Isolation and Identification of Bacterial Biosurfactant Activity from Mangrove Sediments

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ABSTRACT

Background: Ujung Pangkah mangroves are reported to have been contaminated with heavy metals. Such heavy metals can induce microorganisms to produce biosurfactants. Biosurfactants with amphiphilic characteristics can lower surface tension. Biosurfactants can be used as antibacterial, antifungal, and antiviral for biomedical purposes. **Purpose:** This study aimed to identify and test the activity of biosurfactant isolates of bacteria from Ujung Pangkah Mangrove sediments, Gresik Regency. **Methods:** Biosurfactant activity test methods include emulsification index, oil spreading, drop collapse, and parafilm test. **Results:** The results of the identification of bacterial isolates in this study obtained the genus Bacillus sp. because bacterial isolates show rod shape, Gram-positive, aerobic, and have ellipse-shaped endospores on the subterminal. The results of the biosurfactant activity test with the Emulsification index method showed an average result of 54.39% and the results of the biosurfactant activity test with the oil spreading method showed that there was a clear zone. The average result of the clear zone obtained is 54.83 mm. The average result in the parafilm test was 8.02 mm and the drop collapse test showed positive results characterized by falling and spreading of bacterial isolate fermentation broth supernatants.

Keywords: biosurfactant, mangrove sediments, emulsification index, oil spreading, drop collapse, parafilm test

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INTRODUCTION

Veterinary medicine in supporting the development of livestock sub-sector acts as a means for optimizing animal health through disease prevention and treatment and increasing productivity. However, the national veterinary drug industry currently still imports some medicinal raw materials (Ismaya, 2014). The volume of veterinary drug imports in 2016 amounted to 297,468 tons, while in 2015 it was 395,656 tons. Although it has decreased by 17.5%, there is still dependence on imports of medicinal raw materials, so innovation is needed to find new compounds (Alfi, 2017).

One of the efforts that can be done is by utilizing natural resources both plants, animals and marine life as a source of medicinal raw materials. One of them is utilizing bacteria that have the potential to produce beneficial compounds to be developed biotechnologically (Abdelhafiz, *et al.*, 2017).

Bioactive compounds that can be produced by some bacteria in the form of primary metabolites and secondary metabolites. Primary metabolites are compounds produced by bacteria through basic metabolism, used for the growth and development of these bacteria while secondary metabolites are biosynthetic compounds from primary metabolites that are generally produced

by bacteria that are useful for self-defense from the environment and attacks from other organisms (Tabarez, 2005).

Many secondary metabolites are produced by bacteria and applied in the fields of antibiotics, enzymes, biopesticides and biosurfactants. Biosurfactants are biological surface-active agents produced by several microorganisms (Liu, *et al.*, 2010). The using of biosurfactants in biomedicine is as antibacterial, antifungal, antiviral, anti-cancer, immunodulator, anti-oxidant, stimulating dermal fibroblasts, probiotics, and antiparasitic (Geetha, *et al.*, 2011; Donio, *et al.*, 2013).

It is known that certain types of biosurfactants can be produced by several species of bacteria. Bacillus sp. known to produce surfactin, rhamnolipid and lichenysin; Pseudomonas can produce rhamnolipids, glycolipids; Rhodococcus sp. can produce viscosin and trehaloselipids (Fakruddin, 2012).

Research conducted by Secato, *et al.*, (2016) states that biosurfactants can be produced by bacteria in polluted ecosystems. Research conducted by Ranjan (2008) reports that heavy metals as environmental pollutants are generally higher found in mangrove sediments. Kulkarni, *et al.*, (2018) confirmed that copper (Cu), zinc (Zn), manganese (Mn), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) are heavy metals that pollute mangrove ecosystems a lot.

The results of research conducted by the Bandung Institute of Technology together with the Gresik Regency Government (2011) reported that the waters of Ujung Pangkah District were categorized as polluted. The level of marine pollution is at levels 1-5. Some substances found to exceed quality standards include, copper content reaches 0.218 mg / lt (quality standard 0.005mg / lt), ammonia content (NH3), and heavy metals reaches 0.4mg / lt (quality standard 0.3mg / lt) (Rudianto, 2014).

METHODS

Sterilization of tools and materials

Equipment to be used in research such as petri dishes, test tubes, tweezers and erlenmeyer flasks, washed with detergent and then rinsed with aquades, then dried while tips and microtubes are put into their respective containers. The tools are then wrapped in paper and sterilized in an autoclave at 121℃ pressure 2 atm for 15 minutes. Tools such as ose and test tube mouths are sterilized by burning on spirit fire. Laminar Air Flow (LAF) is cleaned by spraying 70% alcohol, dried with tissue then in Ultra Violet (UV) for 15 minutes (Kusdarwati and Sudarno, 2012 in Maulani, 2014).

Rejuvenation of isolates

Rejuvenation of bacterial isolates is carried out by taking one ose bacterial isolate then inoculated on oblique NA media. The inclined NA media was then incubated at a temperature of 37^{o}C for 24 hours (Fernandes, *et al.*, 2017).

Identification of bacterial isolates

1. Macroscopic Examination of Colonies

Examination of bacterial isolate colonies is carried out by taking one ose isolates of bacteria from oblique NA that has been rejuvenated then in a streak quadrant on NA media in a petri dish. NA media in petri dishes is then incubated at 37oC for 24 hours. Macroscopic examination of bacterial isolate colonies can be seen from the color, shape and edges of the colony.

2. Cell Morphology Examination

The identification of bacterial isolates was guided by the flowchart of Bergey's manual of determinative

bacteriology (1948). Identification of bacterial isolates includes examination of cell morphology, namely Gram staining and spore staining. Fermentation of bacterial isolates on MSM media.

Bacterial isolates from oblique NA media were inoculated into 10 ml liquid NB media then incubated with a 150 rpm speed shaker incubator at 37oC for 24 hours. 1 ml of the result of the first NB inoculation is transferred to the second NB media with a volume of 9 ml using a sterile pipette. Furthermore, incubated using a shaker incubator at a speed of 150 rpm at a temperature of 37oC for 24 hours. Next is to measure Optical Density (OD) to have an OD turbidity (absorbance) of 1.5 using a 580 nm spectrophotometer (Fernandes, *et al.*, 2007).

The results of incubation on the second NB media were transferred by 1 ml into 9 ml of MSM solution. Mineral Salt Medium is then fermented at a temperature of 37 ° C for 72 hours at a speed of 150 rpm using a shaker incubator. The fermented broth that has been produced is then taken as much as 10 ml to be centrifuged. Centrifugation is carried out at a speed of 5000 rpm for 30 minutes with a temperature of 4oC. The centrifuged supernatant is then stored in a microtube.

Biosurfactant Activity Test Supernatant Bacterial Isolate Fermentation Broth

1. Emulsification index

The emulsification index test was carried out by mixing 2 ml of kerosene with 2 ml of fermented broth supernatant for 2 minutes. The stability of the emulsion is determined by measuring the height of the emulsion layer after 24 hours. The emulsification index is calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100 (Barakat, *et al.*, 2017).

$$E24 = \frac{\text{Tinggi lapisan emulsi (cm)}}{\text{Tinggi total campuran (cm)}} \times 100\ \%$$

2. Drop collapse test

The drop collapse test is performed in a test tube. Each tube is filled with 1 ml of paraffin liquid, then 20 microliters of supernatant fermented broth is carefully placed into the tube that has contained paraffin liquid. The drop form is visually checked after 1 minute. The collapse and widening of the fermentation broth supernatant on the surface of the oil shows positive results indicating the presence of biosurfactants (Peele, *et al.*, 2016).

3. Oil Spreading Technique.

This technique is done by applying 50 ml of aquades in a petri dish. Next 80 microliters of motor oil dripped in the middle of the cup that has contained aquaades. Then add 10 microliters of supernatant fermented broth over the surface of the oil. A positive result is shown by the formation of a clear zone after dripping with a bacterial fermentation broth supernatant. The diameter of the formed clear zone is then measured using a caliper. (Silva, *et al.*, 2018).

4. Parafilm Test

The parafilm test was carried out by dripping as much as 25 microliters of fermented broth supernatant over the parafilm. Negative control using MSM solution. The test result is positive when the droplet shape of the supernatant expands beyond the negative control (Morita, *et al.*, 2006).

Statistical Analysis

Data analysis was carried out descriptively based on observations of bacterial colony morphology, Gram

staining, spore staining, and Biosurfactant test.

RESULT AND DISCUSSION

Morphological Identification of bacterial isolates

Figure 1 shows colonies of isolates are milky white thatthe results of the morphological identification of bacterial isolate colonies are round-shaped colonies with smooth edges, and elevated elevation.

Figure 2 shows bacterial isolates were incubated in an aerobic incubator to see the ability of bacteria to grow under aerobic conditions. Generally, bacteria are able to grow in aerobic and anaerobic conditions.

Figure 3 shows The results of observations of bacterial isolate cells showed the shape of the rod and the Gram stain showed Gram positive. The results of spore staining using Malachite green and Safranin showed that there were green endospores in the subterminal and red vegetative cells.

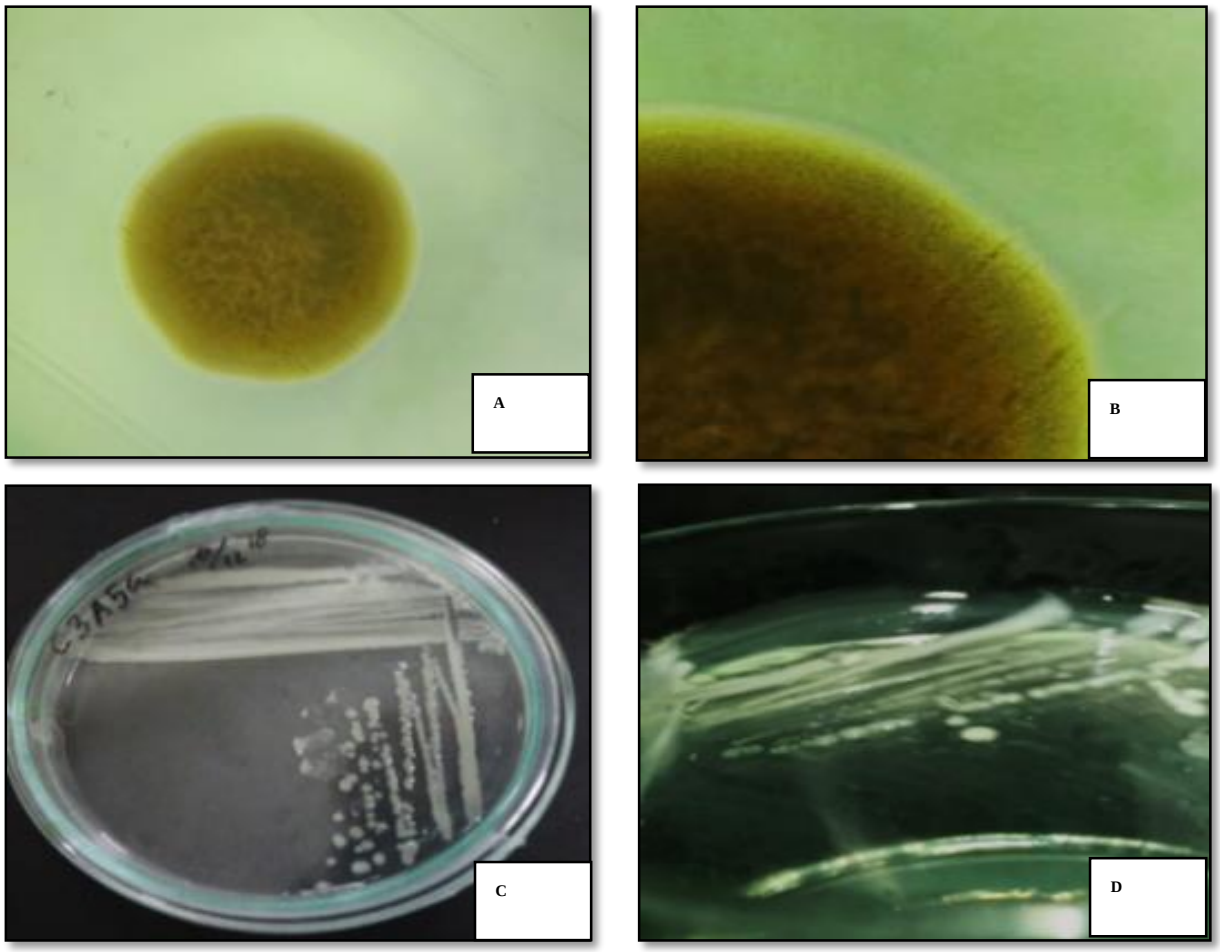

Figure 1. Colony Form Bacterial isolate. Remarks : (A) Form of 24-hour old isolate colony (40x magnification); (B) colony edge shape (40x magnification); (C) Isolate colony color and (D) colony elevation.

Figure 2. Bacterial isolates grown in aerobic incubators.

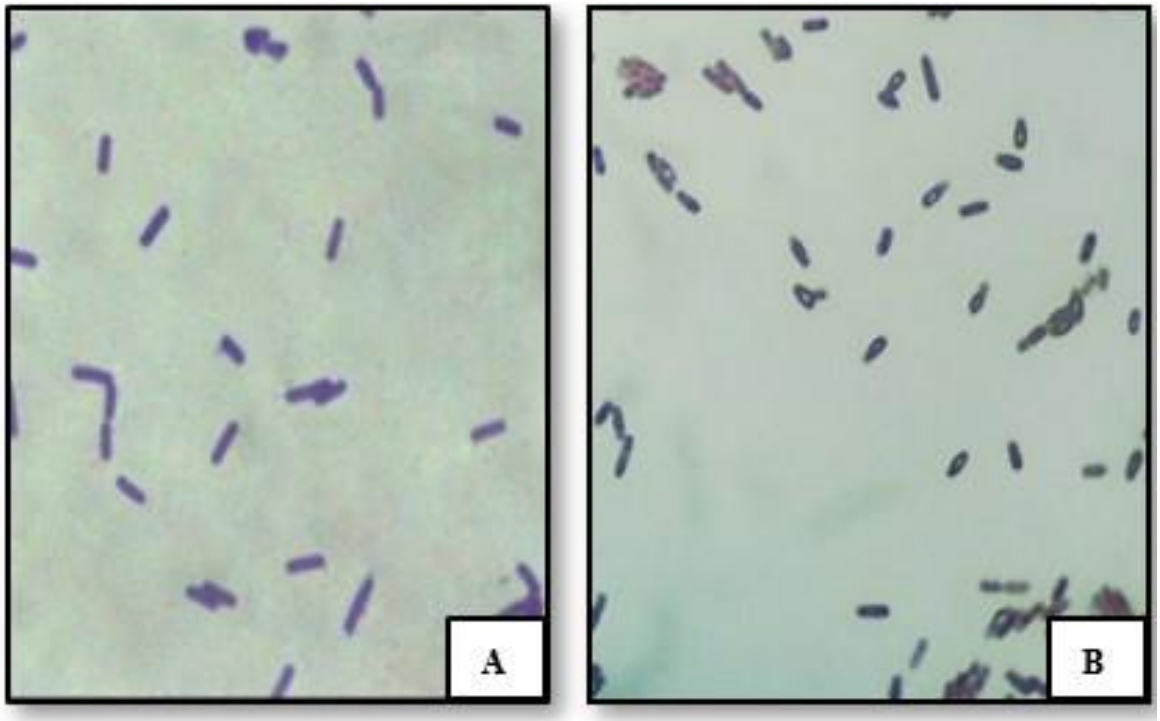

Figure 3. Gram staining and spore staining of bacterial isolates. Description: (A) Gram staining result (1000x magnification) ; (B) Spore staining result (1000x magnification).

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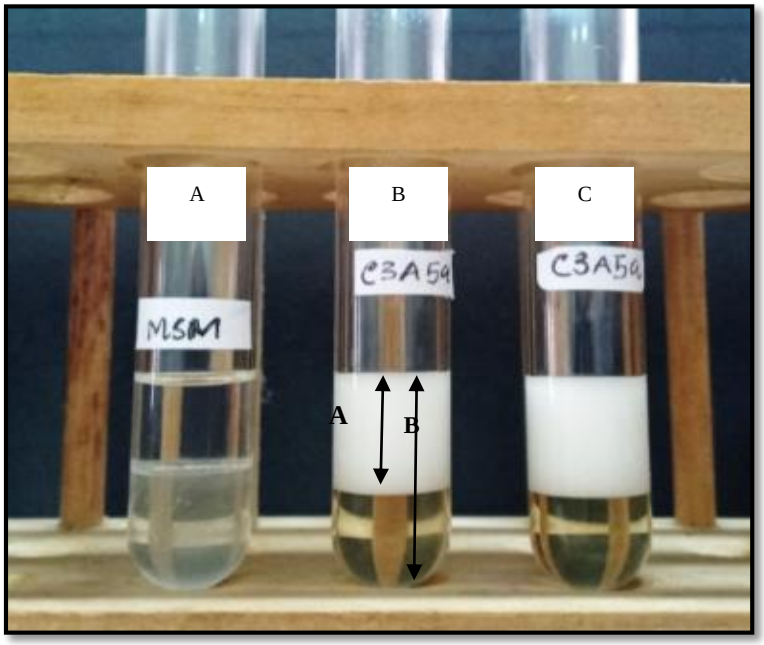

Figure 4. Emulsification index (E24) test of bacterial isolate. Remarks : (A) Negative control of MSM solution; (b) first repetition; (C) second test.

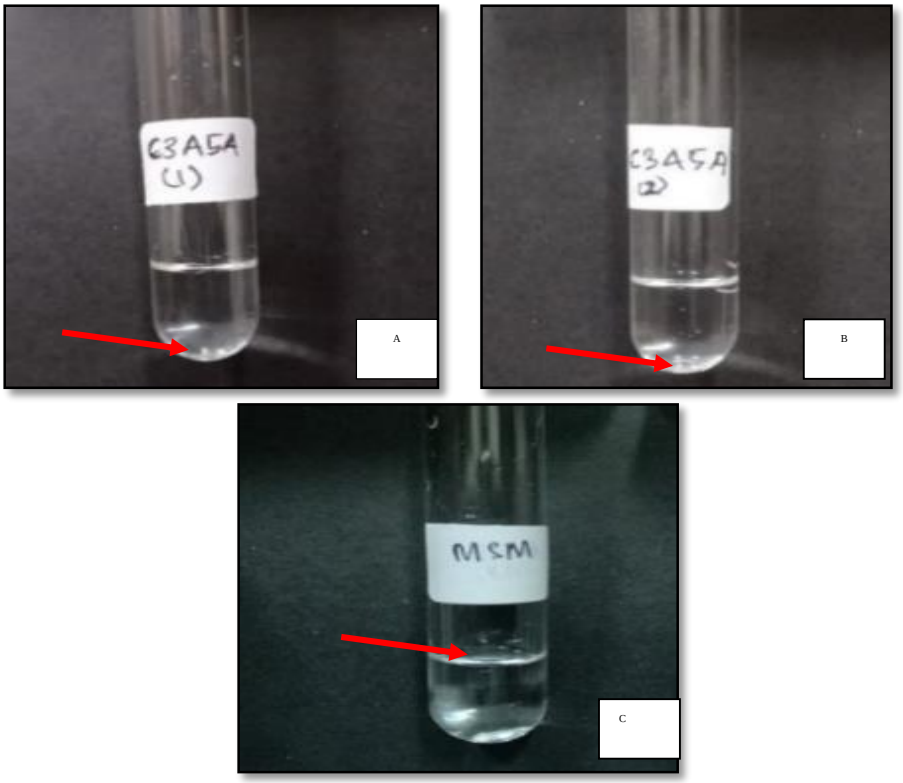

Figure 5. Drop collapse test of bacterial isolates. Remarks : (A) first test; (B) second deuteronomy ; (C) MSM Solution (Control). Remarks : the arrow shows a droplet of supernatant fermented broth bacterial isolate.

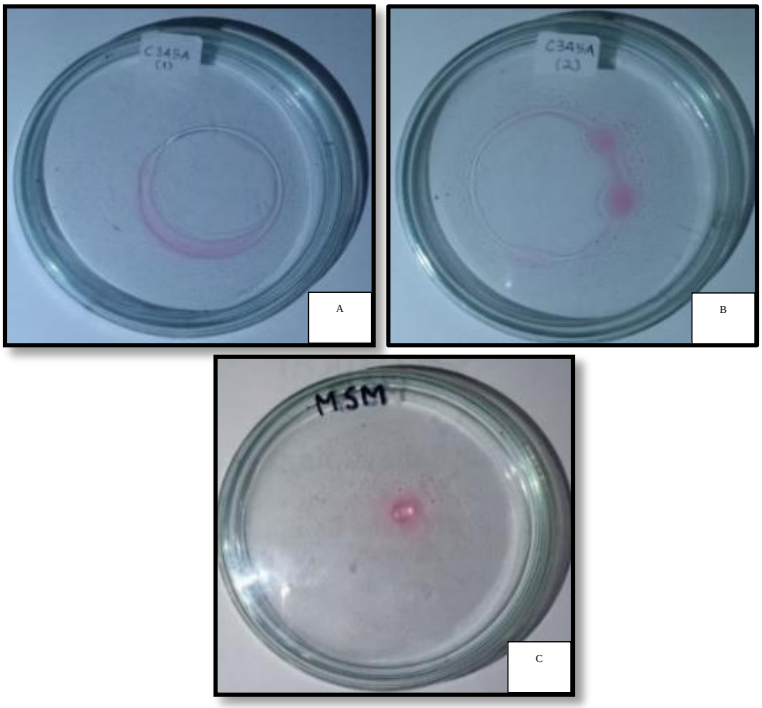

Figure 6. Oil spreding test of bacterial isolate. Remarks : (A) First test; (B) second deuteronomy ; (C) negative control of MSM solution.

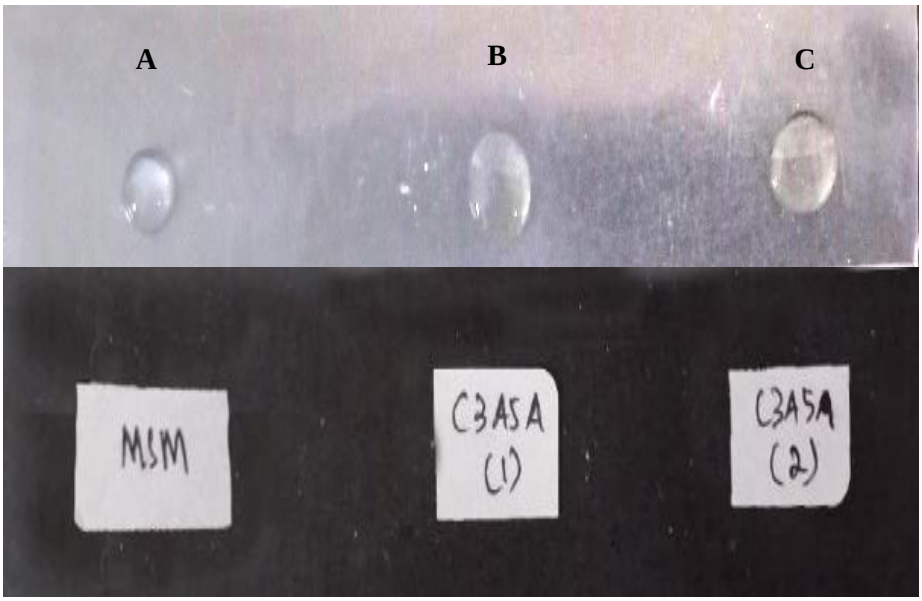

Figure 7. Parafilm test of bacterial isolates. Remarks : (A) MSM solution; (B) supernatant of fermented broth of the first repeat bacterial isolate; (C) supernatant of fermented broth bacterial isolate second repeat.

Table 1. Emulsification value measurement index (E24) bacterial isolate

Bacterial isolates	High emulsion (mm)	High solution (mm)	E24 (%)
Deuteronomy 1	17,93	33,08	54,20 %
Deuteronomy 2	18,30	33,52	54,59%
Negative Cintrol (MSM)	0	33, 24	0
Mean			54,39%

Table 2. Clear zone diameter in oil spreading test

Bacterial Isolates	Diameter Clear Zone (mm)	Negative Control (MSM) (mm)
Deuteronomy 1	52, 81	0
Deuteronomy 2	56,85	0
Mean	54,83	0

Table 3. Measurement of supernatant droplet diameter area in parafilm test

Bacterial Isolates	Droplet Diameter Area (mm)	Control MSM (mm)
Deuteronomy 1	8,2	6,72
Deuteronomy 2	7, 84	6,72
Mean	8.02	6,72

Bacterial Isolate Biosurfactant Activity Test

1. Emulsification index test

The Emulsification index test is intended to determine the ability of biosurfactants to emulsify liquids of different polarities. The emulsification index is related to the concentration of biosurfactants, because the smaller the concentration of biosurfactants, the ability of the compound to emulate crude oil is also reduced (Walter, *et al.*, 2010).

Figure 4 shows the results of the emulsification index test of bacterial isolates showed that there was biosurfactant activity characterized by the formation of emulsions in test tubes.

Table 1 shows the height of the emulsion formed is indicated by the height of layer A in figure 4.4 and the height of layer B indicates the overall amount of liquid in the test tube.

2. Drop collapse test

The drop collapse test is based on the ability of biosurfactants to destabilize liquid droplets. The presence of biosurfactants is indicated by the form of oil droplets that widen after adding bacterial culture supernatants. (Walter, *et al.*, 2010).

Figure 5 shows the results of the Drop collapse test of bacterial isolates showed that there was biosurfactant activity indicated by the fall and widening of the bacterial isolate fermentation broth supernatant at the bottom of the tube, while in the negative

control (MSM) the supernatant did not fall and did not expand.

3. Oil spreading test

The oil spreading test is one of the tests to determine the biosurfactant activity of bacterial isolates. The splitting of the oil film on the surface of the medium indicates the presence of biosurfactants in the supernatant tested.

Kurniati (2016) explained that if the concentration of surfactant has exceeded a certain threshold called critical micelles (CMC), surfactant monomers contained in water will form micelles. This condition will make hydrophobic compounds can enter the hydrophobic core of the micelles so that dissolution occurs. This mechanism is suspected to have caused the rupture of the oil film at the time of testing.

Figure 6 shows the results of the Oil spreading test of bacterial isolates show that there is biosurfactant activity characterized by the formation of clear zones on the oil surface.

Table 2 shows the data from the measurement of the clear zone that the diameter of the clear zone is formed when a supernatant of bacterial isolate fermentation broth is dripped in the middle of the oil and water surface.

4. Parafilm Test

Parafilm tests are carried out by dripping bacterial fermentation supernatants on the surface of hydrophobic parafilms then the hatching diameter is measured using a caliper. The measurement results of bacterial fermentation supernatants were compared with MSM droplet diameters as a negative control. If the droplets of the supernatant dilate larger than the negative control indicates that the supernatant contains biosurfactants (Morita, *et al.*, 2006).

Figure 7 shows the results of biosurfactant activity characterized by the widening of supernatant droplets of bacterial isolate fermentation broth on the surface of the parafilm. Negative control using MSM solution as comparison of droplet area diameter of bacterial isolate supernatant droplets.

Table 3 shows the measurement data are presented. Observation of parafilm test results was carried out after 1 minute of dripping test supernatant on parafilm.

CONCLUSION

Based on macroscopic and microscopic observations, bacterial isolates from mangrove sediments from Ujung Pangkah, Gresik Regency were identified as the genus Bacillus sp.The results of morphological identification of bacterial isolate colonies obtained round colony shapes with smooth edges, elevated elevation and milky white isolate colonies. The results of the identification of bacterial isolate cells obtained rod-shaped, Gram positive and spore-spored cell morphology. Biosurfactant activity test of bacterial isolate by Drop collapse, Oil spreading, emulsification index and parafilm test showed positive results. This indicates that bacterial isolates have the potential to produce biosurfactants.

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Author Contribution

R.S, L.M, and M.P conceptualization. W.T and S.C contributed to the animal study D.R, R.S, and S analyzed data and draft preparation. R.S and M.P writing review and editing. All author have read and agreed to the published version of the

manuscript.

Competing Interest

The authors declare that there is no conflict of interest.

Ethical Approval

In vitro studies or studies of bacteria do not require the approval of an institutional ethics committee.

Data Availability

The article includes data that was used to support the study's conclusions.

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