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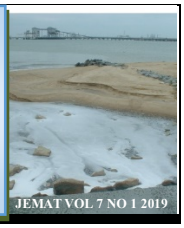

Comparative Screening Methods for the Detection of Biosurfactant-Producing Capability of Antarctic Hydrocarbon-degrading *Pseudomonas* sp.

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ABSTRACT

Four preliminary screening methods for biosurfactant synthesis - drop collapse assay, oil displacement activity, microplate assay and emulsification index (E_{24}) were compared and evaluated for their reliability and ease of use. All screening methods showed positive indications for the synthesis of biological surface-active agents. Nevertheless, partial collapse of the supernatant and low emulsification index (E_{24}) of *Pseudomonas* sp. might signify a low production of biosurfactants. Based on our observation, both drop collapse and oil displacement assay is the fastest, easiest and most reliable analytical routine to be suggested to screen for biosurfactant-producing strains. In the extent for a high throughput screening (HTS), drop collapse assay is the best method for an accurate screening of biosurfactant producers.

INTRODUCTION

Ron and Rosenberg (2001) [1] stated that biological surface-active agents have the capability to perform numerous physiological roles and offer various benefits to their producing strains or 'factories' such as increasing the surface area of water-insoluble substrates by emulsification process, improving the bioavailability of hydrophobic molecules, possess specialised antimicrobial action and also regulate the attachment and detachment of microbes to and from surfaces. Conferring to these distinctive roles, there are numbers of biosurfactant-synthesising microbial cell that can be found in different locations and settings around the world. Previous research had shown that most of the biosurfactant-producing microorganisms were isolated from contaminated locations -particularly hydrocarbon and fuel-contaminated sites [2–8]. Hydrocarbons are known to be water-insoluble, and the presence of biosurfactants help increase the surface area of the substrates to be utilised by the bacteria. The most common bacteria to produce biosurfactants are from the genus *Pseudomonas* with rhamnolipids as the main biological surfactant products. In polar region such as Antarctica, the most common biosurfactant-producing bacteria belongs to genera *Rhodococcus* and *Pseudomonas* [9]. Generally, those bacteria are psychrotolerant that can be obtained from crude diesel contaminants indicating that they can grow in the presence of those substrate [10].

Due to their structurally diverse grouping of bio-active molecules such as lipopeptides, glycolipids or phospholipids, most detection or screening methods for biosurfactant are based on the physical effects of the surfactant [11]. On the other hand, the strains' aptitude to interfere with the hydrophobic interfaces can be studied. While the screening methods can be divided into qualitative and/or quantitative results, qualitative methods are generally sufficient for the first screening of isolates. This study was conducted to find the most sensitive yet reproducible and

simple technique to screen biosurfactant-producing bacteria. The screening methods used in this study include the drop collapse test, oil displacement test, microplate assay and emulsification index (E_{24}).

MATERIALS AND METHODS

Bacterial strain

Bacterial culture used in this study were previously isolated from pristine soil pf Southern Victoria Island, Antarctica. The bacteria were formerly tested for hydrocarbon-degrading capacity and showed positive results for hydrocarbon degradation. The isolate was identified as *Pseudomonas* sp. through 16S rRNA sequence analysis, designated as strain ADL15 and submitted to NCBI under the accession number of KX812776 [12].

Culture growth

Bacterial cultures were grown aerobically in 250 ml Erlenmeyer flask with 100 ml Bushnell-Haas medium and the addition of 1% (v/v) diesel as the carbon source. Culture flasks were maintained in an incubator shaker for 7 days, shaken at 160 rpm at 25°C, before harvested. The bacterial cultures were then centrifuged at 10,000 rpm for 20 min at 4°C to remove the bacterial cell [13]. The supernatant was taken for further analysis.

Screening of isolates for biosurfactant production

Four methods (three qualitative and one quantitative) were carried out to detect the synthesis of bio-active agents such as drop collapse test, oil spread test and emulsification index (E_{24}).

i. Drop collapse test

A glass slide was coated with commercially available diesel oil and fully covered. The glass slide was left for 24 h to allow equilibration at room temperature. Then, 10 µl of the culture supernatant was pipetted very carefully onto the surface of glass slide and left for an hour prior measuring the diameter of the drop. After an hour, the collapse of the drop was assessed based on the diameter whether there was no collapse at all, partial collapse (diameter of collapse is less than 1 cm), near complete collapse (diameter is between 1 cm to 1.5 cm) and complete collapse (diameter is more than 1.5 cm) [13].

ii. Oil spread test

20 ml of distilled water was poured into a clean petri dish. 100 µl of used lubricant oil was slowly placed onto the surface of water to avoid the oil from breaking the water surface. Then, 100 µl of supernatant obtained through centrifugation was placed in the centre of the oil. Formation of the clear area was then observed [14].

iii. Microplate assay

100 µl of culture supernatant was pipetted into a well of 96-well microplate. A graph paper was used as the backing sheet for viewing. The grid image viewed from above the microplate well was observed. Pure water in a hydrophobic well has a flat surface; however, if biosurfactant is present, the concave surface will distort the grid image, taking the shape of the wells.

iv. Emulsification index (E_{24})

2 ml diesel was placed in a test tube with the addition of 2 ml culture supernatant. The mixture was vortexed vigorously for 2 min. The mixture was left to stand for 24 h and the percentage of emulsification index was determined using the following formula:

$$E_{24} = \frac{h\ emulsion}{h\ total} \times 100$$

where: E_{24} is emulsion index after 24 hours, h emulsion is the height of the emulsion layer, h total is the total height of the liquid.

RESULTS AND DISCUSSION

Drop-collapse technique is a sensitive yet rapid method to screen biosurfactant-producing bacteria [15]. The principle behind this test is that the water drop will remain stable because of its polar state which repels against the hydrophobic surface (oil). Conversely, if the droplet contains biosurfactant, the surface tension or interfacial tension between them will be reduced, hence allowing the droplet to collapse and expand on the surface [16]. Drop-collapse test is one of the common methods used to detect the presence of biosurfactant qualitatively or quantitatively since it is simple and practical. The method also requires only minute amount of samples and does not require any specialised equipment [17].

When each replicates of bacterial supernatant was dropped onto the glass slide coated with diesel oil, it collapsed which contrast to the stable water drop. Although considered as a partial collapse, this method indicates a positive production of biosurfactant by the isolate. The results obtained are shown in **Table 1.**

Table 1. Drop-collapse test for bacterial supernatant of *Pseudomonas* sp.

Features	Control (water)	r1	r2	r3
Drop shape	Round	Flattened	Flattened	Flattened
Drop collapse	No collapse	Partial collapse	Partial collapse	Partial collapse
Drop diameter	0.5 cm (maintained)	0.9 cm (after collapse)	0.7 cm (after collapse)	0.7 cm (after collapse)

Oil-spread test, also known as oil displacement test is a method used to measure the diameter of the clear area formed when bacterial supernatant drops onto an oil-water surface [18]. According to Morikawa et al. (2000) [14], the area of oil displacement corresponds to the concentration of biosurfactants in the solution. However, no quantitative study has been conducted on biosurfactants concentration against the activity of oil spreading. The qualitative testing where bacterial supernatant was dropped onto the oily surface showed positive result indicated by the formation of clear area (data not shown). Oil displacement test can be considered as the most popular method for preliminary screening of biosurfactant-producing bacteria as it is rapid and easy to conduct, involves no specialised equipment or apparatus and requires a minute amount of sample [17]. Moreover, it can be applied when the activity and quantity of biosurfactant is low [11]. The effectiveness and reliability of this method has been verified by previous studies [19,20].

Microplate assay is a simple qualitative assay used to observe the grid image formed when microplate containing biosurfactants was viewed using a graph paper as a backing sheet. The assay is based on the change in optical distortion that is caused by surface active compounds in an aqueous solution. Theoretically, pure water in a hydrophobic well has a flat surface. When surfactants are present, it cause 'wetting' at the edge of the well thus causing a concave surface The optical distortion of the grid provides a qualitative assay for the presence of surfactants.[21]. The supernatant of *Pseudomonas* sp. showed positive result as it caused the image to appear distorted (data nit shown). While Chen et al. (2007) [22] validated the efficiency of the microplate method, the drawback of the method is the assay

require a definitive image for the 'correct' optical distortion as an individual may observe the distortion a little bit different from another. Emulsification index is the common method to test the synthesis of biosurfactants. In theory, when an isolate able to produce biosurfactant, a layer (emulsion layer) will be formed between the oil and the supernatant collected through centrifugation process [23]. Theoretically, higher emulsion layer formed will indicates higher quantity of synthesised biosurfactants. Besides, the E_{24} can show the percentage of biosurfactants production during the degradation process. A stable biosurfactants will have increased capability to enhance substrates degradation and bioavailability. Therefore, the degradation rate can be improved [24].

In this study, the emulsion layer formed by the supernatant from *Pseudomonas* sp. indicates the presence of biosurfactants. However, the emulsification index showed a lower percentage when compared to previous study involving the biosurfactant produced by the genus [7,25,26]. The solution containing non-biosurfactants (distilled water) showed negative result where no emulsion layer was observed. **Table 2** showed the emulsification index of the three replicates for the *Pseudomonas* sp. supernatant.

Table 2. Emulsification index (E_{24}) in percentage for *Pseudomonas* sp.

Test tube	E_{24} (%)
r1	20
r2	32
r3	44
Average	32

CONCLUSION

This study has revealed the capacity of the hydrocarbon-degrading isolates, *Pseudomonas* sp. to produce biosurfactant. All tests which include drop collapse assay, microplate assay, oil spreading assay and emulsification index showed positive indication of a biosurfactant synthesis. From the notion of qualitative observation, all method can be considered to be effective with the exception of microplate assay. Meanwhile, only emulsification index could be considered as the acceptable quantitative assay among the four methods assessed hence to comparison should be done. In terms of analysis time, emulsification index requires the longest amount of time to obtain a result while the other three methods can generate findings instantly or within minutes. Last but not least, as the application of a high throughput screening is critically required for a rapid detection of biosurfactant-producing strains, it can be said that only drop collapse and microplate assay is possible in current research as it can be performed in microplates. In conclusion, drop collapse method is considered as the best method to screen biosurfactant-producing bacteria as it is easy, rapid, and can be used in a high throughput screening (HTS).

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