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The potential of indigenous *Bacillus* sp. BT3.1 as an antifungal biopesticide against the plant pathogen *Fusarium oxysporum*

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Abstract. *Nafidiastri FA, Lolita NT, Riqina HS, Aurora BZ, Geraldi A, Nurhariyati T, Supriyanto A, Ni'matuzahroh, Fatimah, Salamun. 2024. The potential of indigenous* Bacillus *sp. BT3.1 as an antifungal biopesticide against the plant pathogen* Fusarium oxysporum. *Biodiversitas 25: 2679-2686.* Biosurfactants are green surfactants that bacteria can produce, one of which is *Bacillus*. This genus can produce lipopeptide-type biosurfactants, namely surfactin, that can inhibit the growth of fungal pathogens. This research aims to determine the biosurfactant activity on molasses substrates and the antifungal activity of isolate BT3.1 against *Fusarium oxysporum*. The biosurfactant activity test method tested hemolytic activity using the spot method, surface tension analysis, and emulsification activity. In addition, an antifungal activity test was also carried out using the swab method. Characterization of isolate BT3.1 was carried out macroscopically and microscopically. The macroscopic characteristics of isolate BT3.1 show that bacteria from the genus *Bacillus* are moderate in size, white, irregular with serrate colony edges, shiny raised elevations, and a shiny surface texture. Microscopic characteristics of the isolate showed Gram-positive bacteria with oval-shaped endospores in the center. Based on the results of the biosurfactant activity test, isolate BT3.1 showed beta-type hemolysis activity, reduced surface tension by 23.3 mN/m on distilled water and 12 mN/m on NB media, and produced an emulsion activity value of around 52%. The results of the production of crude extract of BT3.1 isolate on 2, 4, and 6% molasses substrates showed optimal biosurfactant activity at a concentration of 4% with an incubation time of 48 hours. The antifungal activity of isolate BT3.1 showed that 80% inhibited the growth of *Fusarium oxysporum*, so the indigenous bacteria *Bacillus* sp. BT-3.1 has the potential to be developed as a raw material candidate for antifungal biopesticides against the plant pathogen *Fusarium oxysporum*.

Keywords: Antifungal activity, *Bacillus*, biosurfactant activity, *Fusarium oxysporum*, plant protection

INTRODUCTION

Indonesia is a country with a tropical climate that has relatively high humidity. High humidity can trigger the growth of various microorganisms, one of which is pathogenic and non-pathogenic fungi. Approximately 50 species of fungi cause diseases in people, and another 8,000 species can cause diseases in plants (Fisher et al. 2020). One type of fungus that causes plant disease is *Fusarium oxysporum*, which can infect plants and cause *Fusarium* wilt disease (Sopialena 2015). *Fusarium* wilt disease is characterized by yellowing of the leaves, partial or complete wilting, and the lower stem turning brown, blackish, or yellowish (Ngittu et al. 2014). Plants affected by *Fusarium* wilt include bananas, cabbage, tomatoes, tulips, watermelon, cotton, ginger rhizomes, celery, pea, eggplant, sweet potato, pepper, onion, orange, muskmelon, carnation, strawberry, and lettuce (Husaini et al. 2018; Edel-Hermann and Lecomte 2019). Apart from that, a genus of fungus Colletotrichum can also infect plants and cause anthracnose, with an average disease incidence of 63%. In chili plants in Bali, anthracnose is mostly caused by the fungus *Colletotrichum scovillei*, with an attack percentage reaching 55.55% (Khalimi et al. 2019). Plant diseases caused by these fungi can cause a decrease in crop yields which can lead to crop failure, resulting in large economic losses for farmers.

Many farmers control the growth of fungi on plants using synthetic fungicides made from chemicals. Excessive use of synthetic fungicides can cause various negative effects on living creatures and the environment, such as human health problems, environmental pollution, and the emergence of pathogens resistant to fungicides (Sari et al. 2015). Therefore, to prevent these negative effects, another alternative is needed to reduce synthetic fungicides, especially biological agents (Djaenuddin and Muis 2015). Biological agents widely used are groups of microbes, especially *Bacillus* sp. *Bacillus* sp. is a biological agent to control pathogenic fungi because it can potentially produce biosurfactants (Płaza et al. 2015).

Biosurfactants are green surfactants produced by microbial groups (molds, yeasts, bacteria) using organic materials (Amraini et al. 2022). Biosurfactants can be used in various fields such as agriculture, pharmaceuticals, cosmetics, food processing, and environmental cleaning industries, where they are usually used as a source of raw materials for lubrication, wetting, foaming, and emulsion. Biosurfactants derived from microorganisms are physically and chemically stable, easily decomposed, environmentally friendly, have a low level of toxicity, have resistance to higher temperatures, and have the ability to withstand various pH fluctuations (Kumar et al. 2021).

Based on research conducted by Dunlap et al. 2015, *Bacillus subtilis* has been proven to be an antifungal agent by producing compounds, such as surfactin and iturin, which belong to the lipopeptide group. Lipopeptides are one of the most powerful biosurfactants because they can inhibit the growth of microbes and viruses and act as antitumors (Phulpoto et al. 2020). In addition, lipopeptides have been applied in various fields such as agriculture, medicine, and food sectors. *Bacillus* sp. produces environmentally friendly lipopeptides because they have low toxicity levels and high biodegradability (Youcef-Ali et al. 2014). *Bacillus subtilis* produces other cell wall-degrading enzymes, such as cellulases, pectinases, chitinases, proteases, xylanases, lipases, and amylases (Khan et al. 2018). In addition, based on research by Wang et al. (2020), the species *Bacillus velezensis* was proven to have antifungal activity against *Fusarium graminearum*, which causes malformations in the mycelium and ultra-structural changes.

In this study, the bacteria used was *Bacillus* sp., successfully isolated from Baluran National Park East Java, Indonesia, in research by Findawati et al. (2020). This research aimed to determine the ability of *Bacillus* sp. BT3.1 in producing biosurfactants through hemolytic activity tests, emulsification activity tests, surface tension measurements, and antifungal activity tests against *F. oxysporum in vitro*. Researchers want to prove that *Bacillus* sp. BT-3.1 could potentially be developed as a candidate raw material for antifungal biopesticides against the plant pathogen *F. oxysporum*.

MATERIALS AND METHODS

Media and isolate preparation

Indigenous *Bacillus* sp. BT3.1 was isolated from Baluran National Park, East Java, Indonesia soil in previous research (Findawati et al. 2020). *Fusarium oxysporum* InaCC F642 isolate was obtained from InaCC BRIN. There are five mediums used, namely Nutrient Agar (NA), Nutrient Broth (NB), blood agar, Mineral Salt Medium (MSM) with molasses substrate, and Potato Dextrose Agar (PDA) medium. NA and NB medium were used to preculture bacterial isolates and confirm macroscopic and microscopic characterization, blood agar medium was used to test hemolytic activity, and MSM medium with molasses substrate was used to test emulsification activity and measure surface tension. PDA medium was used to inoculate pathogenic fungi *F. oxysporum* and antifungal activity test. All mediums were sterilized by autoclaving at a temperature of 121°C at 1 atm. Isolate *Bacillus* sp. BT3.1 was inoculated on NA, NB, and blood agar medium with an incubation time of 24 hours and a temperature of 30°C, while in MSM media with a temperature of 30°C and varying incubation times from 0-96 hours. *Fusarium oxysporum* was inoculated on PDA media for test preparation.

Confirmation of *Bacillus* sp. BT3.1

Isolate *Bacillus* sp. BT3.1, which was inoculated on NA medium with an incubation time of 24 hours at 30°C, was characterized macroscopically (shape, color, size, elevation, colony edges) and microscopically (cell shape and Gram type) with Gram staining and endospore staining.

Biosurfactant activity

Biosurfactant activity screening was carried out using three methods, namely hemolytic activity, emulsification activity, and surface tension.

Hemolytic activity

The hemolytic activity test was carried out by inoculating the bacterial *Bacillus* sp. BT3.1 on sterile Blood Agar medium obtained from the Surabaya Laboratory using the spot method was incubated at 37°C for 48-96 hours (Mohanram et al. 2016). Positive indications are observed through forming a clear zone around the colony of *Bacillus* sp. BT3.1.

Emulsification activity

Emulsification activity was carried out to determine the ability of *Bacillus* sp. BT3.1 in emulsifying hydrocarbon solutions (kerosene) in a quantitative way. Isolate *Bacillus* sp. BT3.1 was cultured in NB medium and incubated for 24 hours. The bacterial culture was centrifuged at 3,000 g for 9 minutes. Next, 2 mL of the obtained cell-free supernatant and 2 mL of kerosene were put into a test tube and vortexed for 2 minutes. Emulsification activity was observed at 1 hour (E1) and 24 hours (E24) by measuring the emulsion height (mm) and the total liquid height (mm) using a caliper. Emulsification activity is expressed in the following equation (Ozdal et al. 2017; Datta et al. 2018):

$$\%\, Emulsification = \frac{the\ high\ of\ emultion}{the\ high\ of\ solution\ total} \times 100\%$$

Surface tension

Measurement of surface tension of cell-free supernatant of *Bacillus* sp. BT3.1 was performed using a Kruss 100 tensiometer (Kruss GmbH, Hamburg, Germany) with the Du Nouy ring method. This measurement was carried out with three replications to increase the accuracy of the values obtained. This calculation is carried out using the formula (Chauhan et al. 2013):

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

Where:

γ : sample surface tension
γo : surface tension of distilled water at t°C
θ : the surface tension of the sample is read on the tool
θo : the surface tension of distilled water is read on the tool

Production of biosurfactant on molasses

Biosurfactant production of *Bacillus* sp. BT3.1 was added 2, 4, and 6% molasses on (Mineral Salt Medium) MSM media. MSM media was prepared by dissolving 3 g $(NH_4)2SO_4$, 10 g NaCl, 0.2 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2$, 0.001 g $MnSO_4.H_2O$, 0.001 g H_3BO_3, 0.001 g $ZnSO_4.7H_2O$, 0.001 g $CuSO_4.5H_2O$, 0.005 g $CoCl_2.6H_2O$, and 0.001 g $NaMoO_4.2H_2O$ in 900 mL of distilled water. Phosphate buffer was made by dissolving 5 g KH_2PO_4 and 2 g K_2HPO_4 in 50 mL of distilled water. The Iron buffer was dissolving 0.0006 $FeSO_4.7H_2O$ in 50 mL of distilled water. The three solutions were sterilized by autoclaving for 15 minutes at 121°C, 1 atm. The addition of bacterial culture was 4%. Bacterial density, emulsification activity, and surface tension were measured every 24 hours until 72 hours of incubation.

Antifungal activity

The antifungal activity test was carried out *in vitro* using the swab method to determine the ability of *Bacillus* sp. BT3.1 in suppressing the growth of the pathogenic fungus *F. oxysporum*. Isolate *Bacillus* sp. BT3.1, cultured in an NB medium for 72 hours, was centrifuged. The culture forms tested were cell-free supernatant fraction, sonication pellets fraction, and pure culture. The three types of culture forms were swabbed on PDA media in a petri dish, and then a hole in the middle of the media for the inoculation was made on-site for the *F. oxysporum* block. *Fusarium oxysporum* isolates rejuvenated in PDA media in petri dishes for 5 days were cut by making blocks using a chopper and transferred to the middle of the PDA media that already was swabbed with the fractions. Observations were made every day until the 144 hours of incubation. Measurement of the growth zone of *F. oxysporum* was carried out using the formula calculation (Jasim et al. 2016):

$$\%\ Index\ of\ Growth\ Inhibition = \frac{D1}{D1 - D2} \times 100\%$$

Where:

D1 : diameter of control growth
D2 : diameter of treatment

Data analysis

Data obtained in this study consisted of images of macroscopic and microscopic characteristics of *Bacillus* sp. BT3.1 isolates; clear zone images of hemolytic activity, emulsification activity, and surface tension values on biosurfactant activity; and growth curves of *Bacillus* sp. BT3.1 were analyzed descriptively. Data from the results of emulsification activity and surface tension on biosurfactant production with molasses media and growth inhibition zone on antifungal activity were analyzed statistically and descriptively. We use the normality test (Shapiro-Wilk) and the homogeneity test (Levene test), also continually with the Analysis of Variant tests.

RESULTS AND DISCUSSION

Characterization of *Bacillus* sp. BT3.1

Macroscopic characters *Bacillus* sp. BT3.1 is shown in Figure 1.A. Isolate *Bacillus* sp. BT3.1 has a moderate-sized circular colony shape, white color, serrated edges, raised elevations, and a matte texture. Furthermore, the microscopic characteristics of *Bacillus* sp. BT3.1 belongs to the group of Gram-positive bacteria as seen from the Gram staining results, which are purple and the bacilli shape for the cells (Figure 1.B). The endospore staining shows the isolate *Bacillus* sp. BT3.1 has endospores, as seen from the endospore staining results, which are green with an oval shape and are located in the center (middle of the cell), as shown in Figure 1.C.

Biosurfactant activity

Hemolytic activity

Bacillus sp. BT3.1's hemolytic activity on blood agar media showed positive results. This can be seen from a halo (clear) zone around the colony with β-type hemolysis in Figure 2.

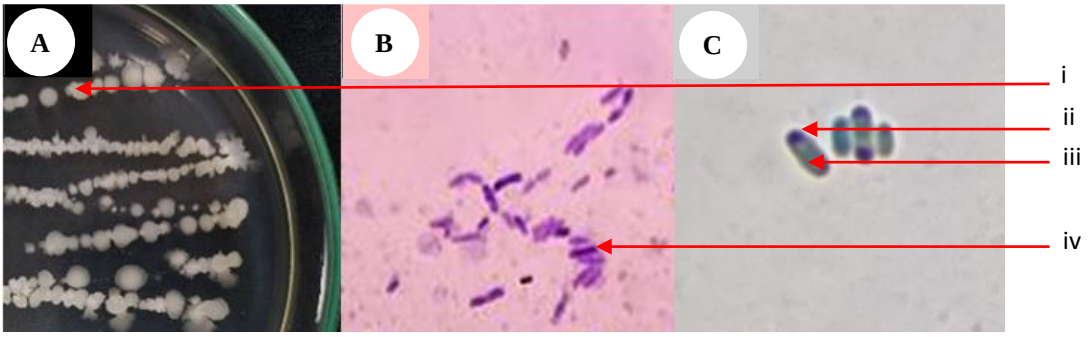

Figure 1. Morphological characteristics of *Bacillus* sp. BT3.1: Colony (A), gram staining (B), endospore staining (C). Colony cell (i), cell (ii), endospore (iii), bacil cell (iv)

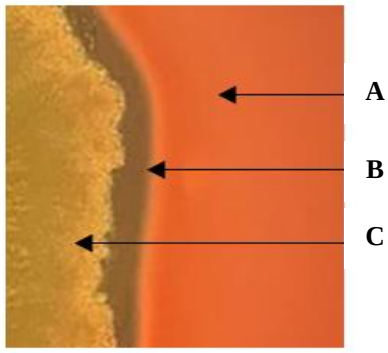

Figure 2. Hemolytic activity of *Bacillus* sp. BT3.1 on blood agar media: A. Blood agar media; B. Clear zone; C. Colony

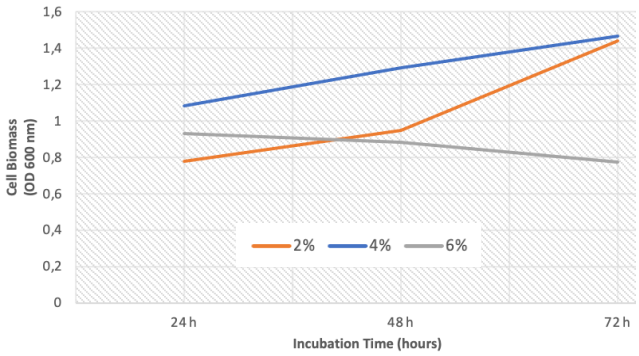

Figure 3. Growth of *Bacillus* sp. BT3.1 on variation of molasses concentration (%) and incubation times 24-, 48-, 72 h. Cells biomass (OD 600nm): x, incubation time (hour): y

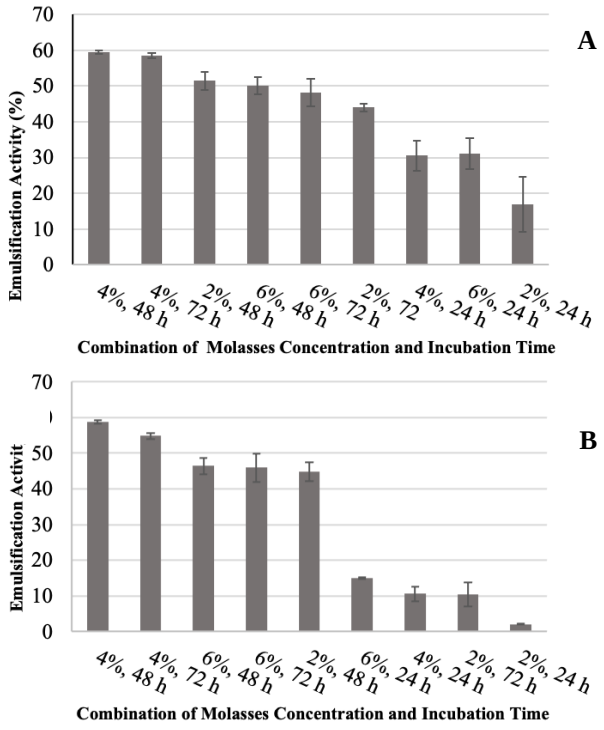

Figure 4. Emulsification activity of *Bacillus* sp. BT3.1 on combination of molasses concentration and incubation time. Observation 1 h (A); and observation 24 h (B)

Table 1. Surface tension of *Bacillus* sp. BT3.1 on screening of biosurfactant activity

Treatment	Surface tension (mN/m)
Aquadest control	72.60 ± 0.00
Nutrient Broth (NB) media control	61.30 ± 0.35
Supernatant-free cells of *Bacillus* sp. isolate BT3.1	49.30 ± 0.24

Emulsification activity

The emulsification activity produced by the supernatant of *Bacillus* sp. BT3.1 using kerosene test oil at 1-hour observation was 53%, and at 24-hour observation, it reached 52%. These results indicate that Bacillus sp. BT3.1 produces biosurfactants that can act as emulsifying agents. Emulsification activity can be identified by forming a foam layer between the substrate and the medium (Arifiyanto et al. 2020).

Surface tension

Based on the results of surface tension analysis presented in Table 1, it is known that the supernatant-free cells of *Bacillus* sp. BT3.1 can reduce the surface tension of the distilled water control by 23.30 mN/m and reduce the surface tension of the NB media control by 12 mN/m. From these results, it can be said that *Bacillus* sp. BT3.1 produces biosurfactants.

Biosurfactant production

The growth phase of Bacillus sp. *BT3.1 on molasses*

Based on the results of biosurfactant screening, *Bacillus* sp. BT3.1 positive can produce biosurfactants, this research can be continued to culture *Bacillus* sp. BT3.1 on AMS with additional molasses for the next step. The growth phase of *Bacillus* sp. BT3.1 on molasses variation such as 2, 4, and 6% showed the difference (Figure 3).

Emulsification activity on molasses

The results of the emulsification activity of the 1-hour and 24-hour observations of *Bacillus* sp. BT3.1 on molasses substrate can be seen in Figure 4. Emulsification activity is said to be stable if it is able to maintain at least 50% at the 24-hour observation (Figure 4.B). Based on Figures 4.A and 4.B, it is known that the emulsification activity of *Bacillus* sp. BT3.1 isolate at 1 hour (E1) and 24 hours (E24) observation using kerosene substrate is the highest of all concentrations (2, 4, and 6%) occurred at 48 hours incubation time.

Surface tension on molasses

Table 2 shows the value of surface tension produced by Bacillus sp. BT3.1 at various molasses concentrations and variations in incubation time. The best surface tension value observed from the low measurement value is shown in 4% molasses substrate with 48 hours incubation time, which is 31.09 mN/m. This is proven by the value of surface tension reduction against the control, which can be seen in Table 3, which is 33.68 mN/m.

Antifungal activity

The antifungal activity test of *Bacillus* sp. BT3.1 against *F. oxysporum* is shown in Figure 5. Of the three culture dosage forms, the lowest *F. oxysporum* growth diameter results were shown in the sonicated pellets fraction. In addition, the sonicated pellet fraction was no less good when compared to the positive control. The positive control used in this study was Score 250EC, a synthetic plant fungicide with the active ingredient difenoconazole. Score 250EC is usually used to control plant diseases caused by pathogenic fungi in chili, potato, tomato, rice, onion, and garlic plants. The growth diameter value of *F. oxysporum* and the level of inhibition are shown in Table 4.

Discussion

Bacillus sp. is one of the bacteria–found in various places. In this research, *Bacillus* sp. BT3.1 was isolated from Baluran National Park, Jawa Timur, Indonesia, on Findawati et al. (2020). *Bacillus* sp. BT3.1 has thick peptidoglycan, so it can be categorized as a Gram-positive bacteria group, maintaining a crystal violet color in its cells (Jawetz et al. 2010). *Bacillus* sp. BT3.1 endospores are the main characteristic differentiating *Bacillus* sp. from other bacteria. *Bacillus* sp. has diverse habitats and can resist heat, ultraviolet, and electromagnetic radiation and produce stable substances. The endospores have good preservation characteristics and can tolerate extreme external environments and long-term survival (Yang et al. 2018).

Biosurfactants are amphipathic molecules that bacteria, mold, and yeast can produce; it applied in various fields, such as agriculture, industry, pharmacy, etc. The biosurfactant activity of *Bacillus* sp. BT3.1 can produce biosurfactants based on their ability to hemolyze blood, reduce surface tension, and form emulsion. Moreover, *Bacillus* sp. BT3.1 showed beta (β) hemolysis, complete or total hemolysis of red blood cells, resulting in a clear zone around the colony. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. Based on research conducted by (Setiani et al. 2020)**,** *B. cereus* forms a clear zone (β-hemolysis), indicating the potential of biosurfactants formed due to contact between bacterial cells and erythrocytes, causing lysis and forming a clear zone. Hemolytic activity of biosurfactants can occur through two mechanisms: normal membrane dissolution at high biosurfactant concentrations or increased membrane permeability to small solutes at low concentrations of biosurfactants due to osmotic lysis (Zaragosa et al. 2010). The inhibition zone formed by observing hemolytic activity indicates a biosurfactant production process. The larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012). The clear zone formed around the colony in the hemolytic activity test indicates the production of biosurfactants.

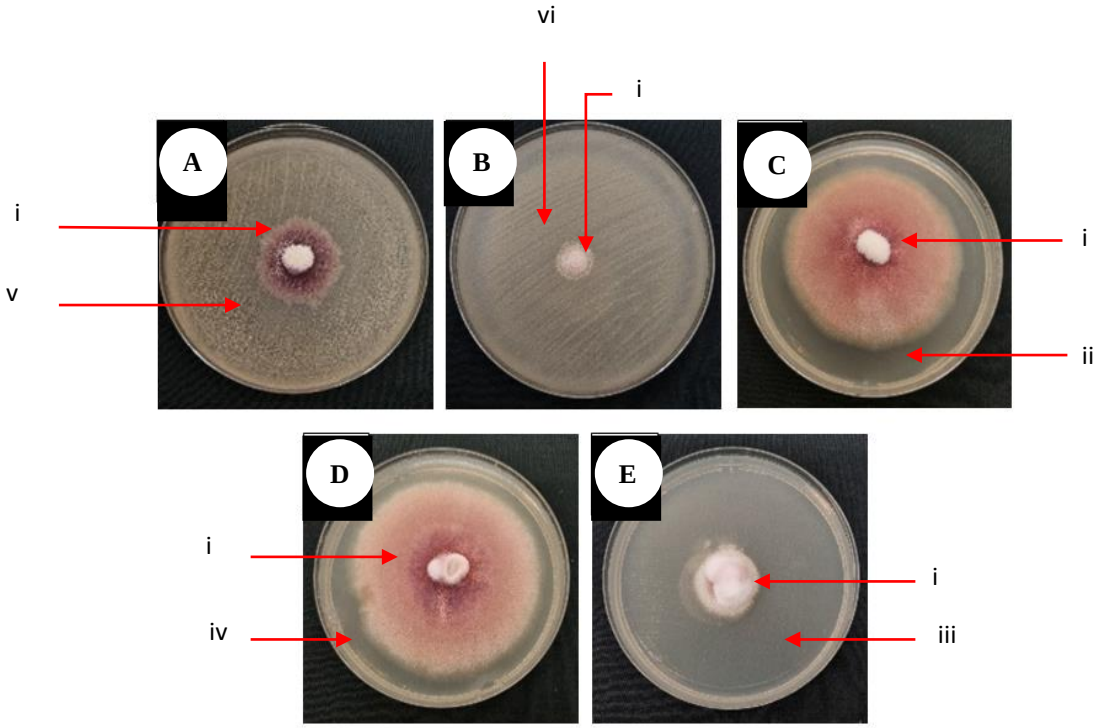

Figure 5. Antifungal activity test results of *Bacillus* sp. BT3.1 against *F. oxysporum* in vitro. A. culture dosage form, B. pellet dosage form, C. supernatant dosage form, D. control (-), E. control (+); (i) *F. oxysporum*, (ii) free-cells supernatant fraction of *Bacillus* sp. BT3.1, (iii) score 250EC synthetic plant fungicide, (iv) nutrient broth, (v) pure culture fraction of *Bacillus* sp. BT3.1, (vi) sonication pellet fraction of *Bacillus* sp. BT3.1

Table 2. Surface tension values of biosurfactant production of *Bacillus* sp. BT3.1 at various molasses concentrations (2, 4, and 6%) and incubation times (24, 48, and 72 hours)

Molasses (%)	Surface tension (mN/m)		
	24 H	48 H	72 H
2	38.14 ± 0.65^{a}	34.73 ± 1.48^{bd}	35.89 ± 0.29^{b}
4	35.11 ± 0.64^{b}	31.09 ± 0.26^{c}	33.80 ± 0.12^{bd}
6	38.33 ± 0.34^{a}	32.32 ± 0.19^{e}	32.38 ± 0.17^{e}

Table 3. Surface tension reduction of *Bacillus* sp. BT3.1 at various molasses concentrations and incubation time

Molasses (%)	Incubation time	Surface tension (mN/m)	Surface tension reduction (mN/m)
2	Control	67.90	0
	24	38.14	29.76
	48	34.73	33.17
	72	35.89	32.01
4	Control	64.77	0
	24	35.11	29.66
	48	31.09	33.68
	72	33.80	30.97
6	Control	64.20	0
	24	38.33	25.87
	48	32.32	31.88
	72	32.38	31.82

Table 4. The antifungal activity test of *Bacillus* sp. BT3.1 against *F. oxysporum* based on growth diameter

Culture of *Bacillus* sp. BT3.1	Growth diameter *F. oxysporum* (mm)	Barrier levels *F. oxysporum*
Sonication culture	26.33	55%
Sonication pellets fraction	11.80	80%
Supernatant fraction	61.03	0%
Negative control	58.58	
Positive control	23.80	

In addition, *Bacillus* sp. BT3.1 also showed emulsification activity and reduced surface tension on biosurfactant activity. Those activities can be observed for 1 hour and 24 hours from the emulsion layer formed between supernatant-free cells and oil, around 53% and 52% (Table 4). According to Willumsen and Karlson (1997), if the emulsification activity value produced by a microbe is more than 50%, then the bacteria is categorized as a good biosurfactant producer like isolate *Bacillus* sp. BT3.1. This isolate can also reduce the surface tension of nutrient broth and aquadest by 11.3 mN/m (Table 1). Bacteria could produce biosurfactants by reducing surface tension values by ≥10 mN/m (Oliveira et al. 2021). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). That's the indication that *Bacillus* sp. BT3.1 can produce biosurfactants.

We continue to produce biosurfactants with the waste substrate of molasses, an intermediate sugar cane product, which are carbon and nitrogen sources. The highest of the growth *Bacillus* sp. BT3.1 on 2% and 4% molasses showed at 72 hours. In comparison, on 6% molasses, the highest of the growth *Bacillus* sp. BT3.1 showed at 24 h. In 2% and 4% concentration molasses substrate, the log phase (exponential phase) continues to increase from the incubation time of 24 hours to the final incubation time (72 hours), while in 6% concentration molasses substrate, the exponential phase occurs at the beginning of the incubation time (24 hours) and continues to decrease the number of bacterial populations significantly until the final incubation time (72 hours) (Figure 3). This is supported by another research using isolates of *B. licheniformis* TR7 and *B. subtilis* SA9 stated that there was an increase in biosurfactant production at 4% molasses concentration with optimum at 48-51 hours and maximum biosurfactant production occurred at 72 hours when the cells were in stationary phase, the addition of molasses concentration more than 4% will reduce biosurfactant production. This is also supported by Al-Dhabi et al. (2020), in their research using isolates of *B. subtilis* stated that there was an increase in biosurfactant production with molasses optimum at 72 hours. In this case, we can say that *Bacillus* sp. BT3.1 can use and break down molasses very well as the substrate.

The emulsification activity of *Bacillus* sp. BT3.1 isolate was highest at 4% concentration within 48 hours of incubation, 59.42% at an hour of observation (E1), and 58.78% at 24 hours (E24). The decrease in emulsification activity measurement results at 1 hour and 24-hour observations at all incubation times indicates that the emulsion produced is unstable (Figure 4). This is because *Bacillus* sp. BT3.1 isolate produces biosurfactants that are only surface active molecules to reduce surface tension. Biosurfactants can be grouped into low and high molecular weights based on their weight.

According to Banat et al. (2014) and Elazzazy et al. (2015), low molecular weight biosurfactants such as glycolipids, phospholipids, and lipopeptides efficiently reduce surface tension. High molecular weight biosurfactants such as proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers are more effective in stabilizing oil-in-water emulsions (emulsifiers). This occurs due to the mechanism of action of surfactants as emulgators, namely by reducing the surface tension between water and oil so that a film layer is formed on the surface of the dispersed globule phase. In addition, the structure and components of biosurfactants can also affect emulsification activity. Biosurfactants from the surfactin-type lipopeptide group are composed of cycloheptapeptides with amino acids bound to fatty acids of different chains. This structure causes biosurfactants to have amphiphilic properties and a good level of homogeneity in polar and non-polar solvents; this amphiphilic nature composes the emulsion layer on biosurfactants (Kurniati 2016).

Bacteria are said to have the potential to produce biosurfactants if they can reduce surface tension >10 mN/m. The results obtained in this study are not too much

different from the other results, which stated that in 2% molasses, *B. licheniformis* TR7 produced a surface tension value of 34.5 mN/m and in *B. subtilis* SA9 of 32.96 mN/m. At 4% molasses, *B. licheniformis* TR7 produced a surface tension value of 30.4 mN/m and *B. subtilis* SA9 of 29.75 mN/m. At 6% molasses, *B. licheniformis* TR7 produced a surface tension value of 30 mN/m and *B. subtilis* SA9 of 30.43 mN/m (Saimmai et al. 2011). Our result differed from Umar et al. (2021) in that *B. subtilis* SNW3 indicated a surface tension value of 41 mN/m on molasses 2% after 96 hours of incubation. In addition, our results are different from research by Verma et al. (2020) also thought that the surface tension of *B. subtilis* RSL-2 on 1, 3, and 5 % molasses were 39 mN/m, 24.46 mN/m, and 28 mN/m. According to Ni'matuzahroh et al. (2017), the difference in biosurfactant production results of *B. subtilis* 3KP with unstable molasses substrate can be caused by differences in the composition and concentration of nutrients contained therein. This is related to the sugar processing compounds where the molasses is obtained. The difference in sugar content in molasses, the main carbon source for the growth of *B. subtilis* 3KP, greatly affects the productivity of biosurfactants produced. In this case, *Bacillus* sp. BT3.1 can be used as a candidate biological agent for green biocides in controlling the growth of fungal colonies based on their ability to reduce surface tension in biosurfactant activity.

Table 4 and Figure 5 show that the biosurfactant produced by *Bacillus* sp. BT3.1, which can act as an antifungal agent that can originate from inside or be secreted outside the cell, is related to separating *Bacillus* sp. BT3.1 between pellets and supernatant fractions. Compounds secreted from the cells are located in the supernatant, while compounds inside the cells are in the pellet. Therefore, separated pellets are sonicated to destroy the bacterial cell walls so that the compounds inside the cells can escape. Sonication is a method for lysing cells using ultrasonic waves with a frequency higher than human hearing (above 20,000 Hz). These ultrasonic waves will cause an increase in temperature, affecting the bacterial cell walls to open and release active compounds produced by the bacteria (Fransiska et al. 2012).

According to Rochmawati et al. (2020), biosurfactant compounds potentially act as antifungals derived from the lipopeptide group, namely iturin, fengycin, and surfactin. Fungal growth can be inhibited by iturin through damaging cell membranes, changing cell membrane permeability, and eliminating K + ions. In addition, iturin and fengycin are thought to cause lysis of conidia and hyphae and can inhibit toxin formation in fungi (Rochmawati et al. 2020).

This study concludes that *Bacillus* sp. BT3.1 can produce biosurfactants based on clear zones formed in hemolytic activity, emulsion layer on emulsification activity, and reduce surface tension. This bacteria can break down and use molasses as a substrate, which can be observed on the growth curve for various incubations. This condition leads to *Bacillus* sp. BT3.1 to produce biosurfactants at low cost and this study revealed that *Bacillus* sp. BT3.1 optimal to produce biosurfactants on 4% molasses for 48 H incubation time. *Bacillus* sp. BT3.1 has potential as an antifungal agent from antagonistic tests against *Fusarium oxysporum* as a fungi pathogen, shown from the diameter of the fungal pathogen, which can't grow well. Further research can be developed for antifungal tests on various types of plant pathogenic fungi and optimization of biosurfactant production by taking into account environmental factors, such as pH, temperature, and substrate, and continued with in-vivo tests on plants to demonstrate its ability before being released as a commercial green product. In addition, it is also necessary to test the safety level of these products against microbes and non-target organisms in water and agriculture. This will help many farmers to improve their horticultural products.

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