Biosurfactant production and diesel-fuel removal by isolated bacteria from Kalimas Estuary, Madura Strait

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Abstract. This experiment was done to evaluate isolated bacteria in the production of biosurfactant and biodegradation of diesel-fuel. Diesel-fuel degrading bacteria were isolated from Kalimas Estuary, Surabaya using three-stage enrichment culture in Bushnell Haas Mineral Salts medium with 1% diesel fuel. Purified isolates were subjected to both oil-displacement and E24-emulsification tests to assess the high activity of produced biosurfactant. Selected isolates were next used in biodegradation analysis of diesel-fuel using a gravimetric total petroleum hydrocarbon test. The potential isolates were identified based on their 16S rDNA sequences. Twenty-eight purified isolates were obtained from the water samples and confirmed as gram-negative hydrocarbonoclastic bacteria. Among them, isolate K8 demonstrated the widest diameter on diesel-fuel displacement test by 3.5 cm of oil-free zone. It was also superior in emulsifying diesel-fuel with 9.4% of E24 index. Isolate K8 was capable of degrading 57% of 1 mL of diesel fuel within 14-day incubation. The isolate was potential in both producing biosurfactant and degrading diesel fuel. Isolate K8 was identified as Pseudomonas aeruginosa with 99.74% sequence similarity.

Keywords: diesel fuel, hydrocarbonoclastic bacteria, Kalimas Estuary

1. Introduction

Hydrocarbon is ranked as one of the most dangerous pollutants today due to its difficulty to control. Its pollution in the aquatic environment has become a global problem in both developed and developing countries [1]. The total volume of world's oil spill to the ocean recorded in a last decade was approximately 1,000 tonnes per year. This number is indeed much lower than the two previous decades which reached ten times [2]. In Indonesia, marine oil spill accidents are quite frequent and several major cases were destructive to the marine and coastal ecosystem. The People's Coalition for Fisheries Justice (KIARA) research and development center recorded significant 37 oil spills in the last two decades and one of the worst occurred recently in Balikpapan Bay which spilled around 5,600 tonnes oil [3,4].

For an archipelagic country, ocean transports are very popular for delivering cargos, people, fisheries catch as well as crude oil and its derivatives. Those activities make Indonesian seawater susceptible to hydrocarbon contamination from spilled oil and fuel. Moreover, seawater also receive hydrocarbon load from land-activities carried by river runoffs. Wastes from anthropogenic activities such as petroleum-based lubricants, municipal and industrial discharge, and automobile wastes are main sources of hydrocarbon pollution into river water bodies [5]. One of the rivers categorized as
polluted is the Kalimas River that flows among densely populated and industrial areas at Surabaya. The river with a length of 13.7 km is a branch of the Brantas River, the longest river at East Java. Runoff of polluted Kalimas River meets Madura Strait seawater at the Kalimas Estuary [6].

Certain bacteria have the ability to degrade hydrocarbons by consuming them as nutrients that can be utilized by the cells. The energy contained in the hydrocarbon bonds can be converted by the cells to ATP as the energy source for cellular processes. In addition, it can also be used as a carbon source for biosynthesis in building cell components. This ability is an advantage for humans as a promising solution to overcome hydrocarbon contamination in the environment [7].

Many studies have been carried out to obtain potential bacteria that are effective to degrade hydrocarbons. Various types of bacteria were isolated from several polluted locations on land and in the sea, however, the estuary location has received little attention [8,9,10]. In this study, hydrocarbon degrading bacteria were isolated from estuary of the Kalimas River. The estuary has unique characteristics since it is a meeting area of pollutants brought from the river and hydrocarbons from adjacent seaport. We used diesel fuel as a model of hydrocarbon since it is liquid (C9-C25) and stable at ambient temperatures with boiling point temperature of 200-350°C [11]. The isolated bacteria were assessed for their properties on producing biosurfactant and degrading diesel fuel.

2. Material and Method

2.1 Water Sample Collection
Water samples were taken from the surface of three different locations at the Kalimas Estuary, Surabaya and stored on ice during transport to the laboratory. The water physico-chemical parameters as pH, temperature, salinity, and conductivity were measured on site.

2.2 Isolation of Diesel Fuel Degrading Bacteria
Ten milliliters of seawater samples were transferred in 100 mL of Bushnell Haas Mineral Salts (BHMS) medium with the composition/liter: 0.2 g MgSO4.7H2O; 0.02 g CaCl2; 0.2 g KH2PO4; 1.8 g K2HPO4; 1 g NH4NO3; 0.05 g FeCl3 and 2% NaCl (pH adjusted to 7.0 ± 0.1). Diesel fuel was added as much as 1% (v/v) as the only carbon source. The inoculated medium was rotary-incubated at 28°C, 120 rpm for 7 days. The culture (10 mL) were then inoculated into 100 mL of BHMS medium under the same medium composition and incubation condition. This enrichment procedure was continued until the third stage. The culture was spread on Bushnell Haas Mineral Salts Agar (BHA) medium pre-added with 25 μL of diesel fuel and then was incubated at 28°C for five days [12]. Morphologically different bacterial colonies were subcultured by streaking on BHA medium pre-added with 25 μL of diesel fuel. Colony purification was accomplished by quadrant streaking on Nutrient Agar medium for more distinction than cultured on BHA medium.

2.3 Diesel Fuel Displacement Test
Bacterial isolates were prepared by subculturing the purified colonies into 20 mL Nutrient Broth (NB). Optical densities of the isolates were then equalized according to the smallest value. Two milliliters of the cultures were inoculated into 18 mL NB and rotary-incubated for 24 hours. The cultures (10 mL each) were centrifuged at 6000 g, 4°C for 15 minutes. Each supernatant as much as 20 μL was dripped into a petri dish containing 20 mL of distilled water and 50 μL of diesel fuel covering the water surface. If biosurfactant is present in the supernatant, diesel fuel will displaced to form an oil-free clearing zone [13].

2.4 Diesel Fuel Emulsification Test
The capacity of bacterial isolates to form oil emulsions was determined using the emulsification index (E24) according to Budsabun (2015) and Thavasi et al. (2011) with modifications [13,14]. Experimental design used was a completely randomized design (CRD) with one-way ANOVA
analysis (a = 0.05) and done in triplicate. The bacterial cultures were prepared and centrifuged as in the previous step. The supernatant as much as 4 mL was transferred into a test tube and followed by 6 mL of diesel fuel. The tube was vortexed at high speed for 2 minutes and the mixture was left to stand for 24 hours at 28°C. The emulsification index (E24) was calculated from the ratio of the height of the emulsion layer to the total height of the solution [7].

2.5 Generating Bacterial Growth Curves
Growth curve was prepared by inoculating bacterial isolate into BHMS medium with 1% (v/v) diesel fuel and rotary-incubated for 72 hours. Optical densities of the isolates were then equalized according to the smallest value. Six milliliters of bacterial culture were transferred into 54 mL of BHMS medium with 1% (v/v) diesel fuel. The culture bottles were then incubated on a rotary-incubator at 28°C, 120 rpm, for 5 days. Every certain time the culture was sampled and subjected to measurement of cell optical density using a spectrophotometer. The data were converted to cell number according to a pre-made standard curve.

2.6 Diesel Fuel Degradation Test
The selected bacterial isolates were tested for their activity in degrading diesel fuel. This test used a completely randomized design (CRD) with one-way ANOVA analysis (a = 0.05) and done in triplicate. Isolates were grown in 20 mL BHMS medium with 1% (v/v) diesel fuel then rotary-incubated for 60 hours. Optical densities of the isolates were then equalized according to the smallest value. Ten milliliters of culture were transferred to 100 mL of BHMS medium containing 1% (v/v) diesel fuel. At before and after the addition of 1% (v/v) diesel fuel, BHMS medium and the flask together was weighed to determine the initial weight of the added diesel fuel. The inoculated medium was then rotary-incubated for 14 days. As a control, uninoculated BHMS medium with 1% (v/v) diesel fuel was used. The degraded diesel fuel was determined gravimetrically.

2.7 Gravimetric Analysis of Total Petroleum Hydrocarbons (TPH)
Gravimetric analysis of TPH levels was carried out according to the method of Panda et al. (2013) with modifications[12]. Incubated culture was added with 1 mL of 1 N HCl to stop the bacterial activity. The next step, 50 mL of the sample was mixed with 30 mL of n-hexane in a separatory funnel and shaken for 5 minutes then left to stand until the n-hexane solution was separated from the water phase. The n-hexane layer contains residual diesel fuel that has not been degraded. The water phase at the bottom was removed, while the n-hexane layer was transferred in a 50 mL polypropylene tube using a pipette. The tubes were then centrifuged at a speed of 6000 g, for 15 minutes for further separation. The upper layer (n-hexane) was aspirated using a pipette and passed through a glass filter-funnel covered by double-layered filter papers to which 0.5 g of Na2SO4 has been added to capture the remaining water. The filtrate was collected in a 250 mL flask which its empty weight had been determined. This procedure was repeated until all volume of the medium was extracted. The flask was heated in the oven at 50°C for 48 hours until the n-hexane fully evaporated. The flask was removed from the oven and allowed to cool until it was ready to be weighed to determine the weight of the remaining diesel fuel in it for calculation of degradation percentage.

2.8 Identification of Bacterial Isolates
Selected bacterial isolates used in the degradation test were identified based on the 16S rDNA sequence. The DNA was extracted using FastDNA™ SPIN Kit for Soil, MP Biomedicals, LLC. Part of the gene was amplified using universal primers set of 27f (5'-AGAGTTGATCMTGGCTCAG-3’) and 1492r (5'-TACGGTTACCTTGTTACGATT-3’) [15]. The sequenced fragment was aligned and identified using online BLAST based on NCBI database. A phylogenetic tree was constructed using MEGA 6 with Neighbour-Joining algorithm [16].

3. Result and Discussion
3.1 Physico-Chemical Parameters of Water Samples
The average value of the water samples’ pH, temperature, salinity, and conductivity were 7.1 ± 0.11, 29.8 ± 0.45°C, 20 ± 1.06 ‰, and 32.29 ± 13.93 mS/cm, respectively. The salinity value was positioned within a range of brackish water (5-30 ‰), while the conductivity value was also within the range of brackish water (1-80 mS/cm) [17,18].

3.2 Diesel Fuel Displacement Test
The isolation obtained 28 isolates and all of them were gram negative. The isolates were used on diesel fuel displacement test. Almost all of the isolates could displace diesel fuel on the water surface. The clearing zone diameter varied from 0.5 cm to 3.5 cm but only three isolates had the widest diameter, namely N3, N8 and K8. The wide clearing zone reflects the high concentration of biosurfactant produced [13]. According to Budsabun (2015), most oil-degrading bacteria release biosurfactants to reduce the interfacial tension of water to improve solubility of oil in water phase [14].

3.3 Diesel Fuel Emulsification Test
A capacity to emulsify diesel fuel is one of the prerequisites to improve the rate of oil biodegradation since it can increase the bioavailability of oil to contact the bacterial cell surface. The emulsification test was applied upon produced biosurfactants from the 28 bacterial isolates. Among them, 5 isolates were able to preserve emulsion layer for 24 hours, namely K8, K10, N2, N3 and N4 with variations in the emulsification index of 3.2-9.8% (Figure 1). The highest emulsion index (E24) was demonstrated by N3 (9.8%) and K8 (9.4%). The percentages were actually low when compared to other study that achieved 10-65% using BHMS medium [19]. Based on our results of E24 index and the oil displacement test, N3 and K8 were selected to be used for the next step.

![Figure 1. Emulsification index (E24) of the five isolates.](image)

3.4 Growth Curves of Selected Bacterial Isolates
Growth curves were plotted to determine the time when the bacteria grow optimally in BHMS medium by consuming 1% (v/v) diesel fuel. The growth curves of N3 and K8 is presented in Figure 2. Both of them show a period of exponential growth up to 108 hours so that the middle logarithmic phase was at the 60th hour. Therefore for biodegradation test, 60 hours was used as incubation time during preparation of inoculum.
Figure 2. Growth curves of isolate N3 and K8 in BHMS medium with 1% (v/v) diesel fuel.

3.5 Diesel Fuel Biodegradation Test
The result of diesel fuel biodegradation test was presented in Figure 3. The graph shows that N3 and K8 within 14 days removed 43 and 57% of 1% (v/v) diesel fuel, respectively. The results of a previous study conducted by Panda et al. (2013) demonstrated 50% of 0.5% (v/v) diesel fuel is removed by Pseudomonas aeruginosa for 20 days [12]. Hence, the values expressed by both N3 and K8 make the isolates potential for efficient diesel-fuel removal.

Figure 3. Percentage of diesel fuel degraded by selected isolates in BHMS medium with 1% (v/v) diesel fuel for 14 days.

3.6 Identification of Selected Isolates
Identification of N3 and K8 isolates based on the 16S rDNA sequence revealed both of them were Pseudomonas aeruginosa (Figure 4). The similarity index of the isolates with the closest strain from the NCBI database was 99.83% and 99.74%, respectively. The species was known to be effective in degrading C10-C26 n-alkanes hydrocarbons since it has several key genes such as the alkane 1-monoxygenase gene, rubredoxin, rubredoxin reductase, alcohol dehydrogenase, aldehyde
dehydrogenase and acyl-CoA synthetase [20]. Moreover, released biosurfactants by the bacteria significantly disperse hydrocarbons thereby increasing their bioavailability for bacterial uptake.

Figure 4. Phylogenetic tree of 16S rDNA sequences presents position of N3 and K8 among reference strains. The tree was generated using the Neighbor-Joining algorithm. Bootstrapping values more than 50% are indicated near the nodes. The scale bar corresponds to 1% estimated sequence divergence.

4. Conclusion
Potential hydrocarbon degrading bacteria were isolated from brackish water of Kalimas Estuary. Isolate N3 and K8, both identified as *Pseudomonas aeruginosa*, showed consistent promising results for activity tests of produced biosurfactant and diesel-fuel biodegradation. For the next experiment, the isolates will be assessed for their ability to degrade more complex and toxic hydrocarbon compounds in combination with heavy metals.

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