Screening of biosurfactant producing bacteria from hydrocarbon contaminated soil

T H Kurniati*, S Rahayu, D Sukmawati and W Maharani

Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jl. Pemuda No. 10 Rawamangun, East Jakarta, Indonesia 13220

*trihandayanik@gmail.com

Abstract. Biosurfactants are surface-active molecule produced by microorganisms and have several advantages over the chemical surfactants. The objective of this research was to obtain biosurfactant-producing bacteria from hydrocarbon contaminated soils in Jakarta Bay and to observe their biosurfactant activity through emulsification capacity and surface tension reduction capability. Overall, 19 isolates were screened for biosurfactant production by hemolysis assay, drop collapse assay and oil spreading assay. The result showed that 13 strains gave positive results on the screening tests and were determined as biosurfactant-producing bacteria. Three strains (TKY 3, TKY 14 and TKY 19) had the best biosurfactant activity. The lowest surface tension value was achieved by two strains (TKY 19 and TKY 14) with the same value of 37.84±1.45 mN/m.

1. Introduction
Biosurfactants are the amphiphilic compounds produced by microorganisms. These are produced by a variety of microorganisms such as bacteria, fungi, and yeast. They have both hydrophobic and hydrophilic domains and can decrease the surface tension or the interfacial tension of two different phase system.

Microbial surfactants are more effective and versatile than many synthetic surfactants owing to their selective action, biodegradable nature and stability at high temperature. There are numerous applications of bio-surfactant as paralleled to their chemically synthesised counterparts such as in food, health care and cosmetic, medical industry and also as environment pollution control [1].

In the previous study, isolation of biosurfactant producing bacteria was done from hydrocarbon contaminated soil in the Jakarta Bay region. Based on the isolation results, 19 bacterial isolates were obtained. The bacteria isolated have not yet known for their ability to produce biosurfactant compounds [2]. The advantage of biosurfactants as described above has encouraged researchers to obtain biosurfactant producing bacteria. Through this study, the ability of 19 isolates of these bacteria to produce biosurfactants was investigated.
2. Methods

2.1. Screening of biosurfactant-producing bacteria
Bacterial isolates were screened to get bacteria that were able to produce biosurfactants. The methods used include:

2.1.1. Hemolysis test. Bacterial isolates were streaked on the surface of Blood Agar and incubated at 30°C for 48 hours. Hemolysis activity is indicated by the presence of clear zones around the colony [3].

2.1.2. Drop collapse test. A two μl of used motor oil was applied to the well of 96-well microplate and allowed to equilibrate for 24 h. Bacterial culture, 48 h in Nutrient Broth was centrifuged at 12,000 g for 5 min to remove cells. Five μL of the cell free culture broth was transferred to the oil and with the aid of a magnifying glass the drop size was observed after 1 min [4]. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative. A Tween 20 solution and a distilled water were used as positive and negative control.

2.1.3. Oil spreading test. Sterile distilled water was put into a 10 mL petri dish followed by adding 15 μL of used motor oil to form a thin layer on the surface of water. Supernatant from bacterial culture was then added 10 μL to the oil surface [5]. If the supernatant contains biosurfactant, the used oil motor will separate and form a clear zone.

2.2. Determination of biosurfactant activity by surface tension measurement
Surface tension testing was done by growing bacteria in a mineral salt medium. This medium containing (g/L): 3.0 g of KH₂PO₄, 6.0 g of Na₂HPO₄, 1.0 g of NH₄Cl, 0.5 g of NaCl, 1.0 mL of 1M MgSO₄, and 2.5 mL of a trace element solution (g/L): 23 mg of MnCl₂·2H₂O, 36 mg of CoCl₂·6H₂O, 30 mg of MnCl₂·H₂O, 31 mg of H₃BO₃, 10 mg of CuCl₂·2H₂O, 30 mg of Na₂MoO₄·2H₂O, 20 mg of NiCl₂·6H₂O and 50 mg ZnCl₂ with pH 7.0. The surface tension of the culture supernatant was carried out using the capillary method [6].

2.3. Characterization of biosurfactant-producing bacterial isolates

2.3.1. Macroscopic and microscopic characterization. Colony observation was carried out by growing bacterial isolates in a Nutrient Agar (NA) medium and incubated for 24 hours. The growing colonies were observed in shape, color, edge of the colony and surface of the colony. The cell shape and Gram type of each isolate were observed using a microscope at 1000X magnification.

2.3.2. Biochemical characterization of biosurfactant producing bacteria. The selected isolates were used for biochemical test including Indole production, motility, citrate utilization and sugar fermentation test.

3. Result and discussion
Biosurfactant consist of many types based on their chemical nature, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids [7]. Only single method was insufficient to detect biosurfactant producing bacteria [8]. Therefore, combination of various screening methods is required to understand the ability of microbe in biosurfactant production. Based on screening tests performed, 13 from 19 isolates which give positive result in more than one screening methods considered as biosurfactant producing bacteria (Table 1).
Table 1. Screening result of biosurfactant-producing bacteria.

| Isolates | Screening method |
|----------|------------------|
|          | haemolysis test | oil spreading test | Drop collapse test |
| 1        | -               | +                   | -                 |
| 2        | -               | -                   | -                 |
| 3*       | +               | +                   | +                 |
| 5        | -               | +                   | -                 |
| 6        | -               | -                   | -                 |
| 7*       | +               | +                   | -                 |
| 8        | -               | -                   | -                 |
| 9        | -               | +                   | -                 |
| 10*      | -               | +                   | +                 |
| 11*      | -               | +                   | +                 |
| 12*      | -               | +                   | +                 |
| 13*      | -               | +                   | +                 |
| 14*      | -               | +                   | +                 |
| 15*      | -               | +                   | +                 |
| 16*      | -               | +                   | +                 |
| 17*      | -               | +                   | +                 |
| 18*      | -               | +                   | +                 |
| 19*      | -               | +                   | +                 |
| 20*      | -               | +                   | +                 |

*: bacterial isolates that give positive result on more than one test

The hemolysis test is generally carried out as a pre-elimination screening on bacteria to determine its ability to produce biosurfactant [9,10]. The hemolysis test was done because it was easily observed and did not require a long time. Biosurfactant producing bacteria will form a clear zone around the colonies on the blood agar media. The drop collapse test relies on the destabilization of liquid droplets by surfactants. It has several advantages such as rapid and easy to carry out, requires no specialized equipment and just a small volume of sample [11]. If the supernatant droplets contain biosurfactants, there will be a decrease in interface tension on the supernatant and a hydrophobic surface. As a result, the shape of the supernatant droplets will be wide [12]. The largest diameter among the 19 isolates was 3.01 mm produced by isolates 16. Oil spreading test or sometimes referred to as an oil displacement assay has advantage that it can detect biosurfactants with low activity and quantity [11]. The oil spreading test results are stated positively when a clear zone is formed on the supernatant droplets in the oil layer (Figure 1). The clear zone is formed because the hydrophobic part of the oil and hydrophilic in biosurfactant fuses, then causes pressure between the hydrophobic and hydrophilic parts. This condition causes interface tension to decrease, the oil layer breaks and a clear zone is formed [13].
Figure 1. Oil spreading test result. (a) clear zone formed on supernatant droplet.

Because of one bacterial isolate (number 7) did not grow in further analysis, activity of biosurfactant through surface tension measurement was carried out on 12 isolates. There was a difference in the ability of biosurfactant bacteria to reduce surface tension (Figure 2).

Figure 2. Surface tension value of biosurfactant-producing bacteria.

The lowest surface tension value was produced by isolates 19 and isolates 14 with a surface tension value of 37.84 ± 1.45 mN / m. [14]. The main criteria used to select biosurfactant producing bacteria is its ability to reduce surface tension up to 40 mN / m or lower [15]. Based on those criteria, isolates 3, 14 and 19 have the best biosurfactant activity of 12 biosurfactant producing bacterial isolates. These three isolates were then tested for biochemical characteristics (Table 2).

Table 2. Biochemical test result of biosurfactant producing bacteria.

| Isolate code | Indole production | Motility | Citrate utilization |
|--------------|-------------------|----------|-------------------|
| 3            | -                 | +        | -                 |
| 14           | -                 | +        | +                 |
| 19           | -                 | -        | +                 |
Motility tests showed that bacterial isolates number 3 and 14 were motile which was characterized by the spread of growth in the media. Isolate 19 is a non-motile isolate, characterized by no spread of growth. Biosurfactant producing bacterial isolates capable of using citrate as the only source of carbon and ammonium ions as single nitrogen are isolates 14 and isolates 19. This is indicated by changes in the color of the media from light green to blue [16].

The ability of bacterial isolates in sugar fermentation was done in five kind of sugar. The results showed that three isolates (isolates number 3, 14 and 19) did not ferment mannitol and sucrose characterized by no change in the color of the medium to yellow [17].

Table 3. Sugar fermentation test result of biosurfactant producing bacteria.

| Isolate code | Mannitol | Glucose | Lactose | Sucrose | Maltose |
|--------------|----------|---------|---------|---------|---------|
| 3            | -        | +       | +       | -       | +       |
| 14           | -        | +       | -       | -       | +       |
| 19           | -        | +       | -       | -       | -       |

Changes in acidity in liquid medium are caused by isolates using sugar in the media as an energy source so that it is broken down into organic acids and produces an acidic environment [17].

4. Conclusions
There were 12 isolates of biosurfactant-producing bacteria gained from hydrocarbon contaminated soil in the Jakarta Bay region. Isolates 3, 14 and 19 have the best surface tension activity. Diverse biochemical characteristics were identified in biosurfactant producing bacteria. In summary, screening by several methods is an approved strategy for discovering new biosurfactant-producing bacteria.

References
[1] Singh V 2012 Biosurfactant–Isolation, production, purification & significance Int J Sci Res Pub. 2 (7) 1–4
[2] Kurniati T H 2016 Bakteri Penghasil Biosurfaktan Dari Lingkungan Tercemar Limbah Minyak Dan Potensinya Dalam Mendegradasi Hidrokarbon Aromatik Polisiklik (HAP) (Thesis)
[3] Carrillo P G, Mardaraz C, Pitta-Alvarez S I and Giulietti A M 1996 Isolation and selection of biosurfactant-producing bacteria World J Microbiol Biotechnol 12 (1) 82–4
[4] Jain D K, Collins-Thompson D L, Lee H and Trevors J T 1991 A drop-collapsing test for screening surfactant-producing microorganisms J Microbiol Methods 13 (4) 271–9
[5] Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y and Imanaka T 1993 A new lipopeptide biosurfactant produced by Arthrobacter sp. strain MIS38 J Bacteriol. 175 (20) 6459–66
[6] Oliveira J G de and Garcia-Cruz C H 2013 Properties of a biosurfactant produced by Bacillus pumilus using vinasse and waste frying oil as alternative carbon sources Brazilian Arch Biol Technol. 56 (1) 155–60
[7] Rodrigues L, Banat I M, Teixeira J and Oliveira R 2006 Biosurfactants: potential applications in medicine J Antimicrob Chemother 57 (4) 609–18
[8] Satpute S K, Bhawas B D, Dhakephalkar P K and Chopade B A 2008 Assessment of different screening methods for selecting biosurfactant producing marine bacteria
[9] Walter V, Syldatk C and Hausmann R 2010 Screening concepts for the isolation of biosurfactant producing microorganisms (Springer: Biosurfactants) pp 1–13
[10] Youssef N H, Duncan K E, Nagle D P, Savage K N, Knapp R M and McInerney M J 2004 Comparison of methods to detect biosurfactant production by diverse microorganisms J Microbiol Methods 56 (3) 339–47
[11] Płaza G A, Zjawiony I and Banat I M 2006 Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soils J...
[12] Bodour A A and Miller-Maier R M 1998 Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms *J Microbiol Methods* **32** (3) 273–80

[13] Techaoei S, Lumyong S, Prathumpai W, Santiarwarn D and Leelapornpisid P 2011 Screening characterization and stability of biosurfactant produced by *Pseudomonas aeruginosa* SCMU106 isolated from soil in northern Thailand *Asian J Biol Sci.* **4** (4) 340–51

[14] Thavasi R, Sharma S and Jayalakshmi S 2011 Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria *J Pet Environ Biotechnol S.* **1** 1–6

[15] Cooper D G and Zajic J E 1980 *Surface-active compounds from microorganisms* (Elsevier: Advances in Applied Microbiology) pp 229–53

[16] Cappuccino J G and Sherman N 1996 *Microbiology: a laboratory manual*

[17] Murray P R, Baron E J, Jorgensen J H, Landry M L and Pfaller M A 2007 *Manual of clinical microbiology* 9th ed (Washington DC)