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## Characterization of biosurfactant production by indigenous bacteria from Sungai Dungun estuary, Terengganu by surface activity and emulsification test

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## Characterization of biosurfactant production by indigenous bacteria from Sungai Dungun estuary, Terengganu by surface activity and emulsification test.

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**Abstract.** Natural surface-active agents or biosurfactants are derived particularly from bacteria and yeasts. These natural bio compounds have several advantages over synthetic surfactants such as more environmentally friendly, biodegradable, biocompatible, and low toxicity. Therefore, many efforts have focused on investigating biosurfactants including the isolation of new potent biosurfactant producers. In the present study, twelve potent biosurfactant producing bacteria were successfully isolated from oil-contaminated water and sediment samples from Sungai Dungun estuary by using Minimal Salt Media (MSM) with the addition of 1 % (v/v) engine oil. From characterization tests, one isolate named as DSB7 demonstrated the highest activity among others by semi-quantitative tests i.e., oil spreading technique and drop collapse test. Besides, it has the lowest value of 38.48 mN/m in water surface tension measurement and emulsification index of 53.57 % and 30.0 % for kerosene and used engine oil respectively. As for the molecular identification of the isolate, it showed the highest similarity to *Pseudomonas aeruginosa* based on 16S rRNA sequence analysis.

### 1. Introduction

Biosurfactants are amphiphilic biological compounds that derived mostly from bacteria and yeasts. These bio compounds have been classified into five main groups i.e., glycolipids, lipopeptides, phospholipids, and fatty acids, polymeric and particulate biosurfactants which produced by microorganisms from genera *Pseudomonas*, *Bacillus*, *Corynebacterium*, *Rhodococcus*, *Micrococcus*, *Acinetobacter*, *Archromobacter*, *Flavobacterium*, *Proteobacteria*, *Candida bombicola*, *Candida apicola*, *Rhodotorula bogoriensis* and *Pseudozyma* [1–4]. The fundamental properties of surfactants are the efficacy to reduce surface and interfacial tension of two immiscible phases and to produce emulsification between water and hydrocarbon [5]. When surfactants are added into the solution, the surfactant monomers will gradually occupy the surface resulted to surface tension reduction until one point called Critical Micelle Concentration (CMC). At CMC point, the chemical interactions between polar and non-polar groups become weak which leads to the spontaneous aggregation of the



monomers into micelles, vesicles, or lamellae [5]. Thus, the further increment of surfactants above CMC point will no longer decrease the surface or interfacial tension.

The presence of biosurfactants in the environment is essential for microbial survival, growth, and reproduction as well as for the removal of hydrophobic pollutants. The marine environment is one of the promising sources for biosurfactant producing bacteria [6]. Hence, the present study was aimed to isolate and characterize the potent biosurfactant producing bacteria from the marine environment and Sungai Dungun estuary was chosen for the source of bacterial isolation. Sungai Dungun became a fishing boat harbor and it is located near to the local wet market and food stores, by which it might be polluted by oil spillage from the boats and the contaminants from wastewater drainage. Microorganisms have a special communication system called a quorum-sensing system to interact with their surroundings [5]. The same bacterial species from different origins may have different gene expression including biosurfactant production, in response to the environmental changes [2,7,8]. Therefore, the bacterial isolates from Sungai Dungun were expected to produce newly potent biosurfactants.

## 2. Materials and methods

### 2.1. Isolation of biosurfactant producing bacteria

Soil samples were collected from three areas with different textures as follows; Area A (sandy), Area B (sandy), and Area C (loamy), while water samples were collected close to the river cliff in all sampling areas. These samples were kept in the clean plastic bag and bottle at room temperature before processed in the laboratory within a week. Biosurfactant producing bacteria were isolated using Minimal Salt Media (MSM) containing (g/L):  $\text{Na}_2\text{HPO}_4$  (6.78),  $\text{KH}_2\text{PO}_4$  (3.0),  $\text{K}_2\text{HPO}_4$  (3.0),  $\text{NH}_4\text{Cl}$  (1.0),  $\text{NaCl}$  (0.5),  $\text{NaNO}_3$  (0.5), yeast extract (1.0) and trace elements of 1.0 M  $\text{CaCl}_2$  (0.015), 0.1 M  $\text{MgSO}_4$  (0.05), supplemented with 1 % (v/v) of 50 % glycerol and 1 % (v/v) of commercial engine oil (Shell Advance SAE40- AX3) with pH of 6.8. For the soil sample, one gram of soil was inoculated in 100 mL of MSM broth and incubated at 30 °C, 150 rpm for 48 hours. The broth cultures were serially diluted with sterile distilled water before spread on fresh MSM agar and incubated again at 30 °C for 48 hours. For the water sample, the process was direct to serial dilution and spread plate as the same procedure as for the soil sample. Colonies with distinctive characteristics were selected, re-streaked several times on new MSM agar. Pure colonies were maintained on Nutrient agar at 4°C.

### 2.2. Identification of bacterial isolates

Potent biosurfactant producers were identified using Gram's stain technique and further characterization by 16S rRNA gene sequencing. Bacterial DNA was extracted by the G-spin Genomic Extraction kit and the isolated gDNA were used as templates for the amplification of 16S rRNA by Polymerase Chain Reaction (PCR) using universal primers 68F (5'- TNA NAC ATG CAA GTC GAR -3') and 1392R (5'-ACG GGC GGT GTG TRC -3'). The thermal profiling for PCR was set as follows; (a) initial denaturation (95 °C, 5 minutes), (b) denaturation (95 °C, 90 seconds), (c) annealing (54 °C, 30 seconds), and (d) extension (72 °C, 30 seconds), with 30 times of reaction cycles throughout the process. Then, the PCR products were purified by using the MEGAquick-spin™ Total Fragment DNA Purification kit (iNtRON Biotechnology, Inc.) before sent to First Base Laboratories Sdn. Bhd. for sequencing service. The obtained DNA sequencing results in format (.ab1) or (.seq fasta) were analyzed using the Basic Local Alignment Search Tool (BLAST) to find the similarity sequences that match the Genbank databases in National Center for Biotechnology Information (NCBI) through the webpage ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)).

### 2.3. Screening assays for biosurfactant production

#### 2.3.1. Preparation of inoculum and cell-free supernatant.

Bacterial inoculum was prepared by inoculating a single colony into 10 mL of Nutrient broth and incubated at 30 °C, 150 rpm until the absorbance measurement (OD<sub>600</sub>) reached approximately 0.5. To prepare cell-free supernatant, 1% (v/v) of inoculum was inoculated into 100 mL of MSM broth supplemented with 1% (v/v) of engine oil. The broth culture was incubated at 30 °C, 150 rpm for 48 hours before it was centrifuged at 10,000 rpm for 10 minutes to get the supernatant.

#### 2.3.2. Characterization assays

Two types of hydrocarbons were used in the characterization tests i.e., used engine oil and kerosene. Sodium Lauryl Sulfate (SLS), an anionic synthetic surfactant with a concentration of 1% (v/v) was used as a positive control, while distilled water was used as negative control. All assays were carried out at room temperature.

(a) *Oil spreading test.* Into a petri dish, 100  $\mu$ L of used engine oil was dropped onto the water surface (25 mL) and left to equilibrate for 1 minute. Then, 20  $\mu$ L of cell-free supernatant was dropped onto the center of the thin oil layer. The diameter of the clear zone formed was measured in unit centimeter (cm) after 30 seconds in triplicate readings.

(c) *Drop collapse test.* A clean glass slide was coated with used engine oil and left to equilibrate at room temperature for one hour. Then, 20  $\mu$ L of cell-free supernatant was dropped on the oil-coated surface. After 1 minute, the condition of the drop was observed whether it collapsed or remain beaded.

(e) *Surface tension measurement.* Water surface tension reduction was measured by using tensiometer SIGMA 700 ATTENSION based on Du-Nouy-Ring method. Cell-free supernatant containing biosurfactant was used and the measurement was taken in duplicate readings.

(f) *Emulsification index, EI<sub>24</sub>.* To a sterile test tube (18 x 150 mm), 3 mL of oil was added and left to equilibrate for several minutes. Then, 3 mL of supernatant was added into the same test tube (ratio 1:1). The mixture was vortexed for two minutes and left at room temperature for 24 hours. The percentage of the emulsion layer was measured by dividing the height of emulsion layer by the total height of all liquid layers, multiplied by 100.

## 3. Results and discussion

### 3.1. Potent biosurfactant producing bacteria

Twelve bacterial strains designated as DSA1, DSA6, DSB7, DWA5, DWA9, DWB5, DWC1, DWC2, DWC4, DWC7, DWC8, and DWC9 were successfully isolated from Sungai Dungun estuary. Most of the isolates were Gram-negative bacillus except for one Gram-positive which is DSA 1. Based on BLAST analyses, the 16S rRNA gene sequence for bacterial isolates were found to match bacteria from NCBI databases with percentages of identity as follows: DSA1 (*Bacillus albus*, 99%), DSA6 (*Acinetobacter venetianus*, 98%), DSB7 (*Pseudomonas aeruginosa*, 99%), DWA5 (*Acinetobacter seifertii*, 93%), DWA9 (*Pseudomonas plecoglossicida*, 97%), DWB5 (*Chryseobacterium cucumeris*, 87%), DWC1 (*Aeromonas dhakensis*, 98%), DWC2 (*Aeromonas caviae*, 98%), DWC4 (*Aeromonas caviae*, 99%), DWC7 (*Delftia lacustris*, 99%), DWC8 (*Pseudomonas protegens*, 99%) and DWC9 (*Alcaligenes faecalis*, 98%). Gram-negative biosurfactant producers are ubiquitous in high hydrocarbon contaminated sites since biosurfactants are essential in enhancing the bioavailability and biodegradability of hydrophobic compounds in the environment [9]. Common bacteria that are frequently isolated from the region with high hydrocarbon residues including in genera *Pseudomonas*,

*Sphingomonas*, *Acinetobacter*, *Alcaligenes*, *Micrococcus*, *Bacillus*, *Flavobacterium*, *Arthrobacter*, *Alcanivorax* *Mycobacterium*, *Rhodococcus* and *Actinobacteria* [10]. Besides, the isolation was carried out in an estuary region, whereby the seawater and river are naturally mixed resulted in brackish water. *Serratia*, *Alcanivorax*, *Arthrobacter*, *Rhodococcus*, *Brevibacterium*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Pontibacter* *Fictibacillus*, and *Halobacterium* are some examples of biosurfactant producers that inhabited in the marine environment [6,11]. It was noticed that some of the bacterial isolates were included in the corresponding genera. However, limited literature were found regarding *Chryseobacterium* sp as biosurfactant producers which might indicate the fewer investigations on the capability of the bacteria in producing biosurfactant.

### 3.2. Characterization assays

All isolates were primarily screened for biosurfactant production through the oil spreading test. In this test, biosurfactants from all twelve isolates showed clear zones indicated the displacement of the oil layer by the biosurfactants (Table 1). The highest diameter of the clear zone was 4.0 cm which demonstrated by biosurfactant from DSB7. The oil spreading technique is a more sensitive method and require only a low concentration of biosurfactant compared to other screening tests [12]. Therefore, it was suitable to be used as a preliminary assay to screen biosurfactant production from bacterial isolates in the present study. The drop collapse test is another simple assay based on the interfacial tension reduction between water and oil. The drop collapse test is reported to have corroboration with the oil spreading test [13]. Biosurfactants that can displace the thin oil layer in the oil spreading test will also spread the supernatant drops on the oil-coated glass slide. However, in this study, not all isolates produced biosurfactants that can collapse the drops of the supernatant on the oil-coated glass slide. One of the reasons might be caused by the relatively low sensitivity of drop collapse test compared to the oil spreading technique since a significant concentration of biosurfactant must be present to collapse the aqueous drops [12]. The surface activity by produced biosurfactant in this study was quantified via surface tension measurement using Du-Nouy Ring technique, whereby the measurement of detachment force is proportional to the surface or interfacial tension [12]. A good biosurfactant can reduce the surface tension of pure water from 72 mN/m to less than 30 mN/m [8]. In this study, biosurfactant from DSB7 showed significant surface tension reduction when it gave the lowest value of 38.48 mN/m. This range value of surface tension measurement also had shown by biosurfactant from *P.aeruginosa* Pa24 grown on vegetable oils when it lowered the surface tension to 30 mN/m [16].

Biosurfactants can be categorized into two classes i.e., low molecular weight (LMW) and high molecular weight (HMW) compounds. LMW biosurfactants are more effective in surface activity, while HMW biosurfactants which also known as bioemulsifiers have roles as dispersing agents in emulsification activity [7,9]. DSB7 was the only isolate that produced biosurfactant with the greatest result in characterization tests based on surface activity in the present study which presumed this isolate to be LMW compounds. Both LMW and HMW biosurfactants require different screening methods to determine their dissimilarities in respect of surface activity and emulsification efficacy [17]. Therefore, DSB7 was primarily chosen based on the effectiveness in reducing surface tension with surface tension measurement as the ultimate test before it was evaluated for emulsification activity.

**Table 1.** Characterization assays for biosurfactant production by bacterial isolates

Isolates	Oil spreading technique (cm)	Drop collapse test	Surface tension measurement (mN/m)
DSA1	3.7	++	46.94
DSA6	3.3	-	52.62
DSB7	4.0	++	38.48
DWA5	1.9	+	44.66
DWA9	2.7	-	63.47
DWB5	3.0	++	50.82
DWC1	1.5	-	57.94
DWC2	1.5	-	55.97
DWC4	2.2	-	61.87
DWC7	3.2	-	52.78
DWC8	3.9	++	62.27
DWC9	3.7	+	60.50
SLS (1%)	5.0	++	23.00

Emulsification is a mixing process of heterogeneous solution, in which one phase as smaller droplets dispersed in the other phase solution. Once added into the immiscible solution, biosurfactants will accumulate at intermediate between aqueous and oil phases, thus reduce the interfacial tension of interphases. Subsequently, the interfacial mass exchange will occur in the surface and lead to the solubilization of dispersed organic compounds into the aqueous solution through micelles [18]. The emulsification activity of bacterial isolates in this study was evaluated by Emulsification Index (EI) in unit percentage (%). The higher EI indicates the better emulsification activity by the bacteria. It was noticed that the biosurfactant from DSB7 had higher EI towards kerosene compared to used engine oil with index values of 53.57 % and 30.0 % respectively (Table 2). Kerosene and used engine oil are different in terms of molecular structure and carbon chain length. In this study, DSB7 demonstrated the greater emulsifying activity towards kerosene or light hydrocarbon oil that have shorter carbon chains rather than used engine oil. This result was contrary to BSW10, a biosurfactant produced by *P.aeruginosa* W10, where the emulsification index was higher towards used engine oil with index value of 88.7 % [19]. It is well known that *Pseudomonas aeruginosa* can produce glycolipid biosurfactant that can emulsify various types of hydrocarbons [20]. However, biosurfactant production is substrate-dependent and it can be altered by carbon and nitrogen sources [21]. Besides, emulsification activity is determined by the hydrophilic-lipophilic balance (HLB) of the molecular compounds [22]. Hence, the contrast result in the emulsification index might be due to different groups of biosurfactants produced by DSB7.

**Table 2.** Emulsification activity by isolate DSB7

Samples	Emulsification Index, EI <sub>24</sub> (%)	
	Kerosene	Used engine oil
DSB 7	53.57	30.0
SLS (1%)	62.47	62.96

#### 4. Conclusion

Twelve potent biosurfactant producing bacteria from Sungai Dungun were successfully isolated by using MSM with the addition of 1 % (v/v) engine oil as carbon source. The molecular identification by 16S rRNA gene sequencing showed that these isolates matched seven genera of bacteria in NCBI databases including *Pseudomonas*, *Acinetobacter*, *Chryseobacterium*, *Aeromonas*, *Delftia*, *Bacillus*, and *Alcaligenes* by BLAST analysis. Limited literature were found regarding biosurfactant production by *Chryseobacterium* sp. which indicates the fewer studies on the capability of this isolate in producing biosurfactant. Meanwhile, isolate DSB7 which hit the highest identity to *Pseudomonas aeruginosa* was selected as the best potent biosurfactant producer since it showed the lowest surface tension reduction of water with a value of 38.48 mN/m. This isolate has higher emulsification activity towards kerosene rather than used engine oil. Therefore, it can be concluded that this isolate was more effective in an emulsifying light hydrocarbon oil.

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#### References

- [1] Ahmad Z, Arshad M, Asghar H N, Sheikh M A and Crowley D E 2016 Isolation, screening and functional characterization of biosurfactant producing bacteria isolated from crude oil contaminated site *Int. J. Agric. Biol.* **18** 542–8
- [2] Banat I M, Franzetti A, Gandolfi I, Bestetti G, Martinotti G, Fracchia L, Smyth T J and Marchant R 2010 Microbial biosurfactants production, applications and future potential *Appl. Microbiol. Biotechnol.* **87** 427–44
- [3] Liu J F, Mbadinga S M, Yang S Z, Gu J D and Mu B Z 2015 Chemical structure, property and potential applications of biosurfactants produced by *Bacillus subtilis* in petroleum recovery and spill mitigation *Int. J. Mol. Sci.* **16** 4814–37
- [4] Shoeb E, Ahmed N, Akhter J, Badar U, Siddiqui K, Ansari F A, Waqar M, Imtiaz S, Akhtar N, Shaikh Q U A, Baig R, Butt S, Khan S, Khan S, Hussain S, Ahmed B and Ansari M A 2015 Screening and characterization of biosurfactant-producing bacteria isolated from the Arabian Sea coast of Karachi *Turkish J. Biol.* **39** 210–6
- [5] Steinbuechel A 2011 *Biosurfactants : From genes to applications* vol 20, ed G. Soberon-Chavez (Heidelberg Dordrecht London New York: Springer)
- [6] Kubicki S, Bollinger A, Katzke N, Jaeger K E, Loeschcke A and Thies S 2019 Marine biosurfactants: biosynthesis, structural diversity and biotechnological applications *Mar. Drugs* **17** 1–30
- [7] Das P, Mukherjee S, Sivapathasekaran C and Sen R 2010 Microbial surfactants of marine origin: potentials and prospects *Biosurfactants : Advances in experimental medicine and biology* vol 672, ed R Sen (Texas: Landes Bioscience) pp 88–101
- [8] Satpute, S.K., Bhuyan, S.S., Pardesi, K.R., Mujumdar, S.S., Dhakephalkar, P.K., Shete A M et

- al. 2006 *Biosurfactants*
- [9] Md F 2012 Biosurfactant: Production and Application *J. Pet. Environ. Biotechnol.* **03** 1–5
- [10] Obayori O S, Ilori M O, Adebusoye S A, Oyetibo G O, Omotayo A E and Amund O O 2009 Degradation of hydrocarbons and biosurfactant production by *Pseudomonas* sp. strain LP1 *World J. Microbiol. Biotechnol.* **25** 1615–23
- [11] Schultz J and Rosado A S 2019 Extreme environments: a source of biosurfactants for biotechnological applications *Extremophiles* **24** 189–206
- [12] Walter V, Syldatk C and Hausmann R 2010 Screening concepts for the isolation of biosurfactant producing microorganisms *Biosurfactants : Advances in experimental medicine and biology* vol 672, ed R Sen (Texas: Landes Bioscience) pp 1–13
- [13] Sabnis S and Juvale V 2016 Enrichment and Isolation of Biosurfactant Producers from Marine Environment *Int. J. Curr. Microbiol. Appl. Sci.* **5** 730–40
- [14] Fracchia L, Cavallo M, Martinotti M G and Banat I M 2012 Biosurfactants and Bioemulsifiers Biomedical and Related Applications – Present Status and Future Potentials *Biomedical Science, Engineering and Technology* ed D N Ghista (Crotia: InTechOpen) pp 325–70
- [15] Santos D K F, Rufino R D, Luna J M, Santos V A and Sarubbo L A 2016 Biosurfactants: Multifunctional biomolecules of the 21st century *Int. J. Mol. Sci.* **17** 1–31
- [16] Bhat R, Dayamani K J, Hathwar S, Hedge R and Kush A 2015 Exploration on production of rhamnolipid biosurfactants using native *Pseudomonas aeruginosa* strains *J. Biosci. Biotechnol.* **4** 157–66
- [17] Uzoigwe C, Burgess J G, Ennis C J and Rahman P K S M 2015 Bioemulsifiers are not biosurfactants and require different screening approaches *Front. Microbiol.* **6** 1–6
- [18] Kaczorek E, Pacholak A, Zdarta A and Smułek W 2018 The Impact of Biosurfactants on Microbial Cell Properties Leading to Hydrocarbon Bioavailability Increase *Colloids and Interfaces* **2** 35
- [19] Chebbi A, Hentati D, Zaghden H, Baccar N, Rezgui F, Chalbi M, Sayadi S and Chamkha M 2017 Polycyclic aromatic hydrocarbon degradation and biosurfactant production by a newly isolated *Pseudomonas* sp. strain from used motor oil-contaminated soil *Int. Biodeterior. Biodegrad.* **122** 128–40
- [20] Ndibe T O, Eugene W C and Usman J J 2019 Screening of Biosurfactant-Producing Bacteria Isolated from River Rido, Kaduna, Nigeria *J. Appl. Sci. Environ. Manag.* **22** 1855
- [21] Al-Araji L, Rahman R N Z R A, Basri M and Salleh A B 2007 Minireview : Microbial Surfactant *Asia-Pacific J. Mol. Biol. Biotechnol.* **15** 99–105
- [22] Yamashita Y and Sakamoto K 2016 Hydrophilic-Lipophilic Balance (HLB): Classical Indexation and Novel Indexation of Surfactant *Encycl. Biocolloid Biointerface Sci.* **1** 570–4