S rRNA primers, in the form of primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was
78 conditioned as follows: initial denaturation of 94ºC for 2 minutes, denaturation of 92ºC for 30 seconds, annealing 55ºC for
79 30 seconds, elongation of 72ºC for 1 minute, final elongation of 72ºC for 5 minutes, 35 cycles. The PCR product were
80 visualized through an electrophoresis process using 1% agarose gel followed by Ethidium Bromide staining and observed
81 in ultraviolet light. The PCR samples were sent to the 1st Base DNA Sequencing Service Malaysia. Amplikon was
82 sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The data were also
83 analyzed for their relation by building a phylogenetic tree using MEGA version 6 software (Tamura et al. 2013).

84 **Detection of Biosynthesis Surfactin gene**
85 In this stage, we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of
86 detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is
87 the primer used. The *srf*A-D gene primers are self-designed on the page ThermoFisher Scientific Oligo Perfect Primer
88 Designer cloning application.

89 **Biosurfactant Screening Activity**
90 **Hemolytic test**
91 Hemolytic test was carried out by culturing the bacterial isolate *Bacillus* sp. ES4.3 in sterile Blood Agar medium that
92 obtained from the Surabaya Laboratory. Isolate *Bacillus* sp. ES4.3 was inoculated into the Blood Agar medium by spot
93 method. After that, it was incubated at 37ºC for 24 to 48 hours. The positive results of this test can be observed in the
94 hemolysis zone and the color changes that occur around the bacterial colony.
95 **Emulsification activity**
96 Emulsification activity was carried out to determine the ability of *Bacillus* sp. ES4.3 in emulsifying liquid
97 hydrocarbons, i.e. kerosene and diesel fuel. The bacteria were cultured in liquid NB media and incubated for 24 hours.
98 Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by with
99 kerosene and used diesel fuel in different test tubes, and homogenized using vortex at high speed for 2 minutes. Stability
100 was measured after 1 hour. Emulsification activity is determined by index calculation (E24 or EI). This calculation is done
101 through the formula by Ozdal et al. (2017).

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

E24 : emulsification activity on 24 hours
HE : high of the emultion 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).

$$g = go\frac{q}{qo}$$

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". In Figure 1. showed the band of DNA from PCR result on agarose gel 1%.

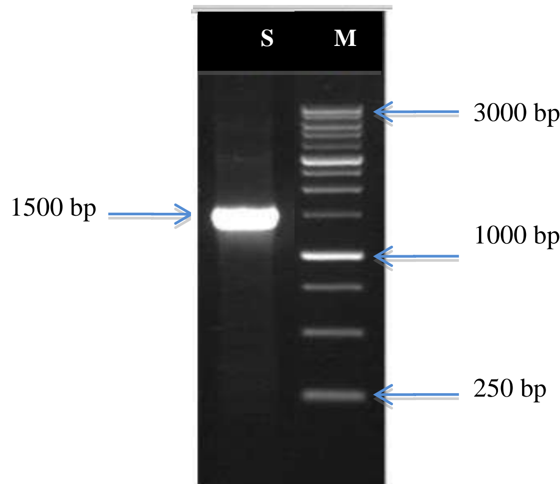


Figure 1. Electrophoresis result of DNA *Bacillus velezensis* ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker)

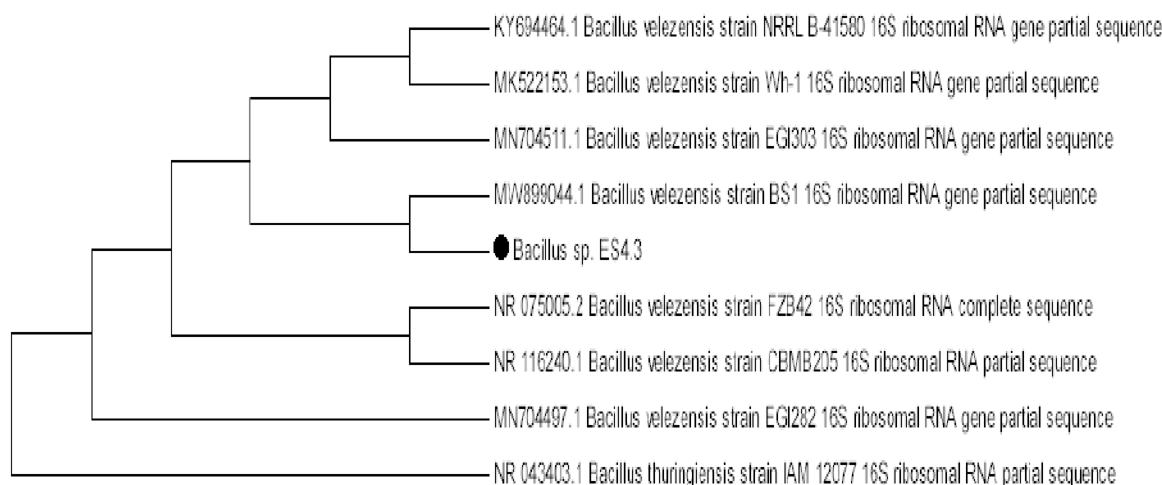
The PCR result in Figure 1. showed the band of 16S rRNA gene from *Bacillus* sp. ES4.3 isolate. When it matched with size order of DNA marker, the size of the band measuring 1500bp.

The result of nucleotide Basic Local Alignment Search Tools (BLASTn) analysis in Table 1. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

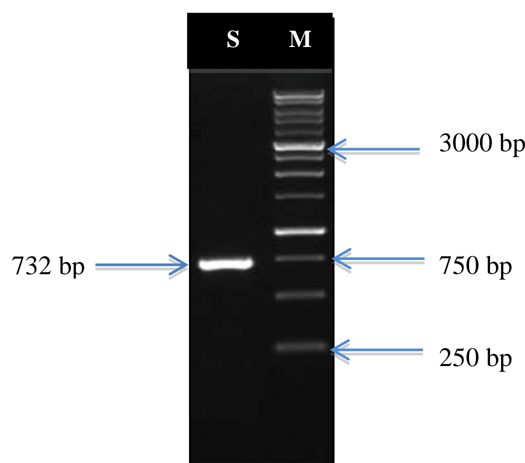
156 **Table 1.** The Results of Basic Local Alignment Search Tools (BLAST) of *Bacillus* sp. ES4.3.
157

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%

158
159 **Analysis of Phylogeni Tree**
160 Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus velezensis.*These
161 bacteria are used to calculate evolutionary distances and construct phylogenetic trees. The bootstrap test (1000
162 replications) is shown next to the branch. The consensus procedure was used to produce a bootstrap phenogram in the
163 phylogeny tree, which is analyzed by Neighbor-Join Method.
164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181



182 **Figure 2.** Phylogeni tree of *Bacillus velezensis* ES4.3 and another bacteria of *B. velezensis* strains
183
184 **Analysis of Biosynthesis Surfactin Gene**
185 Figure 3. shows that the sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to
186 determine the similarity of the *Srf*A-D gene *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted
187 to determine the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 with another nucleotide of the
188 *srf*A-D gene *Bacillus* in GenBank. Based on the BLASTp results, the amino acids in the *srf*A-D gene of *Bacillus* sp. ES4.3
189 have the highest similarity with the surfactin thioesterase *srf*A-D biosynthesis from *Bacillus amyloliquifaciens* group
190 bacteria in Genbank by 99.86%. On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B.*
191 *velezensis* belong to the same clade as a *B. amyloliquifaciens.*
192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207



208 **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
209 band measuring 732 bp. (S = Sample; M = Marker)

210 **Screening of Biosurfactant Activity**
211 **Hemolytic Activity**
212 Hemolytic activity can be identified on Blood Agar media with 24 hours observation by looking at the clear zone
213 around the microbial colonies (Carillo et al. 1996). Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4.
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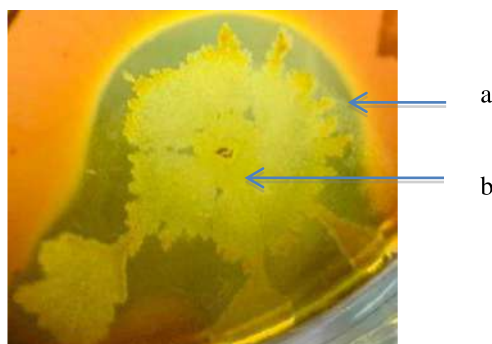

227 **Figure 4.** The clear zone is formed from the hemolytic activity of the *Bacillus* sp. ES4.3 isolate on Blood Agar media. Notes: a) halo
228 zone, b) Colony of *Bacillus* sp. ES4.3.

229 **Emulsification Activity**
230 Table 2. shows that the emulsification activity of the cell-free supernatant of *Bacillus* sp. ES4.3 used kerosene and
231 diesel fuel at 1 hour and 24 hours observation. The emulsification activity of *Bacillus* sp. ES4.3 in kerosene showed an
232 increase, while in diesel fuel it showed a decrease.
233
234 **Table 2.** Results of emulsication activity of supernatant of *Bacillus* sp. ES4.3 on kerosene and diesel fuel.
235

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

236 **Surface Tension**
237 Table 3. shows that the surface tension value of the culture supernatant *Bacillus* sp. ES4.3, when it compared with the
238 surface tension values of the distilled water control, media control, and Tween control, the value of the culture supernatant
239 of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water control, and 3.91
240 mN/m from the Tween control.
241
242 **Table 3.** The surface tension value of the culture supernatant isolate *Bacillus* sp. ES4.3 with an incubation time of 2 days.
243

Treatment	Surface Tension (mN/m)
Distilled Water Control	72.00 ± 0.00
NB Control	59.64 ± 0.12
Supernatant *Bacillus* sp. ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

244

245 **Discussion**
246 The results of DNA isolation from *Bacillus* sp. ES4.3 showed the presence of a DNA band with a size of 1500 bp when
247 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
248 DNA barcode for bacterial species. 16S rRNA gene sequencing serves as an approximation method for rapid and accurate
249 bacterial identification. A bacterium is said to represent the same genus if it has a similarity index above 95% and
250 represents the same species if it is above 97% (Srinivasan et al. 2015; Johnson et al. 2019).
251 The *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with the *Bacillus velezensis* strain FZB42 (GenBank access
252 number NR_075005.2). These results are different from conventional identification results through observations of
253 macroscopic, microscopic, and physiological characters that have been carried out by Salamun et al. (2020), which stated
254 that *Bacillus* sp. ES4.3 is *Bacillus sphaericus* with a comparable coefficient calculation (Ss) of 76.12%.
255 Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *Bacillus velezensis.* This shows
256 that *Bacillus* sp. ES4.3 has a close relationship with *B. velezensis* BS1 and *B. velezensis* FZB42. It can be seen from the

257 location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that
258 could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis*
259 BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the
260 research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and
261 siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei*, a
262 pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis*
263 *cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* isolated from lettuce. In addition,
264 according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as
265 *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study
266 of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic
267 tree, *Bacillus thuringiensis* is an outgroup.
268 The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the
269 similarity of the *srf*A-D gene in *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine
270 the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 and the nucleotide gene *srf*A-D *B. velezensis*
271 in GenBank. Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin
272 biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity
273 with 99.86%. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *Bacillus* sp. ES4.3. This
274 sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B.*
275 *velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *Bacillus* sp.
276 ES4.3, identified as *B. velezensis* FZB42. The *srf*A-D gene is known to produce thioesterase, which is presumed to be
277 involved in the lactonization process (Satpute et al. 2010).
278 The hemolytic activity can be identified by looking at the clear zone around the microbial colonies (Carillo et al. 1996).
279 Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4. Carillo et al. (1996) stated that there is a relationship
280 between hemolytic activity and biosurfactant production. In the Blood Agar medium, *Bacillus* sp. ES4.3 was inoculated.
281 The presence of hemolytic activity, which is indicated by the formation of a halo zone around the colony, means the
282 biosurfactants were produced.
283 From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis,
284 which is indicated by the visible clear zone as a result of the lysis of all red blood cells and the release of hemoglobin from
285 the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because
286 biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane
287 antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different
288 mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and
289 the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant
290 concentration is low causing osmotic lysis (Zaragoza et al. 2010). The clear zone in the Blood Agar media corresponded to
291 changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of
292 cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result,
293 biosurfactants can directly interact with membrane lipids that enter the membrane and form pores in the larvae midgut and
294 destroy the membrane through detergent-like interactions (Butko 2003).
295 The results of the emulsification activity can be seen in Table 2. These results indicate that there are differences in the
296 results of the emulsification activity of the supernatant *Bacillus* sp. ES4.3 on kerosene and diesel fuel substrates. Better
297 properties are characterized by a greater emulsion index value, which means that the surfactant has large emulsion
298 stability. The results also showed a decrease in the value of emulsification activity after 24 hours, which indicates a
299 relatively stable emulsification activity. This research proves that the biosurfactant product can be categorized as a
300 bioemulsifier. The occurrence of emulsification activity in *Bacillus* sp. ES4.3 is indicated by the formation of foam, which
301 creates a layer in the tube. The foam layer was then measured to calculate the emulsification activity value (Ni'matuzahroh
302 et al. 2017). The emulsification index value indicates the stability of the emulsion, and the line above 50% showed good
303 biosurfactant producers (Willumsen and Karlson 1997).
304 The mechanism by which surfactants work as emulgators is by reducing the tension between the surface of water and
305 oil, so that a film layer is formed on the surface of the dispersed globules phase. Hence, emulsification activity is related
306 to the parameters of biosurfactant production because it is a very good emulsifier. The indicators of biosurfactants that are
307 produced by microorganisms can be seen in the emulsification activity of the media. Emulsification activity can be seen by
308 the formation of an emulsion that looks like a bubble between the substrate and the media (Arifiyanto et al. 2020).
309 The surface tension value of the culture supernatant *Bacillus* sp. ES4.3 can be seen in Table 3. When compared with
310 the surface tension values of the distilled water control, NB media control, and tween control, the value of the culture
311 supernatant of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water
312 control, and 3.91 mN/m from the tween control. Bacteria have the potential to produce biosurfactants if they can reduce
313 the surface tension value ≥ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause
314 these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension
315 (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant
316 produced by bacterial isolates during the growth process (Arifiyanto et al. 2020). The decrease in surface tension can affect

317 the entomopathogenic activity of *Aedes aegypti* larvae which causes low oxygen underwater, so that the larvae spiracles
318 continue to open and make it death (Geetha 2010).
319 This study concluded that *Bacillus* sp. ES4.3 which was identified as *Bacillus velezensis* ES4.3, has a biosynthesis
320 surfactin gene. The biosurfactant activity was indicated by the formation of clear zones, the formation of emulsions, and a
321 decrease in surface tension in the values of 21.38 mN/m from the NB media control and 33.74 mN/m from the distilled
322 water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus velezensis* ES4.3 has
323 the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus velezensis*
324 ES4.3 has the potential to be developed as a biocontrol in disease vectors and other fields of medicine, agriculture,
325 pharmaceuticals, and waste treatments. Further research that can be done to produce surfactin include the production and
326 optimization of biosurfactant production, as well as characterization of the resulting biosurfactant product.

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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

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id
[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.
[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.
[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *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 detected by isolating the DNA and visualizing 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 99.86 for % ID with the *srf*A-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 media control and 33.74 mN/m from the distilled water control. The ability of *Bacillus* sp. ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti.* Thus, *Bacillus* sp. ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

Running title: Biosurfactant Activity of *Bacillus* sp. ES4.3

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, petroleum industries, and 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, which is 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) which are 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), which is 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

49 bacterial growth, it breaks down the membranes or disintegrate it through physicochemical interactions (Deleu et al. 2013),
50 suppressing fungi by encouraging the colonization of beneficial bacteria (Jia et al. 2015), and triggers a systemic resistance
51 (Cawoy et al. 2014).
52 Detections the activity of biosurfactants can be used by (i) hemolytic activity tests, (ii) methods to analyze the surface
53 activity, emulsifying activity, and surface tension/interface tension (Plaza 2014). In addition, identification of bacterial
54 strains was also carried out, including 16S rRNA gene analysis and detection of biosynthesis surfactin gene, namely *srf*A-
55 D. This methodology ensures that phenotypic and genotypic features are considered (Das et al. 2013), provided an
56 overview of the role and importance of molecular genetics and the gene regulatory mechanisms behind surfactin
57 biosynthesis of various microbes that have commercial importance.
58 The method that commonly used for screening and genetic identification of bacterial isolates that have the potential to
59 rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in
60 surfactin biosynthesis, which called *srf*A-D (Mulligan et al. 2014). This study was to determine the name of the indigenous
61 *Bacillus* sp. ES4.3, the surfactin biosynthesis gene, and the potential activity for biosurfactant produced by *Bacillus* sp.
62 ES4.3 isolated from the breeding sites of Dengue Hemorrhagic Fever in Surabaya, East Java, Indonesia.

63 **MATERIALS AND METHODS**

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

Commented [U1]: for

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71 **Identification of 16S rRNA gene**
72 Identification of 16S rRNA gene was initiated by culturing isolates of *Bacillus* sp. ES4.3 into 20 mL of Luria Bertani
73 medium, incubated at 35ºC with agitation at 120 rpm for 48 hours. Furthermore, to obtain DNA, extraction was carried out
74 using the CTAB method (Ausubel et al. 2003) with DNA Wizard Genomic DNA Purification Kit (Promega). DNA purity
75 and concentration values were measured using Multiskan GO on λ 260 nm and λ 280 nm. Hereafter, 16S rRNA gene
76 amplification was carried out using Eppendorf Mastercycler equipment. This process begins by adding GoTaq Green
77 Master Mix and 16S rRNA primers, in the form of primers 27F and 1492R. The Polymerase Chain Reaction (PCR) was
78 conditioned as follows: initial denaturation of 94ºC for 2 minutes, denaturation of 92ºC for 30 seconds, annealing 55ºC for
79 30 seconds, elongation of 72ºC for 1 minute, final elongation of 72ºC for 5 minutes, 35 cycles. The PCR product were
80 visualized through an electrophoresis process using 1% agarose gel followed by Ethidium Bromide staining and observed
81 in ultraviolet light. The PCR samples were sent to the 1st Base DNA Sequencing Service Malaysia. Amplikon was
82 sequenced and analyzed for similarity with GenBank data using BLASTn NCBI (Altschul et al. 1997). The data were also
83 analyzed for their relation by building a phylogenetic tree using MEGA version 6 software (Tamura et al. 2013).

84 **Detection of Biosynthesis Surfactin gene**
85 In this stage, we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of
86 detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is
87 the primer used. The *srf*A-D gene primers are self-designed on the page ThermoFisher Scientific Oligo Perfect Primer
88 Designer cloning application.

Commented [U3]: To identify surfactin gene, the same procedures for 16SrRNA identification were performed but by using *srf*A-D gene primers

89 **Biosurfactant Screening Activity**
90 **Hemolytic test**
91 Hemolytic test was carried out by culturing the bacterial isolate *Bacillus* sp. ES4.3 in sterile Blood Agar medium that
92 obtained from the Surabaya Laboratory. Isolate *Bacillus* sp. ES4.3 was inoculated into the Blood Agar medium by spot
93 method. After that, it was incubated at 37ºC for 24 to 48 hours. The positive results of this test can be observed in the
94 hemolysis zone and the color changes that occur around the bacterial colony.
95 **Emulsification activity**
96 Emulsification activity was carried out to determine the ability of *Bacillus* sp. ES4.3 in emulsifying liquid
97 hydrocarbons, i.e. kerosene and diesel fuel. The bacteria were cultured in liquid NB media and incubated for 24 hours.
98 Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by with
99 kerosene and used diesel fuel in different test tubes, and homogenized using vortex at high speed for 2 minutes. Stability
100 was measured after 1 hour. Emulsification activity is determined by index calculation (E24 or EI). This calculation is done
101 through the formula by Ozdal et al. (2017).

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

E24 : emulsification activity on 24 hours
HE : high of the emultion 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).

$$g = go \frac{q}{qo}$$

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”. In Figure 1. showed the band of DNA from PCR result on agarose gel 1%.

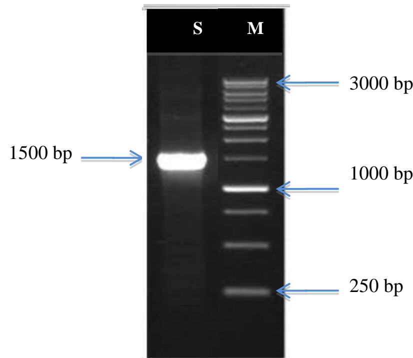

Figure 1. Electrophoresis result of DNA *Bacillus velezensis* ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker)

The PCR result 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

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%

Analysis of Phylogeni Tree

Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus 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 phylogeny tree, which is analyzed by Neighbor-Join Method.

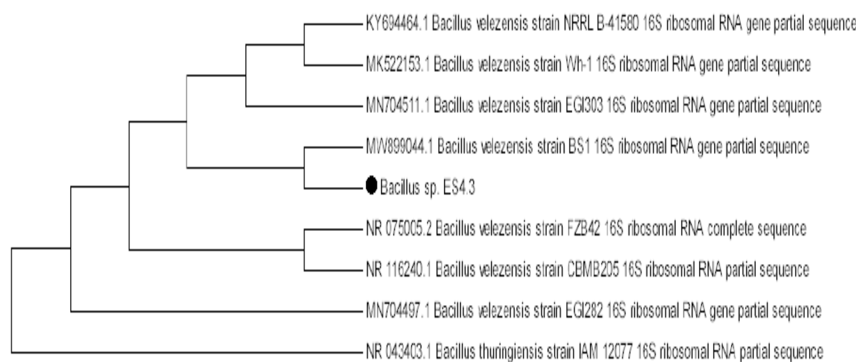

Figure 2. Phylogeni tree of *Bacillus velezensis* ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

Figure 3. shows that the sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *Srf*A-D gene *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 with another nucleotide of the *srf*A-D gene *Bacillus* in GenBank. Based on the BLASTp results, the amino acids in the *srf*A-D gene of *Bacillus* sp. ES4.3 have the highest similarity with the surfactin thioesterase *srf*A-D biosynthesis from *Bacillus amyloliquifaciens* group bacteria in Genbank by 99.86%. On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B. velezensis* belong to the same clade as a *B. amyloliquifaciens.*

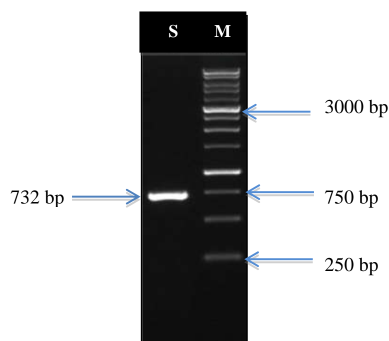

Figure 3. Electrophoresis results of the *srf*A-D gene in *Bacillus velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 732 bp. (S = Sample; M = Marker)

210 **Screening of Biosurfactant Activity**
211 **Hemolytic Activity**
212 Hemolytic activity can be identified on Blood Agar media with 24 hours observation by looking at the clear zone
213 around the microbial colonies (Carillo et al. 1996). Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4.
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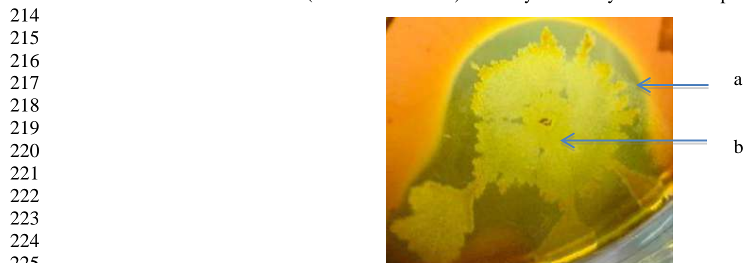

227 **Figure 4.** The clear zone is formed from the hemolytic activity of the *Bacillus* sp. ES4.3 isolate on Blood Agar media. Notes: a) halo
228 zone, b) Colony of *Bacillus* sp. ES4.3.

229 **Emulsification Activity**
230 Table 2. shows that the emulsification activity of the cell-free supernatant of *Bacillus* sp. ES4.3 used kerosene and
231 diesel fuel at 1 hour and 24 hours observation. The emulsification activity of *Bacillus* sp. ES4.3 in kerosene showed an
232 increase, while in diesel fuel it showed a decrease.
233
234 **Table 2.** Results of emulsication activity of supernatant of *Bacillus* sp. ES4.3 on kerosene and diesel fuel.
235

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

236 **Surface Tension**
237 Table 3. shows that the surface tension value of the culture supernatant *Bacillus* sp. ES4.3, when it compared with the
238 surface tension values of the distilled water control, media control, and Tween control, the value of the culture supernatant
239 of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water control, and 3.91
240 mN/m from the Tween control.
241
242 **Table 3.** The surface tension value of the culture supernatant isolate *Bacillus* sp. ES4.3 with an incubation time of 2 days.
243

Treatment	Surface Tension (mN/m)
Distilled Water Control	72.00 ± 0.00
NB Control	59.64 ± 0.12
Supernatant *Bacillus* sp. ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

244

245 **Discussion**
246 The results of DNA isolation from *Bacillus* sp. ES4.3 showed the presence of a DNA band with a size of 1500 bp when
247 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
248 DNA barcode for bacterial species. 16S rRNA gene sequencing serves as an approximation method for rapid and accurate
249 bacterial identification. A bacterium is said to represent the same genus if it has a similarity index above 95% and
250 represents the same species if it is above 97% (Srinivasan et al. 2015; Johnson et al. 2019).
251 The *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with the *Bacillus velezensis* strain FZB42 (GenBank access
252 number NR_075005.2). These results are different from conventional identification results through observations of
253 macroscopic, microscopic, and physiological characters that have been carried out by Salamun et al. (2020), which stated
254 that *Bacillus* sp. ES4.3 is *Bacillus sphaericus* with a comparable coefficient calculation (Ss) of 76.12%.
255 Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *Bacillus velezensis.* This shows
256 that *Bacillus* sp. ES4.3 has a close relationship with *B. velezensis* BS1 and *B. velezensis* FZB42. It can be seen from the

257 location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that
258 could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis*
259 BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the
260 research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and
261 siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei*, a
262 pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis*
263 *cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* isolated from lettuce. In addition,
264 according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as
265 *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study
266 of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic
267 tree, *Bacillus thuringiensis* is an outgroup.
268 The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the
269 similarity of the *srf*A-D gene in *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine
270 the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 and the nucleotide gene *srf*A-D *B. velezensis*
271 in GenBank. Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin
272 biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity
273 with 99.86%. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *Bacillus* sp. ES4.3. This
274 sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B.*
275 *velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *Bacillus* sp.
276 ES4.3, identified as *B. velezensis* FZB42. The *srf*A-D gene is known to produce thioesterase, which is presumed to be
277 involved in the lactonization process (Satpute et al. 2010).
278 The hemolytic activity can be identified by looking at the clear zone around the microbial colonies (Carillo et al. 1996).
279 Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4. Carillo et al. (1996) stated that there is a relationship
280 between hemolytic activity and biosurfactant production. In the Blood Agar medium, *Bacillus* sp. ES4.3 was inoculated.
281 The presence of hemolytic activity, which is indicated by the formation of a halo zone around the colony, means the
282 biosurfactants were produced.
283 From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis,
284 which is indicated by the visible clear zone as a result of the lysis of all red blood cells and the release of hemoglobin from
285 the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because
286 biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane
287 antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different
288 mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and
289 the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant
290 concentration is low causing osmotic lysis (Zaragoza et al. 2010). The clear zone in the Blood Agar media corresponded to
291 changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of
292 cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result,
293 biosurfactants can directly interact with membrane lipids that enter the membrane and form pores in the larvae midgut and
294 destroy the membrane through detergent-like interactions (Butko 2003).
295 The results of the emulsification activity can be seen in Table 2. These results indicate that there are differences in the
296 results of the emulsification activity of the supernatant *Bacillus* sp. ES4.3 on kerosene and diesel fuel substrates. Better
297 properties are characterized by a greater emulsion index value, which means that the surfactant has large emulsion
298 stability. The results also showed a decrease in the value of emulsification activity after 24 hours, which indicates a
299 relatively stable emulsification activity. This research proves that the biosurfactant product can be categorized as a
300 bioemulsifier. The occurrence of emulsification activity in *Bacillus* sp. ES4.3 is indicated by the formation of foam, which
301 creates a layer in the tube. The foam layer was then measured to calculate the emulsification activity value (Ni'matuzahroh
302 et al. 2017). The emulsification index value indicates the stability of the emulsion, and the line above 50% showed good
303 biosurfactant producers (Willumsen and Karlson 1997).
304 The mechanism by which surfactants work as emulgators is by reducing the tension between the surface of water and
305 oil, so that a film layer is formed on the surface of the dispersed globules phase. Hence, emulsification activity is related
306 to the parameters of biosurfactant production because it is a very good emulsifier. The indicators of biosurfactants that are
307 produced by microorganisms can be seen in the emulsification activity of the media. Emulsification activity can be seen by
308 the formation of an emulsion that looks like a bubble between the substrate and the media (Arifiyanto et al. 2020).
309 The surface tension value of the culture supernatant *Bacillus* sp. ES4.3 can be seen in Table 3. When compared with
310 the surface tension values of the distilled water control, NB media control, and tween control, the value of the culture
311 supernatant of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water
312 control, and 3.91 mN/m from the tween control. Bacteria have the potential to produce biosurfactants if they can reduce
313 the surface tension value ≥ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause
314 these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension
315 (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant
316 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 media control and 33.74 mN/m from the distilled water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus velezensis* ES4.3 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus velezensis* ES4.3 has the potential to 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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Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *Nafidiastri FA, Susetyo~~i~~ RD, Nurhariyati T, Supriyanto A, G~~H~~eraldi A, Ni~~u~~'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: xxxx.* *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 detected by isolating the DNA and visualizing 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 ~~99.86~~100 for % ID with the surfactin biosynthesis thioesterase S~~s~~rfA-D gene on the *Bacillus* ~~*amyloliquefaciens*~~ *amyloliquefaciens* group. The biosurfactant activity 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~~media~~ control and 33.74 mN/m from the distilled water control. The ability of ~~*Bacillus* sp.~~*B. velezensis* ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti*. Thus, ~~*Bacillus* sp.~~*B. velezensis* ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

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, petroleum industries, and 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, which is 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) which are 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), which is 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

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membranes or disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 ~~medium~~ 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 ~~on~~ for biosurfactant activity. The three ~~medium~~ 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 medium 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 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 product were 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. Amplikon 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).

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Detection of Biosynthesis Surfactin gene

In this stage, ~~we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is the primer used.~~ to identify surfactine gene, the same procedures for 16S rRNA identification were performed but by 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

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Hemolytic test was carried out by culturing the bacterial isolate *Bacillus* sp. ES4.3 in sterile Blood Agar medium that 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

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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 ~~media~~ medium and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by 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 ~~or EI~~). This calculation is done through the formula by Ozdal et al. (2017).

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

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

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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).

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

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". In 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

Analysis of Phylogenetic~~i~~ Tree

Figure 2. showed~~s~~ the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus 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~~y~~ tree, which is analyzed by Neighbor-Join Method.

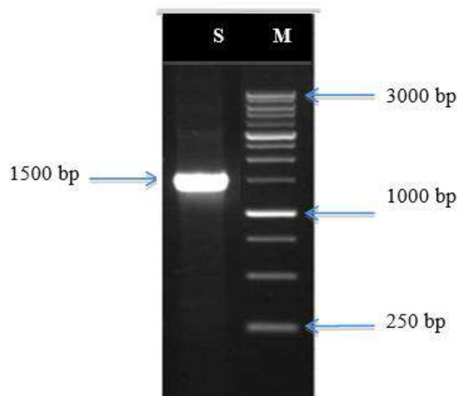

Figure 1. Electrophoresis result of DNA *Bacillus* ~~velezensis~~ sp. ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker).

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%

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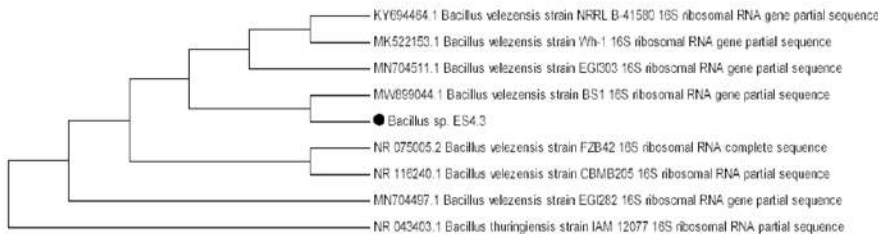

Figure 2. Phylogeneticı tree of *Bacillus* sp. *velezensis* ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

Figure 3. shows that Tthe sequencing results that obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the sS*rf*A-D gene *Bacillus* sp.*B. velezensis* ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the nucleotides protein of the *srf*A-D gene *B. velezensisBacillus* sp. ES4.3 with another nucleotide 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 amino acidsprotein in the *srf*A-D gene of *B. velezensisBacillus* sp. ES4.3 have the highest similarity with the surfactin biosynthesis thioesterase SsrfA-D biosynthesis from *Bacillus amyloliquifaciens amyloliquefaciens* group bacteria in Genbank by 99.86100% (GenBank access number WP_003156383.1). On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B. velezensis* belong to the same clade as a *B. amyloliquifaciens.amyloliquefaciens.*

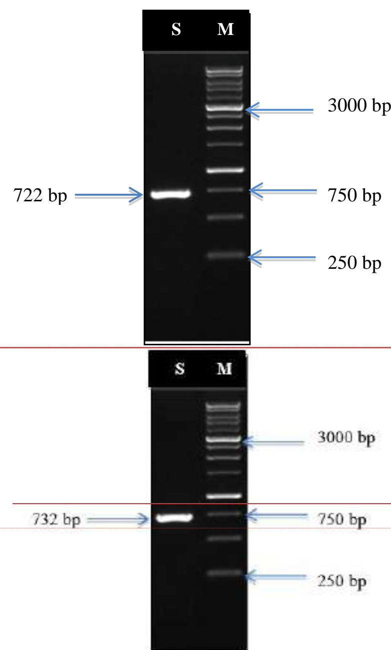

Figure 3. Electrophoresis results of the *srf*A-D gene in *Bacillus B. velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 7232 bp. (S = Sample; M = Marker)

Screening of Biosurfactant Activity

Hemolytic Activity

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

Emulsification Activity

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

Surface Tension

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

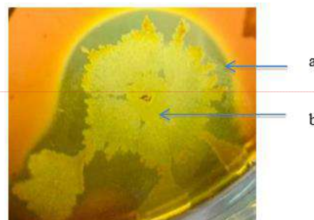

Figure 4. The clear zone is formed from the hemolytic activity of the *B. velezensis Bacillus* sp. ES4.3 isolate on Blood Agar

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medium~~a~~. Notes: a) halo zone, b) Colony of *B. velezensis*~~*Bacillus* sp.~~ ES4.3.

Table 2. Results of emulsication activity of supernatant of *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 on kerosene and diesel fuel.

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

Table 3. The surface tension value of the culture supernatant isolate ~~*Bacillus* sp~~*B. 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 ~~*Bacillus* sp.~~*B. velezensis* ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

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 is said to represent 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 *Bacillus 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 that have been 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 2. show~~s~~ed the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of ~~*Bacillus*~~ *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*, 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* 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 ~~obtained~~ were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *srf*A-D gene in *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the ~~nucleotides~~ protein of the *srf*A-D gene *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 and another~~the~~ ~~nucleotide~~ protein of the gene *srf*A-D *Bacillus* ~~*B. velezensis*~~ 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 *Bacillus amyloliquefaciens* group bacteria in Genbank. ~~Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity with 99.86%.~~ Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of ~~*Bacillus* sp.~~*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* ~~*Bacillus* sp.~~ ES4.3, identified as *B. velezensis* Htq6, with a successfully amplified size of 722 bp. ~~FZB42.~~ 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*~~*Bacillus* sp.~~ 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* ~~*Bacillus* sp.~~ 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 visible clear 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 mediummedia 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 and 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 that there are differences in the results of the emulsification activity of the supernatant *B. velezensis Bacillus* sp. 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 Bacillus* sp. 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 Bacillus* sp. ES4.3 can be seen in Table 3. When compared with the surface tension values of the distilled water control, NB medium media control, and Ttween control, the value of the culture supernatant of this isolate decreased to 21.38 mN/m from the NB media medium control, 33.74 mN/m from the distilled water control, and 3.91 mN/m from the Ttween control. Bacteria have the potential to 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 media medium control and 33.74 mN/m from the distilled water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus B. velezensis* ES4.3 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus B. velezensis* ES4.3 has the potential to 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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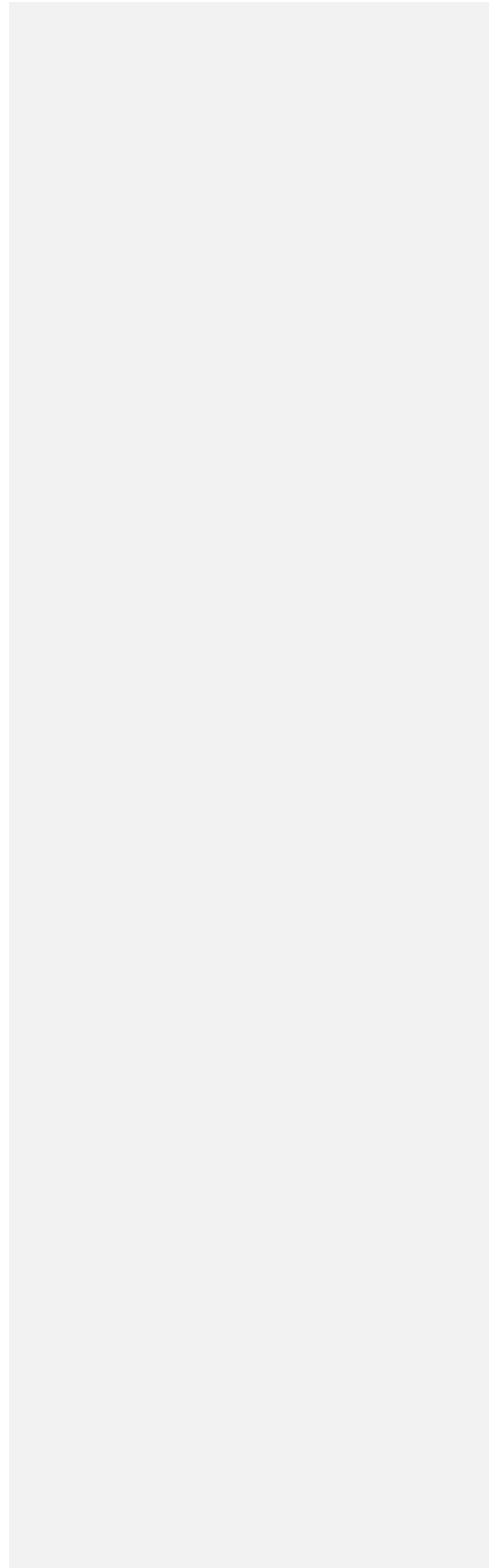

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Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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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: xxxx.* *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 detected by isolating the DNA and visualizing 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 for % ID with the surfactin biosynthesis thioesterase SrfA-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 ability of *B. velezensis* ES4.3 to hemolyzed and reduced 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 vector.

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

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, petroleum industries, and 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, which is 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) which are 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), which is 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 disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 medium 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 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 product were 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. Amplikon 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 but by 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 that 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 by 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).

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

E24 : emulsification activity on 24 hours
HE : high of the emultion 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).

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

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". In 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it 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 *Bacillus 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, which is analyzed by Neighbor-Join Method.

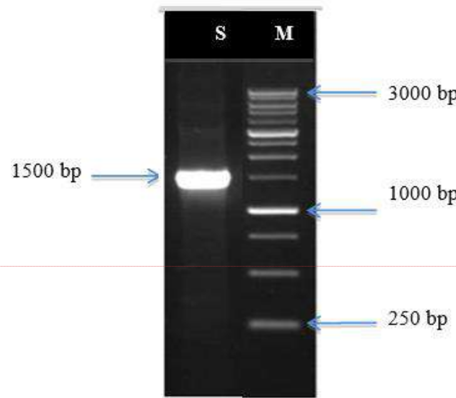

Figure 1. Electrophoresis result of DNA *Bacillus* sp. ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker).

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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%

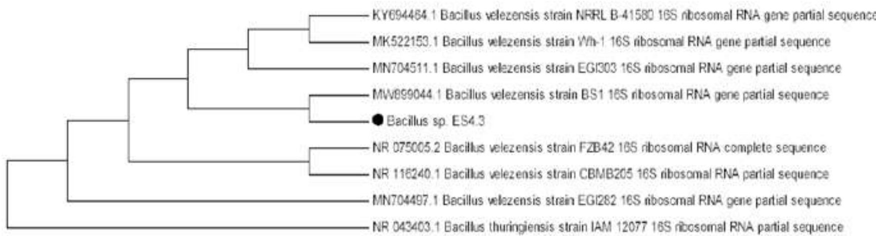

Figure 2. Phylogenetic tree of *Bacillus* sp. ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

The sequencing results that 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 protein of the *srf*A-D gene *B. velezensis* ES4.3 with 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 protein in the *srf*A-D gene of *B. velezensis* ES4.3 have 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), 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 showed a decrease.

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.

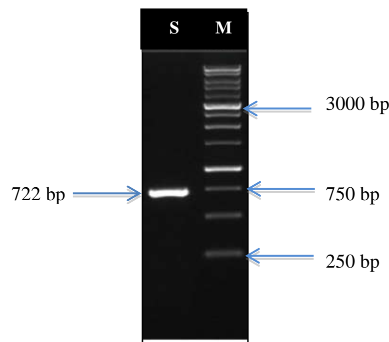

Figure 3. Electrophoresis results of the *srf*A-D gene in *B. velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 722 bp. (S = Sample; M = Marker)

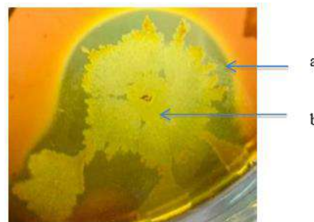

Figure 4. The clear zone is formed from the hemolytic activity of the *B. velezensis* ES4.3 isolate on Blood Agar medium. Notes: a) halo zone, b) Colony of *B. velezensis* ES4.3.

Table 2. Results of emulsication activity of supernatant of *B. 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

Table 3. The surface tension value of the culture supernatant isolate *B. 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

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

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gene sequencing serves as an approximation method for rapid and accurate bacterial identification. A bacterium is said to represent 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 *Bacillus 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 that have been 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 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*, 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* 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 protein of 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 *Bacillus 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 visible clear 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 and 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 that there are 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 have the potential to 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 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *B. velezensis* ES4.3 has the potential to 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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Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id
[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.
[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.
[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *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 detected by isolating the DNA and visualizing 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 99.86 for % ID with the *srf*A-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 media control and 33.74 mN/m from the distilled water control. The ability of *Bacillus* sp. ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti*. Thus, *Bacillus* sp. ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

Running title: Biosurfactant Activity of *Bacillus* sp. ES4.3

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, petroleum industries, and 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, which is 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) which are 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), which is 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

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bacterial growth, it breaks down the membranes or disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 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 in Surabaya, East Java, Indonesia in the previous research (Salamun et al. 2020). We used three medium 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 on biosurfactant activity. The three medium were prepared with distilled water and sterilized using an autoclave at 121°C 1 atm. *Bacillus* sp. ES4.3 was cultured on the different three medium and incubated at 35°C 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 35°C 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 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 product were 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. Amplikon 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, we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is the primer used. 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 that 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 37°C 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 media and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by 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 or EI). This calculation is done through the formula by Ozdal et al. (2017).

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102 $$E24 = \frac{HE}{HS} \; x \quad 100\%$$

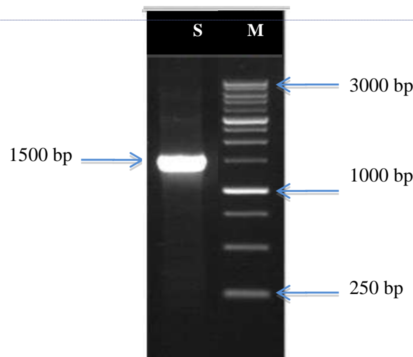
103
104 E24 : emulsification activity on 24 hours
105 HE : high of the emultion layer
106 HS : high of total solution
107
108 **Surface tension**
109 The surface tension of the cell-free culture supernatant obtained by centrifugation was measured using the Kruss 100
110 tensiometer (Kruss GmbH, Hamburg, Germany) by the Du Nouy ring method. Measurements were replicated 3x to
111 improve accuracy and average retrieval. This calculation is done through the formula by Chauhan et al. (2013).
112

113 $$g = go\frac{q}{qo}$$

114
115 γ : the surface tension of the sample
116 γ_o : surface tension standard value of distilled water at t°C
117 θ : the indicated sample value according to the instrument scale
118 θ_o : distilled water value shown according to the instrument scale

119 **RESULTS AND DISCUSSION**

120 **Analysis of 16S rRNA gene**
121 Isolate of *Bacillus* sp. ES4.3 was identified by amplification and sequencing of the 16S rRNA gene using PCR. The
122 sequences of *Bacillus* sp. ES4.3 DNA was analyzed using BioEdit Sequence Alignment Editor software version 7.2.5. and
123 nucleotide Basic Local Alignment Search Tool (BLASTn) that followed by the website of the National Center for
124 Biotechnology Information (NCBI) "http://www.ncbi.nlm.nih.gov". In Figure 1. showed the band of DNA from PCR
125 result on agarose gel 1%.
126
127–142



143 **Figure 1.** Electrophoresis result of DNA *Bacillus velezensis* ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M =
144 Marker)
145
146 The PCR result in Figure 1. showed the band of 16S rRNA gene from *Bacillus* sp. ES4.3 isolate. When it matched with
147 size order of DNA marker, the size of the band measuring 1500bp.
148 The result of nucleotide Basic Local Alignment Search Tools (BLASTn) analysis in Table 1. indicate that this isolate
149 as the *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with *Bacillus velezensis* strain FZB42 (GenBank access number
150 NR_075005.2). This is because the results in Table 1. show the highest % ID is *Bacillus velezensis* strain FZB42 when it
151 compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.
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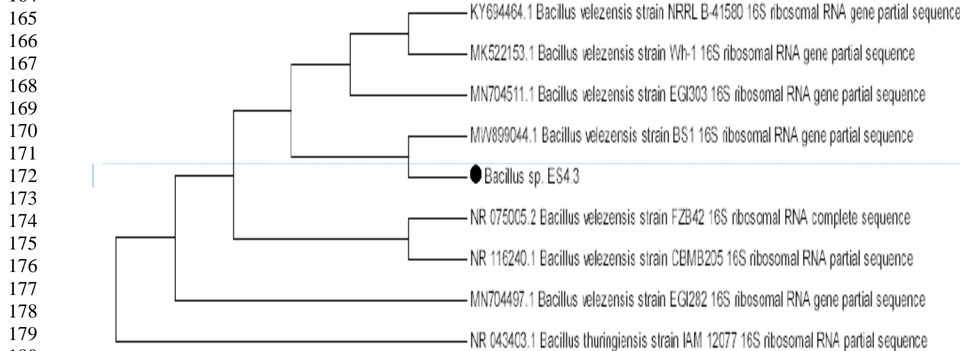
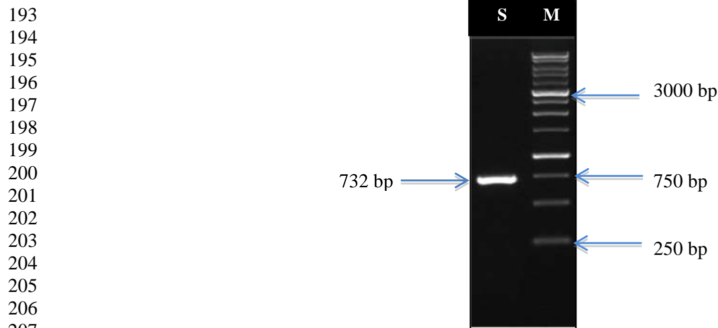
156 **Table 1.** The Results of Basic Local Alignment Search Tools (BLAST) of *Bacillus* sp. ES4.3.
157

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%

158
159 **Analysis of Phylogeni Tree**
160 Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus velezensis*.These
161 bacteria are used to calculate evolutionary distances and construct phylogenetic trees. The bootstrap test (1000
162 replications) is shown next to the branch. The consensus procedure was used to produce a bootstrap phenogram in the
163 phylogeny tree, which is analyzed by Neighbor-Join Method.
164–181



182 **Figure 2.** Phylogeni tree of *Bacillus velezensis* ES4.3 and another bacteria of *B. velezensis* strains
183
184 **Analysis of Biosynthesis Surfactin Gene**
185 Figure 3. shows that the sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to
186 determine the similarity of the *Srf*A-D gene *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted
187 to determine the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 with another nucleotide of the
188 *srf*A-D gene *Bacillus* in GenBank. Based on the BLASTp results, the amino acids in the *srf*A-D gene of *Bacillus* sp. ES4.3
189 have the highest similarity with the surfactin thioesterase *srf*A-D biosynthesis from *Bacillus amyloliquifaciens* group
190 bacteria in Genbank by 99.86%. On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B.*
191 *velezensis* belong to the same clade as a *B. amyloliquifaciens.*
192–207



208 **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
209 band measuring 732 bp. (S = Sample; M = Marker)

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210 **Screening of Biosurfactant Activity**
211 **Hemolytic Activity**
212 Hemolytic activity can be identified on Blood Agar media with 24 hours observation by looking at the clear zone
213 around the microbial colonies (Carillo et al. 1996). Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4.
214–226

227 **Figure 4.** The clear zone is formed from the hemolytic activity of the *Bacillus* sp. ES4.3 isolate on Blood Agar media. Notes: a) halo
228 zone, b) Colony of *Bacillus* sp. ES4.3.

229 **Emulsification Activity**
230 Table 2. shows that the emulsification activity of the cell-free supernatant of *Bacillus* sp. ES4.3 used kerosene and
231 diesel fuel at 1 hour and 24 hours observation. The emulsification activity of *Bacillus* sp. ES4.3 in kerosene showed an
232 increase, while in diesel fuel it showed a decrease.
233
234 **Table 2.** Results of emulsication activity of supernatant of *Bacillus* sp. ES4.3 on kerosene and diesel fuel.
235

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

236 **Surface Tension**
237 Table 3. shows that the surface tension value of the culture supernatant *Bacillus* sp. ES4.3, when it compared with the
238 surface tension values of the distilled water control, media control, and Tween control, the value of the culture supernatant
239 of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water control, and 3.91
240 mN/m from the Tween control.
241
242 **Table 3.** The surface tension value of the culture supernatant isolate *Bacillus* sp. ES4.3 with an incubation time of 2 days.
243

Treatment	Surface Tension (mN/m)
Distilled Water Control	72.00 ± 0.00
NB Control	59.64 ± 0.12
Supernatant *Bacillus* sp. ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

244

245 **Discussion**
246 The results of DNA isolation from *Bacillus* sp. ES4.3 showed the presence of a DNA band with a size of 1500 bp when
247 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
248 DNA barcode for bacterial species. 16S rRNA gene sequencing serves as an approximation method for rapid and accurate
249 bacterial identification. A bacterium is said to represent the same genus if it has a similarity index above 95% and
250 represents the same species if it is above 97% (Srinivasan et al. 2015; Johnson et al. 2019).
251 The *Bacillus* sp. ES4.3 isolate shares 97.66% similarity with the *Bacillus velezensis* strain FZB42 (GenBank access
252 number NR_075005.2). These results are different from conventional identification results through observations of
253 macroscopic, microscopic, and physiological characters that have been carried out by Salamun et al. (2020), which stated
254 that *Bacillus* sp. ES4.3 is *Bacillus sphaericus* with a comparable coefficient calculation (Ss) of 76.12%.
255 Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *Bacillus velezensis.* This shows
256 that *Bacillus* sp. ES4.3 has a close relationship with *B. velezensis* BS1 and *B. velezensis* FZB42. It can be seen from the

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Commented [mm54]: We still use mN/m because it is the unit (international standard) of surface tension
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257 location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that
258 could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis*
259 BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the
260 research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and
261 siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei*, a
262 pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis*
263 *cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* isolated from lettuce. In addition,
264 according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as
265 *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study
266 of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic
267 tree, *Bacillus thuringiensis* is an outgroup.

268 The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the
269 similarity of the *srf*A-D gene in *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine
270 the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 and the nucleotide gene *srf*A-D *B. velezensis*
271 in GenBank. Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin
272 biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity
273 with 99.86%. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *Bacillus* sp. ES4.3. This
274 sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B.*
275 *velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *Bacillus* sp.
276 ES4.3, identified as *B. velezensis* FZB42. The *srf*A-D gene is known to produce thioesterase, which is presumed to be
277 involved in the lactonization process (Satpute et al. 2010).

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

283 From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis,
284 which is indicated by the visible clear zone as a result of the lysis of all red blood cells and the release of hemoglobin from
285 the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because
286 biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane
287 antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different
288 mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and
289 the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant
290 concentration is low causing osmotic lysis (Zaragoza et al. 2010). The clear zone in the Blood Agar media corresponded to
291 changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of
292 cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result,
293 biosurfactants can directly interact with membrane lipids that enter the membrane and form pores in the larvae midgut and
294 destroy the membrane through detergent-like interactions (Butko 2003).

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

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

309 The surface tension value of the culture supernatant *Bacillus* sp. ES4.3 can be seen in Table 3. When compared with
310 the surface tension values of the distilled water control, NB media control, and tween control, the value of the culture
311 supernatant of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water
312 control, and 3.91 mN/m from the tween control. Bacteria have the potential to produce biosurfactants if they can reduce
313 the surface tension value ≥ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause
314 these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension
315 (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant
316 produced by bacterial isolates during the growth process (Arifiyanto et al. 2020). The decrease in surface tension can affect

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Commented [mm61]: We added "*Colletotrichum*" and deleted "*C.*"

Commented [U62]: Full name specie

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Commented [mm65]: We support our discussion about the mechanism of vector death caused by surfactin (Comment 10)

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 media control and 33.74 mN/m from the distilled water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus velezensis* ES4.3 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus velezensis* ES4.3 has the potential to 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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Commented [U67]: What is the name of your strain???

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Commented [U69]: ????? *Bacillus* sp. ES4.3????

Commented [mm70]: Yes, it's true. Because we conclude that our indigenous species name have the potential to be developed as a biocontrol in disease vector

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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

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *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 detected by isolating the DNA and visualizing 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 99.86 for % ID with the *srf*A-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 media control and 33.74 mN/m from the distilled water control. The ability of *Bacillus* sp. ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti.* Thus, *Bacillus* sp. ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

Running title: Biosurfactant Activity of *Bacillus* sp. ES4.3

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, petroleum industries, and 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, which is 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) which are 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), which is 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 disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 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 in Surabaya, East Java, Indonesia in the previous research (Salamun et al. 2020). We used three medium 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 on biosurfactant activity. The three medium were prepared with distilled water and sterilized using an autoclave at 121ºC 1 atm. *Bacillus* sp. ES4.3 was cultured on the different three medium 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 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 product were 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. Amplikon 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, we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is the primer used. 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 that 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 media and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by 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 or EI). This calculation is done through the formula by Ozdal et al. (2017).

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

E24 : emulsification activity on 24 hours
HE : high of the emultion 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).

$$g = go\frac{q}{qo}$$

γ : the surface tension of the sample
γ_o : surface tension standard value of distilled water at t^oC
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". In Figure 1. showed the band of DNA from PCR result on agarose gel 1%.

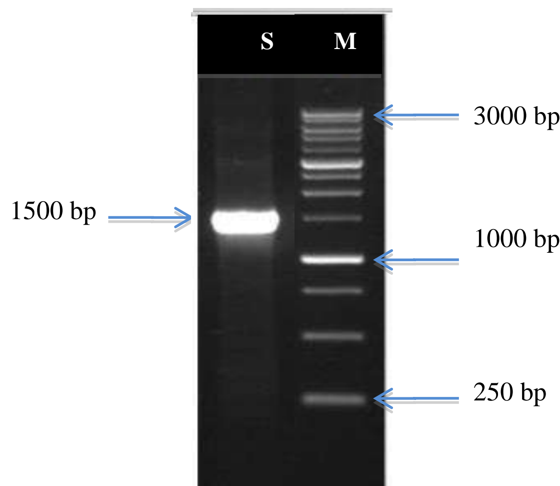

Figure 1. Electrophoresis result of DNA *Bacillus velezensis* ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker)

The PCR result 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

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%

Analysis of Phylogeni Tree

Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus 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 phylogeny tree, which is analyzed by Neighbor-Join Method.

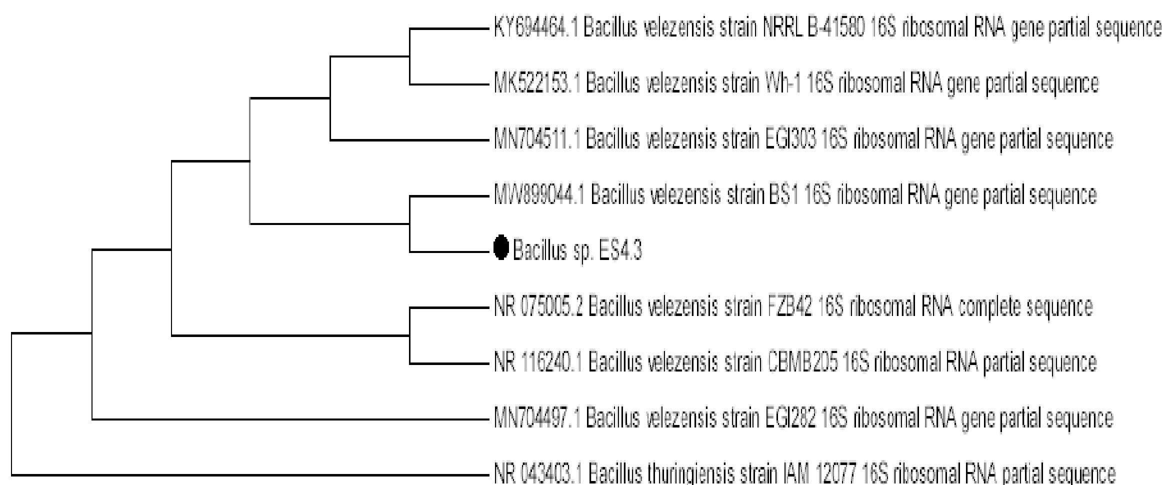

Figure 2. Phylogeni tree of *Bacillus velezensis* ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

Figure 3. shows that the sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *Srf*A-D gene *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 with another nucleotide of the *srf*A-D gene *Bacillus* in GenBank. Based on the BLASTp results, the amino acids in the *srf*A-D gene of *Bacillus* sp. ES4.3 have the highest similarity with the surfactin thioesterase *srf*A-D biosynthesis from *Bacillus amyloliquifaciens* group bacteria in Genbank by 99.86%. On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B. velezensis* belong to the same clade as a *B. amyloliquifaciens.*

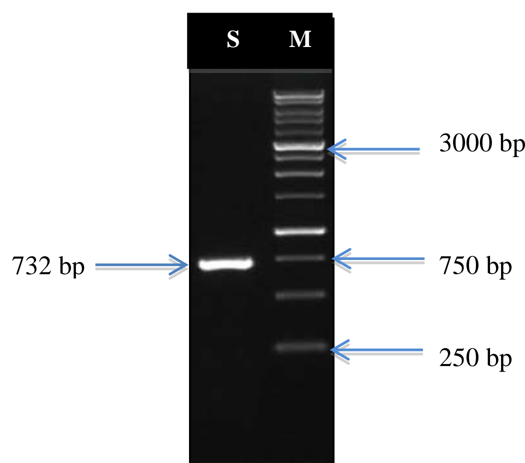

Figure 3. Electrophoresis results of the *srf*A-D gene in *Bacillus velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 732 bp. (S = Sample; M = Marker)

Screening of Biosurfactant Activity

Hemolytic Activity

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

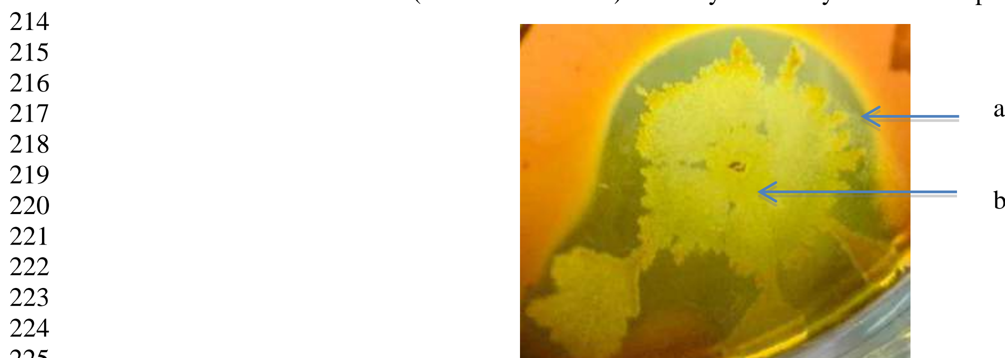

Figure 4. The clear zone is formed from the hemolytic activity of the *Bacillus* sp. ES4.3 isolate on Blood Agar media. Notes: a) halo zone, b) Colony of *Bacillus* sp. ES4.3.

Emulsification Activity

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

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

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

Surface Tension

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

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

Treatment	Surface Tension (mN/m)
Distilled Water Control	72.00 ± 0.00
NB Control	59.64 ± 0.12
Supernatant *Bacillus* sp. ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

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 is said to represent 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 *Bacillus 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 that have been 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 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *Bacillus 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

257 location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that
258 could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis*
259 BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the
260 research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and
261 siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei*, a
262 pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis*
263 *cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* isolated from lettuce. In addition,
264 according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as
265 *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study
266 of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic
267 tree, *Bacillus thuringiensis* is an outgroup.
268 The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the
269 similarity of the *srf*A-D gene in *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine
270 the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 and the nucleotide gene *srf*A-D *B. velezensis*
271 in GenBank. Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin
272 biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity
273 with 99.86%. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *Bacillus* sp. ES4.3. This
274 sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B.*
275 *velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *Bacillus* sp.
276 ES4.3, identified as *B. velezensis* FZB42. The *srf*A-D gene is known to produce thioesterase, which is presumed to be
277 involved in the lactonization process (Satpute et al. 2010).
278 The hemolytic activity can be identified by looking at the clear zone around the microbial colonies (Carillo et al. 1996).
279 Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4. Carillo et al. (1996) stated that there is a relationship
280 between hemolytic activity and biosurfactant production. In the Blood Agar medium, *Bacillus* sp. ES4.3 was inoculated.
281 The presence of hemolytic activity, which is indicated by the formation of a halo zone around the colony, means the
282 biosurfactants were produced.
283 From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis,
284 which is indicated by the visible clear zone as a result of the lysis of all red blood cells and the release of hemoglobin from
285 the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because
286 biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane
287 antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different
288 mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and
289 the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant
290 concentration is low causing osmotic lysis (Zaragoza et al. 2010). The clear zone in the Blood Agar media corresponded to
291 changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of
292 cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result,
293 biosurfactants can directly interact with membrane lipids that enter the membrane and form pores in the larvae midgut and
294 destroy the membrane through detergent-like interactions (Butko 2003).
295 The results of the emulsification activity can be seen in Table 2. These results indicate that there are differences in the
296 results of the emulsification activity of the supernatant *Bacillus* sp. ES4.3 on kerosene and diesel fuel substrates. Better
297 properties are characterized by a greater emulsion index value, which means that the surfactant has large emulsion
298 stability. The results also showed a decrease in the value of emulsification activity after 24 hours, which indicates a
299 relatively stable emulsification activity. This research proves that the biosurfactant product can be categorized as a
300 bioemulsifier. The occurrence of emulsification activity in *Bacillus* sp. ES4.3 is indicated by the formation of foam, which
301 creates a layer in the tube. The foam layer was then measured to calculate the emulsification activity value (Ni'matuzahroh
302 et al. 2017). The emulsification index value indicates the stability of the emulsion, and the line above 50% showed good
303 biosurfactant producers (Willumsen and Karlson 1997).
304 The mechanism by which surfactants work as emulgators is by reducing the tension between the surface of water and
305 oil, so that a film layer is formed on the surface of the dispersed globules phase. Hence, emulsification activity is related
306 to the parameters of biosurfactant production because it is a very good emulsifier. The indicators of biosurfactants that are
307 produced by microorganisms can be seen in the emulsification activity of the media. Emulsification activity can be seen by
308 the formation of an emulsion that looks like a bubble between the substrate and the media (Arifiyanto et al. 2020).
309 The surface tension value of the culture supernatant *Bacillus* sp. ES4.3 can be seen in Table 3. When compared with
310 the surface tension values of the distilled water control, NB media control, and tween control, the value of the culture
311 supernatant of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water
312 control, and 3.91 mN/m from the tween control. Bacteria have the potential to produce biosurfactants if they can reduce
313 the surface tension value $\geq$ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause
314 these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension
315 (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant
316 produced by bacterial isolates during the growth process (Arifiyanto et al. 2020). The decrease in surface tension can affect

317 the entomopathogenic activity of *Aedes aegypti* larvae which causes low oxygen underwater, so that the larvae spiracles
318 continue to open and make it death (Geetha 2010).
319 This study concluded that *Bacillus* sp. ES4.3 which was identified as *Bacillus velezensis* ES4.3, has a biosynthesis
320 surfactin gene. The biosurfactant activity was indicated by the formation of clear zones, the formation of emulsions, and a
321 decrease in surface tension in the values of 21.38 mN/m from the NB media control and 33.74 mN/m from the distilled
322 water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus velezensis* ES4.3 has
323 the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus velezensis*
324 ES4.3 has the potential to be developed as a biocontrol in disease vectors and other fields of medicine, agriculture,
325 pharmaceuticals, and waste treatments. Further research that can be done to produce surfactin include the production and
326 optimization of biosurfactant production, as well as characterization of the resulting biosurfactant product.

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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

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *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 detected by isolating the DNA and visualizing 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 99.86 for % ID with the *srf*A-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 media control and 33.74 mN/m from the distilled water control. The ability of *Bacillus* sp. ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti.* Thus, *Bacillus* sp. ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

Running title: Biosurfactant Activity of *Bacillus* sp. ES4.3

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, petroleum industries, and 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, which is 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) which are 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), which is 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 disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 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 in Surabaya, East Java, Indonesia in the previous research (Salamun et al. 2020). We used three medium 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 on biosurfactant activity. The three medium were prepared with distilled water and sterilized using an autoclave at 121ºC 1 atm. *Bacillus* sp. ES4.3 was cultured on the different three medium 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 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 product were 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. Amplikon 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, we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is the primer used. 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 that 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 media and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by 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 or EI). This calculation is done through the formula by Ozdal et al. (2017).

Commented [U1]: for

Commented [U2]: media

Commented [U3]: To identify surfactin gene, the same procedures for 16SrRNA identification were performed but by using *srf*A-D gene primers

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

E24 : emulsification activity on 24 hours
HE : high of the emultion 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).

$$g = go\frac{q}{qo}$$

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". In Figure 1. showed the band of DNA from PCR result on agarose gel 1%.

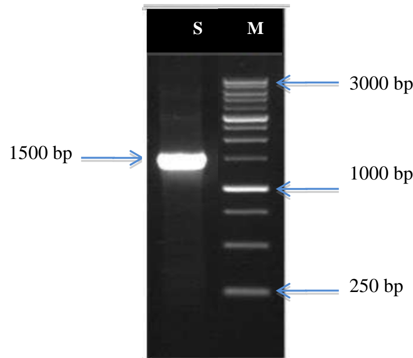

Figure 1. Electrophoresis result of DNA *Bacillus velezensis* ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker)

The PCR result 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

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%

Analysis of Phylogeni Tree

Figure 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus 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 phylogeny tree, which is analyzed by Neighbor-Join Method.

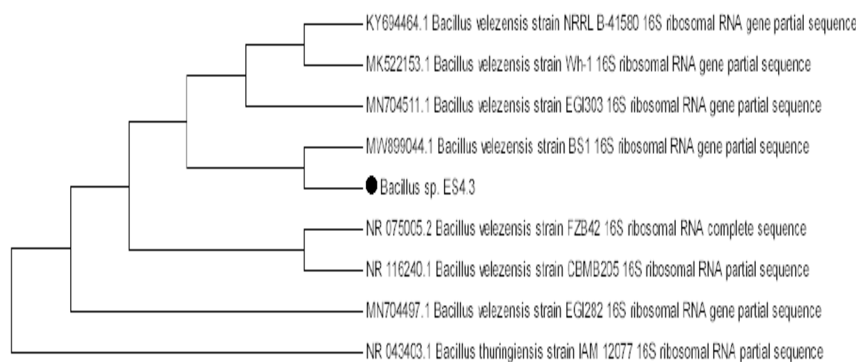

Figure 2. Phylogeni tree of *Bacillus velezensis* ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

Figure 3. shows that the sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *Srf*A-D gene *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 with another nucleotide of the *srf*A-D gene *Bacillus* in GenBank. Based on the BLASTp results, the amino acids in the *srf*A-D gene of *Bacillus* sp. ES4.3 have the highest similarity with the surfactin thioesterase *srf*A-D biosynthesis from *Bacillus amyloliquifaciens* group bacteria in Genbank by 99.86%. On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B. velezensis* belong to the same clade as a *B. amyloliquifaciens.*

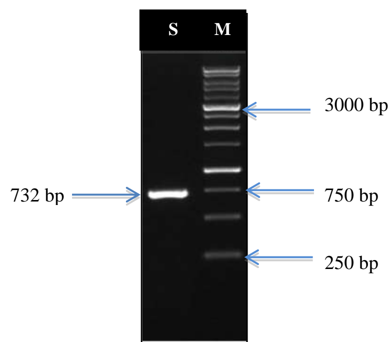

Figure 3. Electrophoresis results of the *srf*A-D gene in *Bacillus velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 732 bp. (S = Sample; M = Marker)

Screening of Biosurfactant Activity

Hemolytic Activity

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

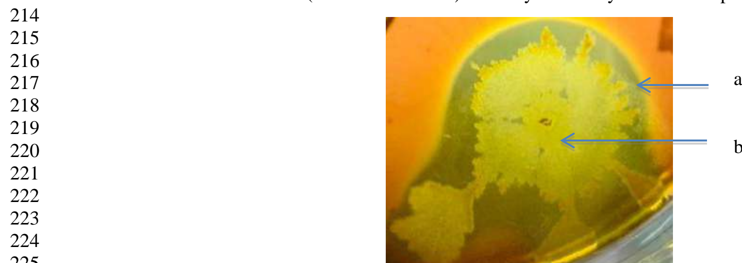

Figure 4. The clear zone is formed from the hemolytic activity of the *Bacillus* sp. ES4.3 isolate on Blood Agar media. Notes: a) halo zone, b) Colony of *Bacillus* sp. ES4.3.

Emulsification Activity

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

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

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

Surface Tension

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

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

Treatment	Surface Tension (mN/m)
Distilled Water Control	72.00 ± 0.00
NB Control	59.64 ± 0.12
Supernatant *Bacillus* sp. ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

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 is said to represent 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 *Bacillus 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 that have been 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 2. shows the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of *Bacillus 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

257 location of the branching between *Bacillus* sp. ES4.3 with *B. velezensis* BS1 and *B. velezensis* FZB42. Another thing that
258 could also be due to the high percent identity or the low nucleotide variation between *Bacillus* sp. ES4.3 with *B. velezensis*
259 BS1 and *B. velezensis* FZB42. This strain was found from pepper fields in Gangwon Province, Korea. Based on the
260 research of Shin et al. (2021), they said that *B. velezensis* BS1 was consistently able to produce cellulase, proteases, and
261 siderophores; and inhibited the growth, appressorium formation, and disease development of *Colletotrichum scovillei*, a
262 pepper anthracnose pathogen. *B. velezensis* BS1 showed a high inhibitory effect on the mycelium growth of *Botrytis*
263 *cinerea* isolated from strawberries, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* isolated from lettuce. In addition,
264 according to Shahid et al. (2021), the antifungal activity of *Bacillus* can also fight other agricultural pathogens, such as
265 *Fusarium oxysporum, Fusarium moniliforme,* and *Colletotrichum falcatum* (Shahid et al. 2021). Other results in the study
266 of Shin et al. (2021) also showed that *B. velezensis* BS1 could promote the growth of chili seedlings. In the phylogenetic
267 tree, *Bacillus thuringiensis* is an outgroup.
268 The sequencing results obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the
269 similarity of the *srf*A-D gene in *Bacillus* sp. ES4.3 with the genes in other bacteria. A test was also conducted to determine
270 the similarity between the nucleotides of the *srf*A-D gene *Bacillus* sp. ES4.3 and the nucleotide gene *srf*A-D *B. velezensis*
271 in GenBank. Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin
272 biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity
273 with 99.86%. Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of *Bacillus* sp. ES4.3. This
274 sample was used to detect surfactin genes by PCR with a primer designed from 732 bp of *srf*A-D gene fragments from *B.*
275 *velezensis*. The PCR screening results showed that the amplification of the *srf*A-D gene fragment was found in *Bacillus* sp.
276 ES4.3, identified as *B. velezensis* FZB42. The *srf*A-D gene is known to produce thioesterase, which is presumed to be
277 involved in the lactonization process (Satpute et al. 2010).
278 The hemolytic activity can be identified by looking at the clear zone around the microbial colonies (Carillo et al. 1996).
279 Hemolytic activity of *Bacillus* sp. ES4.3 can be seen in Figure 4. Carillo et al. (1996) stated that there is a relationship
280 between hemolytic activity and biosurfactant production. In the Blood Agar medium, *Bacillus* sp. ES4.3 was inoculated.
281 The presence of hemolytic activity, which is indicated by the formation of a halo zone around the colony, means the
282 biosurfactants were produced.
283 From these results, it can be seen that the hemolysis type of this bacterial isolate is β-hemolysis or total hemolysis,
284 which is indicated by the visible clear zone as a result of the lysis of all red blood cells and the release of hemoglobin from
285 the cells. Bacteria that cause β-hemolysis are the ones with the most potential to produce biosurfactants because
286 biosurfactants act as hemolysin substances. The hemolysin substance acts as an antibody against erythrocyte membrane
287 antigen which causes hemolysis (Ibrahim et al. 2013). The hemolytic activity of biosurfactants can occur by two different
288 mechanisms. The first one is by dissolving the membrane, which normally occurs at high biosurfactant concentrations and
289 the second one is by increasing the membrane permeability to small solutes that occur when the biosurfactant
290 concentration is low causing osmotic lysis (Zaragoza et al. 2010). The clear zone in the Blood Agar media corresponded to
291 changes in the permeability of the target cell membrane. The ability of these compounds to increase the permeability of
292 cell membranes is caused by the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). As a result,
293 biosurfactants can directly interact with membrane lipids that enter the membrane and form pores in the larvae midgut and
294 destroy the membrane through detergent-like interactions (Butko 2003).
295 The results of the emulsification activity can be seen in Table 2. These results indicate that there are differences in the
296 results of the emulsification activity of the supernatant *Bacillus* sp. ES4.3 on kerosene and diesel fuel substrates. Better
297 properties are characterized by a greater emulsion index value, which means that the surfactant has large emulsion
298 stability. The results also showed a decrease in the value of emulsification activity after 24 hours, which indicates a
299 relatively stable emulsification activity. This research proves that the biosurfactant product can be categorized as a
300 bioemulsifier. The occurrence of emulsification activity in *Bacillus* sp. ES4.3 is indicated by the formation of foam, which
301 creates a layer in the tube. The foam layer was then measured to calculate the emulsification activity value (Ni'matuzahroh
302 et al. 2017). The emulsification index value indicates the stability of the emulsion, and the line above 50% showed good
303 biosurfactant producers (Willumsen and Karlson 1997).
304 The mechanism by which surfactants work as emulgators is by reducing the tension between the surface of water and
305 oil, so that a film layer is formed on the surface of the dispersed globules phase. Hence, emulsification activity is related
306 to the parameters of biosurfactant production because it is a very good emulsifier. The indicators of biosurfactants that are
307 produced by microorganisms can be seen in the emulsification activity of the media. Emulsification activity can be seen by
308 the formation of an emulsion that looks like a bubble between the substrate and the media (Arifiyanto et al. 2020).
309 The surface tension value of the culture supernatant *Bacillus* sp. ES4.3 can be seen in Table 3. When compared with
310 the surface tension values of the distilled water control, NB media control, and tween control, the value of the culture
311 supernatant of this isolate decreased to 21.38 mN/m from the NB media control, 33.74 mN/m from the distilled water
312 control, and 3.91 mN/m from the tween control. Bacteria have the potential to produce biosurfactants if they can reduce
313 the surface tension value ≥ 10 mN/m (Francy et al. 1991). The hydrophobic and hydrophilic groups in biosurfactants cause
314 these compounds to accumulate between the liquid phases, thereby reducing surface tension and interfacial tension
315 (Kapadia and Yagnik 2013). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant
316 produced by bacterial isolates during the growth process (Arifiyanto et al. 2020). The decrease in surface tension can affect

317 the entomopathogenic activity of *Aedes aegypti* larvae which causes low oxygen underwater, so that the larvae spiracles
318 continue to open and make it death (Geetha 2010).
319 This study concluded that *Bacillus* sp. ES4.3 which was identified as *Bacillus velezensis* ES4.3, has a biosynthesis
320 surfactin gene. The biosurfactant activity was indicated by the formation of clear zones, the formation of emulsions, and a
321 decrease in surface tension in the values of 21.38 mN/m from the NB media control and 33.74 mN/m from the distilled
322 water control. The presence of these genes and the biosurfactant activity indicates that the *Bacillus velezensis* ES4.3 has
323 the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *Bacillus velezensis*
324 ES4.3 has the potential to be developed as a biocontrol in disease vectors and other fields of medicine, agriculture,
325 pharmaceuticals, and waste treatments. Further research that can be done to produce surfactin include the production and
326 optimization of biosurfactant production, as well as characterization of the resulting biosurfactant product.

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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

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id

[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.

[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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Abstract. *Nafidiastri FA, Susetyo~~i~~ RD, Nurhariyati T, Supriyanto A, G~~H~~eraldi A, Ni~~u~~'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: xxxx. 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 detected by isolating the DNA and visualizing 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 ~~99.86~~100 for % ID with the surfactin biosynthesis thioesterase S~~s~~rfA-D gene on the *Bacillus* ~~*amyloliquefaciens*~~ *amyloliquefaciens* group. The biosurfactant activity 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~~media~~ control and 33.74 mN/m from the distilled water control. The ability of ~~*Bacillus* sp.~~*B. velezensis* ES4.3 to hemolyzed and reduced surface tension indicated the presence of biosurfactant that can disrupt stability and damage the midgut of *Aedes aegypti*. Thus, ~~*Bacillus* sp.~~*B. velezensis* ES4.3 has the potential to be developed as a biocontrol in disease vector.

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

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, petroleum industries, and 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, which is 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) which are 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), which is 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 disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 ~~medium~~ 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 ~~on~~ for biosurfactant activity. The three ~~medium~~ 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 medium 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 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 product were 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. Amplikon 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).

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Detection of Biosynthesis Surfactin gene

In this stage, ~~we used the DNA that has been obtained on the identification of 16S rRNA gene stage. The procedure of detection biosynthesis surfactin gene same with the procedure of identification 16S rRNA gene, but the only difference is the primer used.~~ to identify surfactine gene, the same procedures for 16S rRNA identification were performed but by 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

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Hemolytic test was carried out by culturing the bacterial isolate *Bacillus* sp. ES4.3 in sterile Blood Agar medium that 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

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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 ~~media~~ medium and incubated for 24 hours. Bacterial cell-free culture supernatant obtained by centrifugation at 5500 rpm for 15 minutes was supplied by 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 ~~or EI~~). This calculation is done through the formula by Ozdal et al. (2017).

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

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

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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).

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

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". In 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it compared with *Bacillus atrophaeus* strain NBRC 15539 and *Bacillus atrophaeus* strain JCM 9070.

Analysis of Phylogenetic~~i~~ Tree

Figure 2. showed~~s~~ the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strain of *Bacillus 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~~y~~ tree, which is analyzed by Neighbor-Join Method.

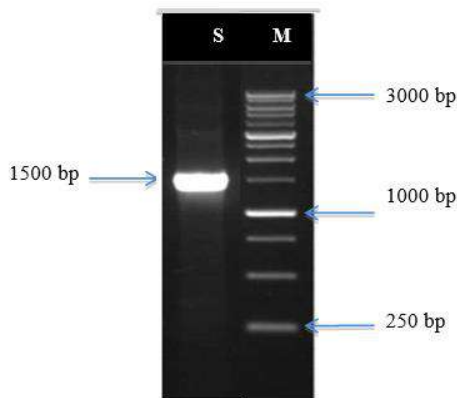

Figure 1. Electrophoresis result of DNA *Bacillus* ~~velezensis~~ sp. ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker).

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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%

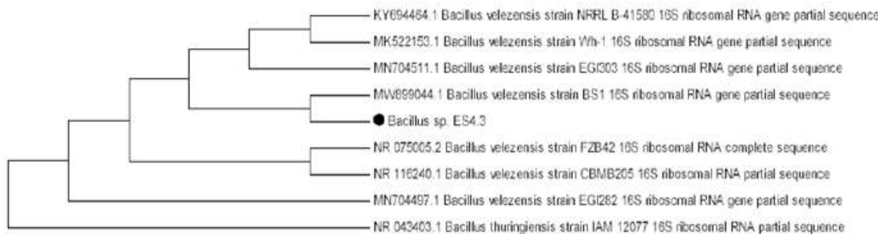

Figure 2. Phylogenetic~~i~~ tree of *Bacillus* sp. ~~*velezensis*~~ ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

~~Figure 3. shows that~~ T~~t~~he sequencing results that obtained were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the ~~s~~S*rf*A-D gene ~~*Bacillus* sp.~~*B. velezensis* ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the ~~nucleotides~~ protein of the *srf*A-D gene *B. velezensis*~~*Bacillus* sp.~~ ES4.3 with another ~~nucleotide~~ 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 ~~amino acids~~protein in the *srf*A-D gene of *B. velezensis*~~*Bacillus* sp.~~ ES4.3 have the highest similarity with the surfactin biosynthesis thioesterase ~~S~~srfA-D ~~biosynthesis~~ from *Bacillus* ~~*amyloliquifaciens*~~ *amyloliquefaciens* group bacteria in Genbank by ~~99.86~~100% (GenBank access number WP_003156383.1). On the research of Rabbee et al. (2019), said that based on phylogenomic analysis *B. velezensis* belong to the same clade as a *B.* ~~*amyloliquifaciens.*~~*amyloliquefaciens.*

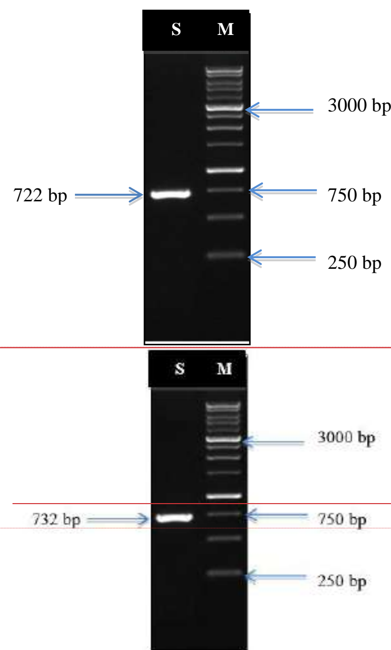

Figure 3. Electrophoresis results of the *srf*A-D gene in ~~*Bacillus*~~ *B. velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 72~~3~~2 bp. (S = Sample; M = Marker)

Screening of Biosurfactant Activity

Hemolytic Activity

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

Emulsification Activity

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

Surface Tension

Table 3. show~~ed~~s that the surface tension value of the culture supernatant *B. velezensis*~~*Bacillus* sp.~~ ES4.3, when it compared with the surface tension values of the distilled water control, NB medi~~um~~a control, and Tween control, the value of the culture supernatant of this isolate decreased to 21.38 mN/m from the NB medi~~um~~a control, 33.74 mN/m from the distilled water control, and 3.91 mN/m from the Tween control.

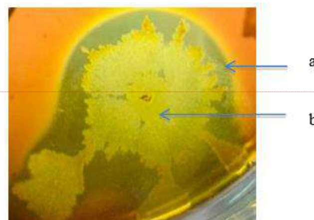

Figure 4. The clear zone is formed from the hemolytic activity of the *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 isolate on Blood Agar

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medi~~a~~um. Notes: a) halo zone, b) Colony of *B. velezensis* ~~*Bacillus* sp.~~ ES4.3.

Table 2. Results of emulsication activity of supernatant of *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 on kerosene and diesel fuel.

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

Table 3. The surface tension value of the culture supernatant isolate ~~*Bacillus* sp~~*B. 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 ~~*Bacillus* sp.~~*B. velezensis* ES4.3	38.26 ± 0.25
Tween Control	34.35 ± 0.07

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 is said to represent 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 *Bacillus 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 that have been 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 2. show~~s~~ed the phylogenetic analysis of *Bacillus* sp. ES4.3 against other strains of ~~*Bacillus*~~ *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*, 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* 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 ~~obtained~~ were analyzed using BioEdit software, BLASTn, and BLASTp to determine the similarity of the *srf*A-D gene in *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 with the genes in other bacteria. A test was also conducted to determine the similarity between the ~~nucleotides~~ protein of the *srf*A-D gene *B. velezensis* ~~*Bacillus* sp.~~ ES4.3 and another~~the~~ ~~nucleotide~~ protein of the gene *srf*A-D *Bacillus* ~~*B. velezensis*~~ 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 *Bacillus amyloliquefaciens* group bacteria in Genbank. ~~Based on the results of BLASTp on the amino acid gene *srf*A-D from *Bacillus* sp. ES4.3, surfactin biosynthesis thioesterase *srf*A-D from the *Bacillus amyloliquifaciens* group bacteria in Genbank has the highest similarity with 99.86%.~~ Figure 3. is the result of electrophoresis of the *srf*A-D gene from DNA samples of ~~*Bacillus* sp.~~*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* ~~*Bacillus* sp.~~ ES4.3, identified as *B. velezensis* Htq6, with a successfully amplified size of 722 bp. ~~FZB42.~~ 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*~~*Bacillus* sp.~~ 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* ~~*Bacillus* sp.~~ 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 visible clear 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~~media~~ 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 and 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 that there are differences in the results of the emulsification activity of the supernatant *B. velezensis* ~~*Bacillus* sp.~~ 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* ~~*Bacillus* sp.~~ 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* ~~*Bacillus* sp.~~ ES4.3 can be seen in Table 3. When compared with the surface tension values of the distilled water control, NB medium ~~media~~ control, and T~~t~~ween control, the value of the culture supernatant of this isolate decreased to 21.38 mN/m from the NB ~~media~~ medium control, 33.74 mN/m from the distilled water control, and 3.91 mN/m from the T~~t~~ween control. Bacteria have the potential to 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 ~~media~~ medium control and 33.74 mN/m from the distilled water control. The presence of these genes and the biosurfactant activity indicates that the ~~*Bacillus*~~ *B. velezensis* ES4.3 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, ~~*Bacillus*~~ *B. velezensis* ES4.3 has the potential to 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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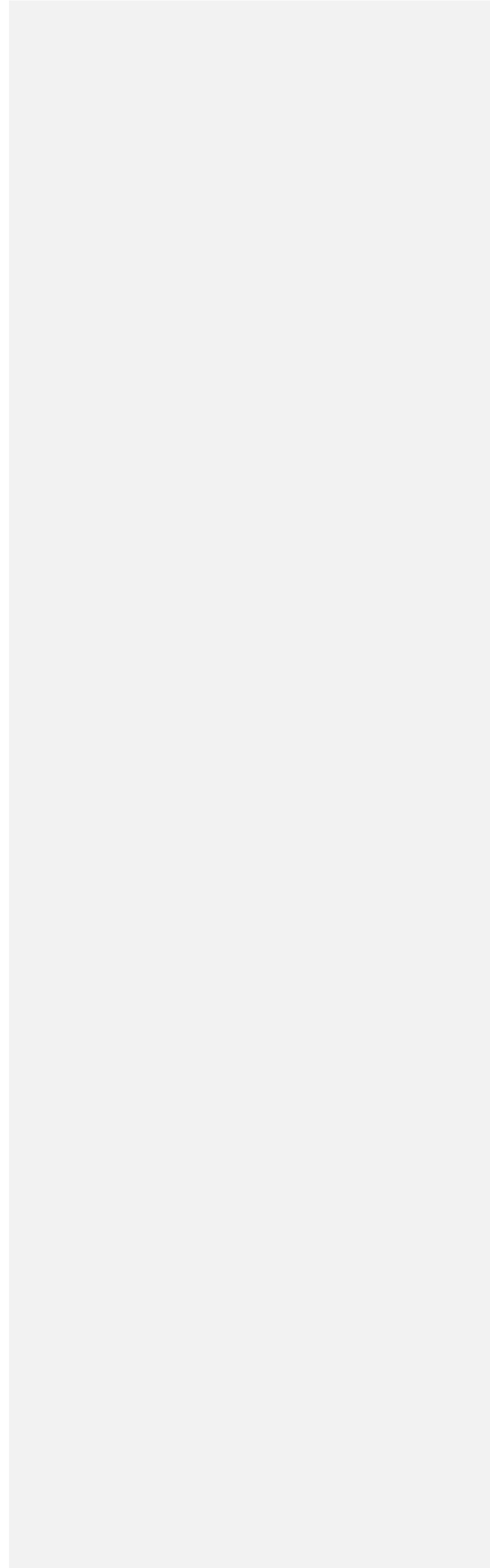

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Biosurfactant Activity of Indigenous *Bacillus* sp. ES4.3 Isolated from Endemic Breeding Sites of Dengue Hemorrhagic Fever Vector in Surabaya, East Java, Indonesia

FARAH AISYAH NAFIDIASTRI[1♥], RIZKY DANANG SUSETYO[1], TRI NURHARIYATI[2], AGUS SUPRIYANTO[2], ALMANDO GERALDI[3], NI'MATUZAHROH[4], FATIMAH[3], SALAMUN[4♥♥]

[1]Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel. +6282233442815, ♥email: farah.aisyah.nafidiastri-2020@fst.unair.ac.id
[2]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.
[3]Research Center for Bio-Molecule Engineering (BioME), Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115.
[4]Research Group for Applied Microbiology, Faculty of Science and Technology, Universitas Airlangga. Jl. Mulyorejo, Surabaya, East Java, Indonesia 60115. Tel./Fax. +6281332198122, ♥♥email: salamun@fst.unair.ac.id

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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: xxxx.* *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 detected by isolating the DNA and visualizing 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 for % ID with the surfactin biosynthesis thioesterase SrfA-D gene on the *Bacillus amyloliquefaciens* group. The biosurfactant activity 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 ability of *B. velezensis* ES4.3 to hemolyzed and reduced 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 vector.

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

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, petroleum industries, and 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, which is 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) which are 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), which is 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 disintegrate 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), provided 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 that commonly used for screening and genetic identification of bacterial isolates that have the potential to rapidly produce biosurfactants is through PCR. PCR was used to identify the 16S rRNA gene and gene that involved in surfactin biosynthesis, which 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 medium 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 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 product were 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. Amplikon 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 but by 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 that 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 by 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).

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

E24 : emulsification activity on 24 hours
HE : high of the emultion 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

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improve accuracy and average retrieval. This calculation is done through the formula by Chauhan et al. (2013).

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

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". In 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. indicate that this isolate as 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 *Bacillus velezensis* strain FZB42 when it 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 *Bacillus 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, which is analyzed by Neighbor-Join Method.

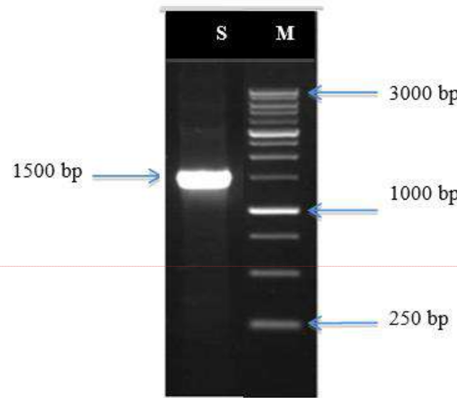

Figure 1. Electrophoresis result of DNA *Bacillus* sp. ES4.3 isolate marked with a band measuring 1500 bp. (S = Sample; M = Marker).

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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%

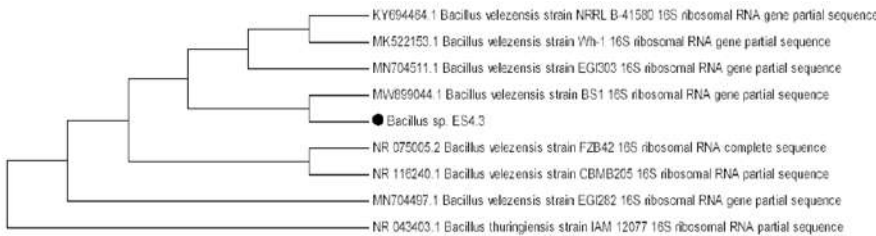

Figure 2. Phylogenetic tree of *Bacillus* sp. ES4.3 and another bacteria of *B. velezensis* strains

Analysis of Biosynthesis Surfactin Gene

The sequencing results that 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 protein of the *srf*A-D gene *B. velezensis* ES4.3 with 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 protein in the *srf*A-D gene of *B. velezensis* ES4.3 have 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), 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 showed a decrease.

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.

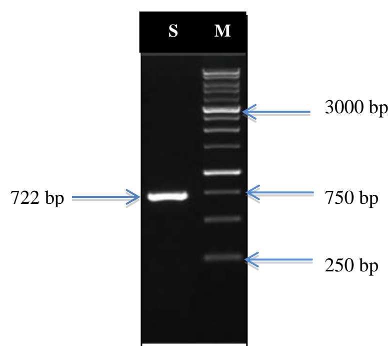

Figure 3. Electrophoresis results of the *srf*A-D gene in *B. velezensis* ES4.3 isolates marked with samples. In the sample, there is a band measuring 722 bp. (S = Sample; M = Marker)

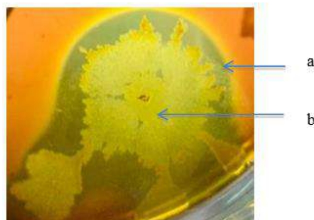

Figure 4. The clear zone is formed from the hemolytic activity of the *B. velezensis* ES4.3 isolate on Blood Agar medium. Notes: a) halo zone, b) Colony of *B. velezensis* ES4.3.

Table 2. Results of emulsication activity of supernatant of *B. 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

Table 3. The surface tension value of the culture supernatant isolate *B. 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

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

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gene sequencing serves as an approximation method for rapid and accurate bacterial identification. A bacterium is said to represent 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 *Bacillus 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 that have been 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 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*, 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* 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 protein of 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 *Bacillus 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 visible clear 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 and 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 that there are 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 have the potential to 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 has the potential to act as a biosurfactant producer evidenced by the resulting biosurfactant activity. Thus, *B. velezensis* ES4.3 has the potential to 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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