Isolation, Characterization, and Identification of Biosurfactant-Production Bacteria from Used Cooking Oil Waste

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Received: 30 July 2025, Revised: 10 September 2025, Available Online: 15 October 2025

Abstract *– Waste cooking oil constitutes a significant environmental concern in Indonesia. It has the potential to be utilized as a substrate for biosurfactant-producing bacteria. Biosurfactants are amphiphilic compounds produced by microorganisms, containing both hydrophilic and hydrophobic components that enable them to dissolve in both water and fats, as well as to reduce surface tension. This study aims to isolate, characterize, and identify biosurfactant-producing bacteria from waste cooking oil. Isolation proccess was conducted using Mineral Salt Medium enriched with cooking oil as an inducer and Carboxy Methyl Cellulose as a carbon source and emulsifier. Two isolates were successfully obtained and purified. Their biosurfactant-producing ability was evaluated through drop collapse test and oil spreading test. One isolate demonstrated superior performance, showing positive activity in both tests, including a 0.7 cm oil spreading zone. Identification involved macroscopic observation, Gram staining, endospore staining, and a catalase test. The isolate exhibited characteristics of a Gram-positive, rod-shaped, spore-forming, catalase-positive bacterium with small, circular, milky-white colonies and a smooth surface. This identification is non-molecular and based on a phenotypic approach according to Bergey's Manual for preliminary bacterial classification. Based on these features, the isolate is presumed to belong to the genus Bacillus, known for its robust biosurfactant production. The findings suggest that waste cooking oil can be a promising source of indigenous biosurfactant-producing bacteria, contributing to both microbial conservation and potential development of eco-friendly biotechnological products.*

Keywords: *biosurfactant, waste cooking oil, isolation*

INTRODUCTION

Waste cooking oil is one of the serious environmental issues in Indonesia. Indonesian people tend to favor fried foods, which leads to relatively high consumption of cooking oil. According to the ICCT (The International Council on Clean Transportation), Indonesia produces approximately 715.000 tons of waste cooking oil/year.

Repeated use of cooking oil can generate harmful compounds, including heavy metals. When discharged into waterways or directly into the environment, waste cooking oil can cause environmental pollution, disrupt aquatic ecosystem balance, and damage the habitats of aquatic organisms. Additionally, waste cooking oil can lead to soil contamination, affect nutrient availability for plants, and harm the soil microbial ecosystem.

Waste cooking oil has potential as a substrate for the growth of bacteria capable of producing biosurfactants. Biosurfactants are amphiphilic compounds produced by microorganisms, either directly within their cell membranes or secreted into the surronding environment (Ambarsari & Chen, 2021). These compounds exhibit dual chemical behavior, being soluble in both water and lipids. Biosurfactants possess both hydrophilic and hydrophobic components and have the ability to reduce surface tension (Amelia & Titah, 2021).

Several bacterial, such as Pseudomonas, Bacillus, and Rhodococcus are capable of utilizing waste cooking oil as a carbon and energy source for growth and biosurfactant production. Biosurfactants exhibit high biodegradability, contributing to the reduction of environmental pollution (Olasanmi & Thring, 2022).

The main advantage of biosurfactants lies in their ability to effectively remove dirt and oil from surfaces and to form stable emulsions between oil and water. This makes biosurfactants highly applicable in the cleaning industry for cleaning equipment and vehicles. In the oil and gas industry, biosurfactants play a role in enhancing oil recovery from hard-to-reach reservoirs. In environmental applications, these compounds are used in bioremediation processes to addres soil and water contamination by oil and hydrocarbons (Chanif et al., 2017).

This research aims to isolate, characterize, and identify biosurfactant-producing bacterial strains from waste cooking oil.

MATERIALS AND METHODS

This study was conducted in the Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Neger Semarang. The research was carried out from October to November 2024.

1. Materials

The tools used in this study included an autoclave, incubator, microscope, test tubes, Erlenmeyer, petri dish, pipette, object glass, micropipette, ose, spirit burner, analytical balance, centrifuge, and filter paper. The materials used are Mineral Salt Medium (composed of 7 grams of $NaNO_3$; 1 gram of K_2HPO_4; 0,5 grams of KH_2PO_4; 0,1 gram of KCl; 0,5 grams of $MgSO_4$; 0,001 gram of $CaCl_2 \cdot 2H_2O$; 0,001 gram of $FeSO_4$; 2 L of aquadest; and 0,1 gram of yeast extract), Gram staining reagents, malachite green, oil, parafilm, hydrogen peroxide, agar, cooking oil, and carboxymethyl cellulose.

2. Method and research design

This research is quantitative descriptive study involving a series of methods and techniques for data collection. The primary methods used in this study include bacterial isolation, drop collapse test, oil spreading test, Gram staining, endospore staining, catalase test, and morphological observation. The data obtained are presented in descriptive form and supported by images.

3. Procedures

a. Isolation

The waste cooking oil samples were obtained from household waste. The samples were then filtered to remove large particles and homogenized. A -liter Erlenmeyer was filled with 1 liter of Mineral Salt Medium (MSM) and aquadest up to 1 liter. The medium was then sterilized in autoclave at 121°C for 15 minutes. Cooking oil and carboxymethyl cellulose (CMC) were subsequently added and heated until homogenized. Solid medium was prepared by adding agar to liquid MSM medium, followed by sterilization in autoclave at 121°C for 15 minutes. The sample was inoculated into the liquid MSM medium in test tubes. A total of 200 µL of the sample was inoculated into the solid medium, which had been perforated in the center with a 5 mm diameter cork borer. All samples were incubated at 36°C in incubator for 48 hours. The purification of bacterial isolates was performed using the quadrant streak method on solid MSM medium and incubated at 36°C for 48 hours.

b. Characterization

Liquid bacterial culture was transferred into centrifuge tubes using micropipette. The tubes were centrifuged at 45.000 rpm for 45 minutes.

The drop collapse test was performed by preparing a petri dish lined with a sheet of parafilm. A total of 75 µL of oil was dropped into the parafilm surface and allowed to stabilize for 1 hour. Then, 30 µL of supernatant was dropped onto the surface of the oil.

The oil spreading test was conducted by pouring 75 mL of water into a petri dish. Then, 150 µL of waste oil was added to the surface of the water, forming an oil ring. Subsequently, 30 µL of supernatant was dropped into the center of the oil ring.

c. Identification

Gram staining was initiated by aseptically collecting a colony near the clear zone on solid and spread onto object glass. The smear was then heat-fixed over a Bunsen flame. Gram A was applied to the smear and left for 1 minute, followed by the addition of Gram B. The slide was then rinsed with Gram C for 20 seconds. Gram D was added and allowed to air dry. A drop of immersion oil was added to the smear and observed under a microscope. For endospore staining, the smear was air-dried and heat-fixed over a Bunsen flame.

Endospore staining was initiated by air-drying the smear and heat-fixing it over a Bunsen flame. Malachite green solution was excessively applied to the smear and left for 1 minute. The smear was then heated over a Bunsen flame until the dye evaporated, for approximately 30 seconds. The slide was rinsed with running water and air-dried, then counterstained with safranin for 30 seconds. The smear was rinsed again with running water, dried, and observed under a microscope.

The catalase test was performed by aseptically collecting a colony and spreading it onto object glass. The smear was then heat-fixed over a Bunsen flame. One drop of hydrogen peroxide was added to the smear.

4. Data analysis

The technique used to analyze the data obtained from this study was descriptive analysis. The results from all conducted tests were recorded and analyzed descriptively. The collected data included morphological characteristics and various traits of bacteria with potential for biosurfactant production.

RESULT AND DISCUSSION

1. Bacterial Isolation

The presence of biosurfactant-producing bacteria in the liquid MSM medium is shown in Figure 1.

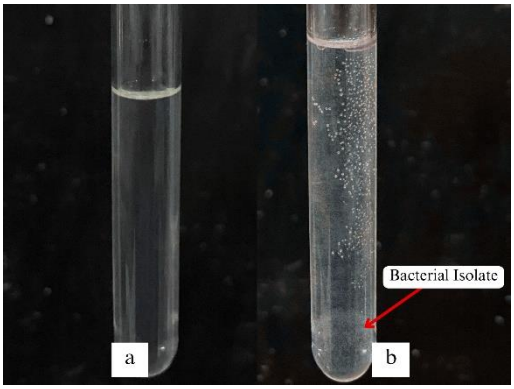

Figure 1. Difference between (a) Liquid MSM medium and (b) Colonies in liquid MSM medium

Figure 1 (b) demonstrates that the MSM medium became turbid and contained small bubbles. These small bubbles are presumed to be dispersed waste cooking oil. Biosurfactants are molecules capable of reducing surface tension and forming stable emulsions, allowing the dispersion of oil within the medium (Santos et al., 2016). The turbidity of the medium is caused by the presence of bacterial cells.

The presence of biosurfactant-producing bacteria in solid MSM medium is shown in Figure 2.

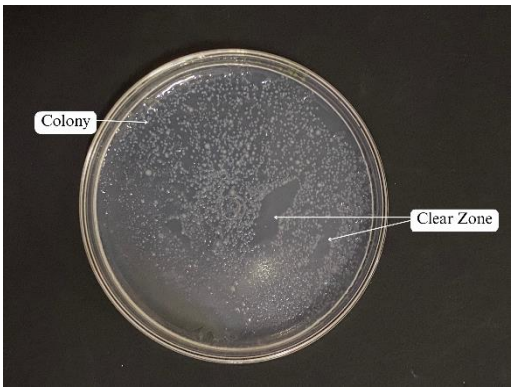

Figure 2. Isolation Results of Samples in Solid MSM Medium

Figure 2 shows colonies spreading across the solid MSM medium after an incubation period of 2 x 24 hours. A clear zone formed around the colonies on MSM indicates biosurfactant activity, as these compounds can reduce surface tension and solubilize the substrate, making the area around the colony transparent. In ordinary bacteria, only colonies are formed without any changes to the medium, so no clear zone appears. Potential biosurfactant-producing colonies are selected based on the presence of a clear zone around the colony on medium, since this indicates extracellular activity related to surfactant production.

2. Characterization

a. Drop Collapse Test

The results of the drop collapse test can be seen in Figure 3.

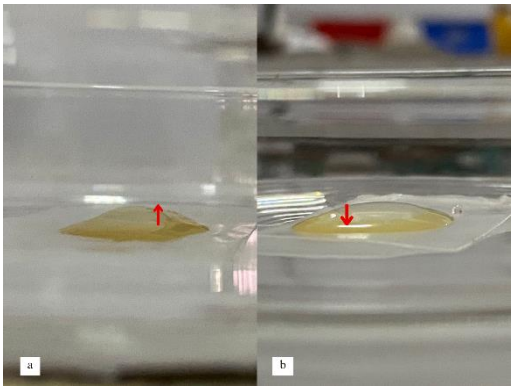

Figure 3. Drop Collapse Test Results (a) Control (b) Sample Supernatant Treatment

Figure 3 shows a difference in droplet height. Biosurfactants possess amphiphilic properties, meaning they contain both hydrophilic and hydrophobic regions. In Figure 3 (a) the control, where aquadest was used, the droplet appears convex. This is due to the hydrophobic

nature of the oil, which cannot interact with the hydrophilic nature of the aquadest. In this condition, cohesive forces (the attraction between like particles) are stronger than adhesive forces, thus surface tension is not reduced.

In contrast, Figure 3 (b) shows a droplet that flattens after being treated with the supernatant. The hydrophilic part of the biosurfactant in the supernatant interacts with the hydrophilic side of the aquadest, while the hydrophobic part binds to the hydrophobic side of the oil. This interaction balances adhesive and cohesive forces, resulting in decreased surface tension, which is evident in the flattened shape of the droplet. This confirms the ability of biosurfactants to spread droplets, demonstrating their effectiveness in lowering surface tension (Shokouhfard et al., 2015).

b. Oil Spreading Test

The results of the oil spreading test can be seen in Figure 4.

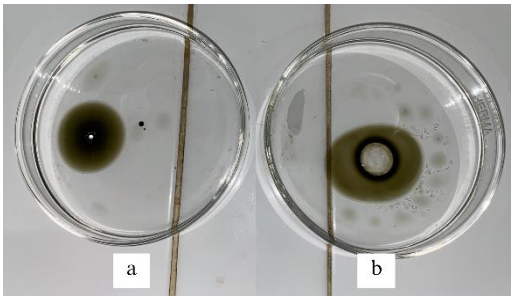

Figure 4. Oil Spreading Test Results (a) Control (b) Sample Supernatant Treatment

A significant difference can be observed in Figure 4 between the used oil substrate treated with aquadest and the one treated with supernatant. The oil spreading test is as simple rapid method to detect biosurfactant activity by observing the displacement zone formed on the surface of used oil. The formation of a clear zone is an indication of the presence of biosurfactants (Thomas et al., 2022). This zone is created due to the interaction between the hydrophilic and hydrophobic regions of the supernatant, which generates surface pressure and reduces the surface tension of the used oil, resulting in a visible spreading zone.

Figure 4 (a) the control treatment using sterile aquadest, which demonstrates no change. Sterile water is unable to reduce surface tension and thus does not affect the interaction between water and the used oil substrate. As a result, the oil remains concentrated in a small area without any spreading.

In contrast, Figure 4 (b) displays spreading zone of used oil on the water surface with a diameter of 0.7 cm. This diameter indicates moderate biosurfactant activity, possibly influenced by the biosurfactant concentration, incubation time, or induction medium effectiveness. Clear zone diameters greater than 0.5 cm were reported among active biosurfactant-producing strains (Ray et al., 2021). After the supernatant was applied to the surface of water containing used oil, a spreading zone became visible. This indicates that the supernatant contains biosurfactants capable of reducing surface tension between oil and water, allowing the oil to spread. Biosurfactants are known to reduce both surface and interfacial tension between oil and water phases (Shaimerdenova et al., 2024).

3. Identification

a. Macroscopic Observation

The macroscopic difference between MSM liquid medium before and after inoculation with biosurfactant-producing bacterial samples can be observed in Figure 5.

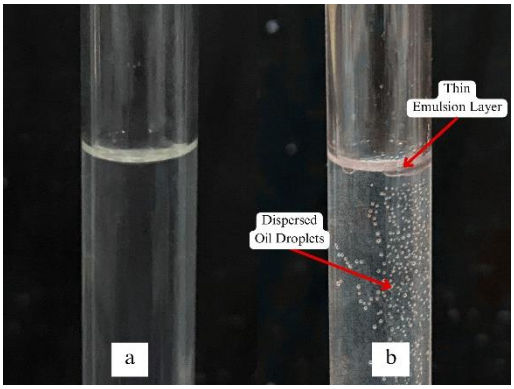

Figure 5. Macroscopic Differences (a) MSM liquid Medium (b) Colonies in MSM Liquid Medium

Figure 5 (a) shows the appearance of the MSM liquid medium before inoculation, where the medium appeared clear, colorless, and free of sediments or surface changes. In contrast, Figure 5 (b) the MSM medium after inoculation and incubation. The medium became turbid, indicated microbial growth. Additionally, dispersed oil droplets and a thin oily film were observed on the surface of the lipid medium.

Biosurfactant-producing bacteria are capable of dispersing oil droplets in aqueous media due to the amphiphilic nature of biosurfactants. These molecules help mix two immiscible phases, such as oil and aquadest by reducing surface tension and stabilizing emulsions. This explains the information of a thin emulsion layer on the surface of the medium following incubation.

The results of bacterial isolation on solid MSM medium are shown in Figure 6.

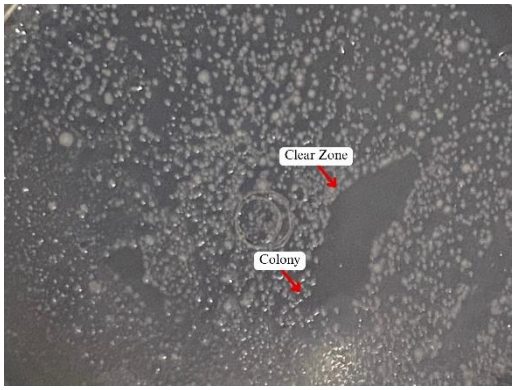

Figure 6. Bacterial Colonies on Solid Medium

Figure 6, the bacterial colonies appear milky white, small, and round with a mucoid texture on the surface of the solid medium. The colonies exhibit a shiny appearance and smooth surface, which are characteristic features of biosurfactant-producing bacteria. A clear zone was observed surrounding the colonies on the solid medium. The clear zone indicates bacterial activity, as biosurfactant produced by the colonies diffuses into the medium and solubilizes the substrate, unlike non-producer bacteria that only form colonies without altering the surrounding medium.

This observation provides initial visual evidence supporting the presence and potential of the isolated bacteria to produce biosurfactants. The formation of the clear zone around the colonies indicates biosurfactant activity by the bacteria (Chigede et al., 2024).

b. Gram Staining

The results of the Gram staining can be seen in Figure 7.

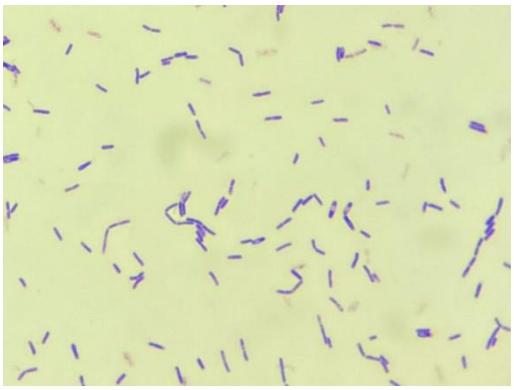

Figure 7. Gram Staining Result

Figure 7 shows bacteria stained dark purple. The bacterial cells appear rod-shaped and are observed as individual cells.

The purple color observed in the staining result indicates that the isolate possesses as thick cell wall with a strong peptidoglycan decolorizer. The composition and surface properties of Gram-positive cell walls, which include lipids and proteins, support biosurfactant production (Nguyen et al., 2020). This morphological characteristic aligns with typical Gram-positive bacteria, which are generally more resistant to extreme environmental conditions and are commonly found in waste that has undergone physical or chemical processes, such as heating.

The results of the Gram staining indicate that the bacteria isolated from waste cooking oil belong to the Gram-positive group. This suggests that the isolates that successfully grew in the selective medium are likely members of the genera Bacillus, Clostridium, Listeria, or Rhodococcus. Gram-positive bacteria, particularly Bacillus, are well known as producers of lipopeptide-type biosurfactants such as surfactin, iturin, and fengycin which exhibit high surface activity and antimicrobial properties (Varjani & Upasani, 2017).

c. Endospore Staining

The results of the endospore staining can be seen in Figure 8.

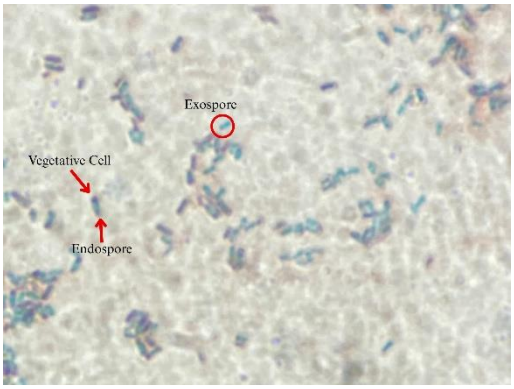

Figure 8. Endospore Staining Result

Figure 8 shows green coloration within and around the bacterial cells, while the red coloration represents the vegetative cells.

The differential staining using malachite green and safranin works based on differences in the permeability of endospore and vegetative cell walls. Endospores, which have thick protective walls, are stained with malachite green through heating to allow dye penetration and retention. After rinsing, the vegetative cells that do not retain the malachite green are counterstained with safranin. As a results, endospore appear green, whereas vegetative cells appear red-pink.

Endospore formation is commonly observed in Gram-positive bacteria, particularly those belonging to genera Bacillus and Clostridium. The function of endospores is to protect bacterial genetic material under extreme conditions such as desiccation, high temperature, or chemical exposure (Setlow & Christie, 2023). The presence of endospores in the tested isolates supports the indication that these bacteria are spore-forming species, which are generally known to be capable of biosurfactant production.

d. Catalase Test

The results of the catalase test can be seen in Figure 9.

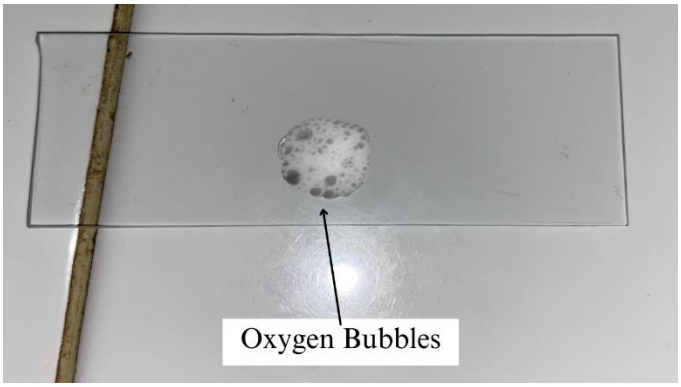

Figure 9. Catalase Test Result

Figure 9 shows the presence of air bubbles. The formation of air bubbles indicates that the bacteria possess active catalase enzymes. The presence of catalase in biosurfactant-producing bacteria may aid in their survival under oxidative environmental conditions. The catalase test is used to determine bacterial oxygen requirements. The appearance of bubbles signifies that the bacteria are aerobic and produce catalase.

Catalase is an enzyme that hydrolyzes peroxides or catalyzes the decomposition of hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O) (Kharisma Swandi et al., 2015). Based on observations, the biosurfactant-producing bacterial isolate from waste cooking oil exhibited a positive catalase reaction, which is typical of bacteria from the genus Bacillus. These bacteria are capable of breaking down hydrogen peroxide, classifying them as aerobic bacteria. This result is consistent with the general characteristics of biosurfactant-producing bacteria, which require oxygen for their metabolic processes.

e. Identification Based on Bergey's Manual

According to the Ninth Edition of Bergey's Manual of Determinative Bacteriology, the combination of three physiological characteristics—Gram-positive staining, endospore formation, and catalase-positive activity—suggests that the isolate belongs to the genus Bacillus. This genus is

well-known for producing lipopeptide-type biosurfactants such as surfactin, iturin, fengycin which exhibit strong surface activity and antimicrobial properties (Zia & Linda, 2023). Therefore, these physiological identification results support the hypothesis that the isolate possesses significant biotechnological potential, particularly for biosurfactant production from local waste sources.

CONCLUSION

Based on the results of this study, it can be concluded that two biosurfactant-producing bacterial isolates were successfully obtained from waste cooking oil. These bacteria exhibited emulsifying activity, as indicated by the spread of supernatant droplets on the substrate surface in drop collapse test and the formation of clear zone in the oil spreading test. The isolates are presumed to belong the genus Bacillus, characterized as Gram-positive, rod-shaped cells, positive for endospore and catalase test, with small, round, smooth-textured colonies growing on the surface of solid media.

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