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ISOLATION AND CHARACTERIZATION OF BIOSURFACTANT-PRODUCING BACTERIA ISOLATED FROM PETROLEUM CONTAMINATED SITES WITH THE POTENTIAL TO BE USED IN BIOREMEDIATION

Noor Shaidatul Lyana Mohamad Zainal, Suhaila Mohd Omar, Mardiana Mohd Ashaari*

Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, Bandar Indera Mahkota, Kuantan, Pahang Darul Makmur, Malaysia.
Corresponding author email: mardianama@iium.edu.my

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ABSTRACT

Surfactants have been widely used to facilitate biodegradation of petroleum pollutant; particularly those are being produced by microorganisms which are also known as biosurfactant. Six bacterial isolates from petroleum contaminated sites were found to be potential hydrocarbon degrader candidates based on their ability to grow on minimal media supplemented with petroleum crude oil as sole carbon source. In addition, they also found to be having potential in producing extracellular biosurfactant based on the results of screening for biosurfactant activity. These isolates were further identified and characterized. Morphological and biochemical characteristics of these isolates have been examined and molecular identification was done by amplifying 16S rRNA gene. Three isolates were identified as the member of the genus *Pseudomonas;* another two isolates were the member of genus *Comamonas* and one isolate from the genus *Stenotrophomonas.* Several qualitative screening methods (microplate assay, oil displacement test, emulsification assay and drop-collapse test) showed variation of results; suggesting the need to support these findings with quantitative screening based on measurement of surface activity. BSP 6 which was found to be closely related to *Comamonas aquatica* was the most promising biosurfactant producer found in this study based on two positive results out of four qualitative screening methods performed.

1. INTRODUCTION

Petroleum oil which are being used extensively in automotive industries contains major contaminant which are polycyclic aromatic hydrocarbons (PAHs). Remediation of these pollutants can be done using physicochemical approaches and also by applying bioremediation strategies which use the ability of indigenous microorganisms to degrade pollutants. However, the biodegradation process of PAH compounds are limited due to their poor availability to the microorganisms, hydrophobicity and low aqueous solubility [1]. During biodegradation process by hydrocarbon-degrading microorganisms, they tend to produce surface-active molecules known as biosurfactant to facilitate assimilation of these insoluble substrates [2].

Biosurfactant which has a structure consisting of hydrophobic tail and hydrophilic head are found to be able to increase the bioavailability of hydrophobic water-insoluble substrate [3] and also changed the properties of the bacterial cell surface [4]. In addition, biosurfactant also can reduce the surface tensions and interfacial tensions as it accumulating the hydrophobic compound in the micelles along the air-liquid and liquid-liquid interfaces, which then improved the mass-transfer of hydrophobic pollutants from a solid or non-aqueous liquid phase into aqueous phase [5]. Therefore, biosurfactant are suitable to be used in wide range of applications including enhancing oil recovery in petroleum industries; in food, cosmetics and pharmaceutical industries and also in bioremediation processes.

Biosurfactants that produced by microorganisms are more environmental friendly and non-hazardous besides having better foaming properties and higher selectivity [4]. They are stable and do not lose their physico-chemical properties at different range of pH, temperatures and salinity [6, 7]. They can be a substitute for synthetic surfactants; which are often toxic and at the same time introduce other types of contamination. Biosurfacatant-producing microorganisms can be isolated from sites contaminated by oil or other substances containing hydrocarbons; where they are indigenous population of those sites [8-11]. They can simply thrive in these polluted environments and at the same time degrade the hydrocarbons which they use as their carbon source. In addition, biosurfactant also can be produced from fermentation of cheap agro-based substrate and waste material [12, 4]. The use of these renewable natural resources can be a solution to the problem of low yield and high production cost of biosurfactant to be commercialized.

Researchers worldwide have been doing a lot of studies on biosurfactant-producing bacteria for bioremediation purposes. For instance, Pacwa-Plociniczak and co-workers were able to isolate *Pseudomonas* sp [13]. P-1 strain from heavily petroleum hydrocarbon-contaminated soil at Czechowice-Dziedzice, Poland. *Bacillus thuringiensis* strain NA2 was isolated from petroleum contaminated soil near oil refinery plant in India, while *Pseudomonas aeruginosa* was found from leakage channels in Indian oil tanks at Surareddypalem [14,15]. In Malaysia, Hamzah and co-workers were able to isolate and characterized biosurfactant-producing bacteria that able to degrade Sumandak and Angsi oil in Sarawak [16]. They are *Acinetobacter iwoffi*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. Four most promising biosurfactant-producers from 11 isolates of Tapis crude oil, which were *Acinetobacter baumanii* UKMP-12T, *Pseudomonas aeruginosa* UKMP-14T, *Rhodococcus* sp. UKMP-5T, and *Rhodococcus* sp. UKMP-7T also have been discovered later by Hamzah and co-workers [17].

In this study, several bacteria have been isolated from petroleum contaminated soil and were further identified and characterized using morphological, biochemical and molecular approaches. Qualitative screening of biosurfactant activity have been conducted for these isolates which consists of several tests including microplate assay, oil displacement test, emulsification assay and drop-collapse test using 1% sodium dodecyl sulfate (SDS) as a positive control and water as a negative control.

2. MATERIALS AND METHODS

2.1 Soil sampling and enrichment culture

The top 15 cm layer of soil was excavated using spade from three sites

adjacent to three different vehicles workshops around Kuantan, Malaysia. Samples were brought to the laboratory stored at 4°C until usage. The soil samples were sieved manually with 1.4 mm size of sieve, in order to remove small rocks, sands, and vegetation. Next, 10g of the soil sample were weighed and mixed into 100mL of maximum recovery diluent before shaking them at 150 rpm for 30 minutes at temperature 30°C. After settling of course materials, enrichment cultures were performed by transferring 1 ml of the soil suspension into 50 ml Bushnell Haas broth supplemented with 1% (v/v) petroleum crude. The suspension was incubated in incubator shaker at 150 rpm for 7 days at temperature 30°C.

2.2 Isolation of bacteria

Serial dilution was performed for the bacterial cultures at 3rd, 5th and 7th day of incubation and spreaded on Bushnell Haas agar plate supplemented with 1% (v/v) petroleum crude. All plates were prepared in triplicate and incubated at 30°C incubator for 5 days as hydrocarbon degraders bacteria are slow proliferator. Pure colonies of bacteria were obtained after several dilution streakings on Bushnell Haas agar plate supplemented with 1% (v/v) petroleum crude. Morphological characteristics, such as the colour, texture, size and shape of these pure colonies were determined and several different isolates having distinct morphological characteristics were chosen for further characterization.

2.3 Characterization of isolates

Selected isolates were characterized based on Gram staining procedure and several biochemical tests to look at their metabolic potential. Biochemical tests performed including catalase and oxidase test and also by using Analytical Profile Index (API) strips (Biomerieux) according to manufacturer's instructions.

2.4 DNA extraction of isolates

DNA extractions of isolates were performed using GF-1 Bacterial DNA Extraction Kit (Vivantis) according to the manufacturer's instructions. The concentrations of DNA extracted were recorded using Nanodrop Spectrophotometer and stored at -20°C.

2.5 Identification of bacterial isolates by PCR amplification

PCR was performed to amplify the 16S rRNA from the extracted DNA using the 27F and 1492R primers [18]. PCR was carried out in 25 µL reaction volume consisting of 12.5 µl 2X PCR master mix (Promega, WI, USA), 2.5 pmoles of each forward and reverse primer and 0.2 µl ultrapure BSA (50 mg/ml, Ambion) using the following conditions: 94°C for 1 min, 57°C for 2 min and 72°C for 2 min for 25 cycles. PCR products were visualized on a 1.2 % (w/v) agarose (Roche Diagnostics) gel and were purified using GF-1 AmbiClean Kit (Vivantis) and sequenced by 1st BASE Laboratories. Sequences obtained were compared against National Center for Biotechnology Information (NCBI) database using BLAST [19].

2.6 Screening of biosurfactant activities

Four screening tests were chosen which were microplate assay, oil displacement test, emulsification assay and drop-collapse test. For each test, the positive control used was SDS 1% (v/v) while the negative control was distilled water. Prior screening, cell free broth was firstly prepared by inoculating culture in 50 mL Bushnell-Haas media enriched with 1% (v/v) crude oil and incubated for 5 days with shaking at 150 rpm and 30°C. Sample was then centrifuged at 9000 rpm for 40 minutes at 4°C. The supernatant was then filtered to remove the oil and stored in 4°C. Microplate assay was performed by pipetting 100 µL of supernatant from each sample into well of the microplate in triplicate [20]. Beneath the microplate, a graph paper was placed in order to observe the distortion image. If the distortion image is visible, the isolate is said to be positive for biosurfactant activity.

Meanwhile, the oil displacement test was performed by filling a small-sized petri dish with 20 mL of distilled water [21]. 20 µL of each crude oil and supernatant were added. After 30-60 seconds, the displacement of oil was observed and said to be positive if the oil only resided at the edge of the petri dish. Emulsification assay was done by adding the ratio of 1:1 of filter sterilized crude oil and supernatant into test tubes [22]. After vortexing for 2 minutes, the tube was left undisturbed for 24 hours and emulsification activity was determined by observing the height of emulsion produced. Finally, the drop-collapse test was conducted by pipetting petroleum crude oil on the lid of the microplate [23]. It was then left undisturbed for 24 hours. After 24 hours, 5 µL of supernatant was added and result was observed after 1 minute. If the supernatant dropped into the crude oil, the test is said to be positive and vice versa if remain beaded.

3. RESULTS

Isolation of bacteria which could degrade hydrocarbon from petroleum contaminated sites was done by enrichment culture method; which minimal media was supplemented with petroleum crude oil as sole carbon source. From the 3 sites, 6 isolates were chosen as they have distinct morphological characteristics. The isolates chosen were designated as BSP 1, BSP 2, BSP 3, BSP 4, BSP 5 and BSP 6. The morphological characteristics of the 6 isolates and their Gram classification are as in Table 1. BSP 4 and BSP 6 were Gram positive bacteria while the others were Gram negative. Further characterization of the isolates was performed using different API kit according to Gram classification and their biochemical properties summarized in Table 2. In addition, identification of isolates based on molecular approach were performed and amplified 16s rRNA gene which the size was approximately 1500 bp can be viewed by gel electrophoresis. The PCR products were sent for sequencing and the closest relatives of the 16S rRNA gene sequences of the bacterial isolated when compared to the NCBI nucleotide database were listed in Table 3. The Gram positive isolates (BSP 4 and BSP 6) were the member of the genus *Comamonas* and majority of isolated Gram negative bacteria was the member of the genus *Pseudomonas* (BSP 1, BSP 2, BSP 3). Another isolate (BSP 5) was the member of the genus *Stenotrophomonas;* with closest hit (95%) to *Stenotrophomonas maltophilla* isolated form insecticide contaminated soil. Some of the isolates were previously isolated from contaminated soil and some from river.

Table 1: Morphological characteristics and Gram staining for selected bacterial isolates

Isolates	Form	Margin	Elevation	Texture	Appearance	Color	Gram-shape	Gram
BSP 1	Irregular	Entire	Raised	Smooth	Dull	Creamy	Bacillus	Negative
BSP 2	Circular	Entire	Convex	Smooth	Shiny	Creamy	Bacillus	Negative
BSP 3	Circular	Erose	Raised	Rough	Shiny	Transparent	Bacillus	Negative
BSP 4	Circular	Entire	Convex	Smooth	Shiny	Dull yellow	Bacillus	Positive
BSP 5	Circular	Entire	Convex	Smooth	Dull	Yellowish	Bacillus	Negative
BSP 6	Circular	Entire	Raised	Smooth	Dull	Dull creamy	Coccus	Positive

Table 2: Biochemical properties of selected bacterial isolates using different types of API system according to Gram classification

Characteristics	Isolate					
	BSP 1	BSP 2	BSP 3	BSP 4	BSP 5	BSP 6
Oxidase	-	-	-	-	-	-
Catalase	+	+	-	-	-	-
Arginine dihydrolase	+	+	-	-	-	-
Tryptophan deamination	+	+	n.d.	n.d.	n.d.	n.d.
Esculin hydrolysis	n.d.	n.d.	-	-	+	-
Gelatin hydrolysis	-	-	-	n.d.	+	n.d.
p-nitrophenyl-βD-galactopyranoside	n.d.	n.d.	-	n.d.	+	n.d.
hydrolysis	-	-	+	n.d.	+	n.d.
Glucose assimilation	-	-	-	+	-	+
Arabinose assimilation	n.d.	n.d.	n.d.	+	n.d.	+
Ribose assimilation	n.d.	n.d.	+	n.d.	+	n.d.
Mannose assimilation	-	-	+	-	+	-
Mannitol assimilation	n.d.	n.d.	-	n.d.	+	n.d.
N-acetyl-glucosamine assimilation	n.d.	n.d.	-	n.d.	+	n.d.
Maltose assimilation	n.d.	n.d.	+	n.d.	+	n.d.
Gluconate assimilation	n.d.	n.d.	+	n.d.	+	n.d.
Caprate assimilation	n.d.	n.d.	-	n.d.	+	n.d.
Adipate assimilation	n.d.	n.d.	+	n.d.	+	n.d.
Malate assimilation	-	-	+	n.d.	+	n.d.
Citrate assimilation	n.d.	n.d.	+	n.d.	+	n.d.
Phenyl-acetate assimilation	-	-	n.d.	+	n.d.	+
Voges-Proskauer reaction						

Note: +: positive; -: negative; n.d.: not determined

Table 3: The closest relatives of the 16S rRNA gene sequences of bacterial isolated in this study

Isolate	Closest hit	Source	Similarity

BSP 1	*Pseudomonas* sp. LZJ-9	Soil	97%
BSP 2	*Pseudomonas putida* strainB4	Biofilms from river	97%
BSP 3	*Pseudomonas putida* strain B4	Biofilms from river	97%
BSP 4	*Comamonas* sp. TS24	Arsenic contaminated soil	97%
BSP 5	*Stenotrophomonas maltophilla*	Insecticide contaminated soil	95%
BSP 6	*Comamonas aquatica*	River	97%

To further explore the ability of these isolates to produce biosurfactant, several qualitative tests were conducted (Figure 1). Out of the 4 tests, only oil displacement test showed positive result for all isolates whilst from microplate assay, only BSP 6 showed positive result. On the other hand, the other two tests (drop-collapse and emulsification assay), all isolates showed negative result for biosurfactant activity. The results were summarized in Table 4. For microplate assay, observation for cell-free supernatant from BSP 6 showed a distortion of image of grids from the graph paper placed underneath the microplate; similar to what can be observed for positive control (1% SDS) (Figure 1(a)). The observation for oil displacement test showed clearing zone formed when each of the isolates' cell-free supernatant were placed on the centre of petroleum crude oil which has been layered on water. All isolates had displaced the oil layer to the edge of the petri dish containing water; and formed this clearing zone; similar to the activity of 1% SDS (Figure 1(b)). However, the diameter of the clearing zone was not measured and therefore correlation between the clearing zone and biosurfactant activity could not be justified yet.

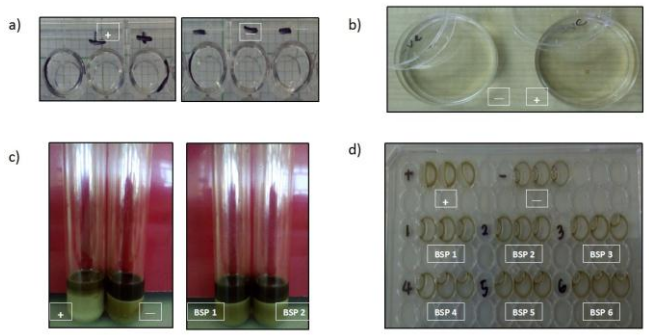

Figure 1: Tests performed to screen biosurfactant activity of isolates. a) Microplate assay; b) Oil displacement test; c) Emulsification assay and d) Drop-collapse test. The + symbol is for positive control (1% SDS) while – symbol is for negative control (distilled water). BSP 1, BSP 2, BSP 3, BSP 4, BSP 5 and BSP 6 are the name of the isolates respectively.

Table 4: Biosurfactant activity screening for selected bacterial isolates

Sample	Oil displacement	Drop-collapse	Microplate assay	Emulsification assay
BSP 1	+	-	-	-
BSP 2	+	-	-	-
BSP 3	+	-	-	-
BSP 4	+	-	-	-
BSP 5	+	-	-	-
BSP 6	+	-	+	-

Note: +: positive; -: negative

As for emulsification assay, addition of positive control (1% SDS) to filter sterilized petroleum crude showed larger height of emulsion being produced compared addition of distilled water to the petroleum crude. However, when each of the cell-free supernatant was added to the petroleum crude, the height of emulsion produced were found to be approximately similar to the height produced by addition of distilled water (Figure 1 (c)). Finally, for drop-collapse test, observation was done to check the destabilization of petroleum crude droplets by cell-free supernatant which might contain biosurfactant. Presence of biosurfactant was found to be spreading or even collapsing the droplets of petroleum crude as exhibited by 1% SDS (Figure 1 (d)) due to the force or interfacial tension between the droplets and the hydrophobic surface was reduced. However for all cell-free supernatant placed on droplets of petroleum crude, the droplets remain stable; indicating the absence of biosurfactant.

4. DISCUSSION

Most microorganisms isolated from petroleum contaminated sites have been shown to be able to degrade hydrocarbons. In this study, *Pseudomonas*, *Comamonas* and *Stenotrophomonas* have been isolated and were found to be able to use petroleum crude as their sole carbon source. *Pseudomonas* is well known hydrocarbon degraders and therefore they can be easily found at petroleum contaminated sites. Scientific literature suggests that the bacterial PAHs degradation in soil is dominated by strains belonging to very limited phylogenetic groups such as *Sphingomonas*, *Burkholderia*, *Pseudomonas*, and *Mycobacterium* [24]. Besides, *Pseudomonas* sp. is the best known bacteria that obtained their carbon and energy sources by utilizing lots of aliphatic and aromatic compound [13]. This genus has wide diversity of metabolic and physiological properties due to the presence of a complex enzymatic system. *Pseudomonas aeruginosa* UKMP-14T had been found to have high percentage of total petroleum hydrocarbon degradation and also exhibited biosurfactant activity [17]. In addition, *Pseudomonas* sp. is also known to produce different types of rhamnolipids; which are one of the major groups of biosurfactant [25].

On the other hand, *Comamonas* sp. was found by Al-Deeb and Malkawi from oil refinery site in Zarga, Jordan and the bacteria were tested positive for biodegradation of diesel [26]. Besides that, Hussein and co-workers also found this strain from contaminated site in Aqaba, Jordan and was confirmed of its ability to degrade crude oil [27]. Recently, a strain with high sequence similarities (98.5 %) to *Comamonas aquatica* had been isolated from agricultural soil and has a high biosurfactant activity [28]. In additon, *Stenotrophomonas* was found in soil and several species have been associated in biodegradation of pesticides [29]. Previous study by Rajaei and co-workers confirmed the ability of this bacteria to degrade crude petroleum by incorporating into the rhizosphere part of wild oat, which a potential for phytoremediation [30]. *Stenotrophomonas* also had been isolated from petroleum and refinery site and was confirmed of its ability to degrade Methyl tert-butyl ether (MTBE)-contaminated water [31]. Furthermore, *Stenotrophomonas maltophilia* was also being used together with other bacteria in biodegradation of oil residues from petroleum refinement and other bioremediation and phytoremediation studies [32, 33].

In terms of screening the activity of biosurfactant produced by bacteria, the principle aim is to find new compounds with strong interfacial activity, low critical micelle concentration (CMC), high emulsion capacity, good solubility and activity in a broad-pH range [34]. The biosurfactant-producing bacteria need to be easily cultivated and producing high yield of biosurfactant to ensure economical and sustainable commercialization. There are many different techniques that can be used; both qualitative and quantitative types of screening. Several qualitative screening methods have been used in this study (microplate assay, oil displacement test, emulsification assay and drop-collapse test) as a preliminary step for screening. Further analysis could be done using more accurate and sensitive quantitative method including surface tension measurement using an instrument known as tensiometer.

The microplate assay was performed due to its advantages such as easy and quick to be performed and also sensitive enough to detect surface-active compounds by using just a small volume of sample [35]. The oil displacement test also is a rapid and easy method to be carried out. In addition, this method can detect even low activity and quantity of biosurfactant present [36]. Meanwhile, emulsification assay can also be reported as quantitative measurement by calculating the emulsion index which correlated to the concentration of biosurfactant present. However, emulsification activity by biosurfactant present does not always correlate with surface activity; therefore this method just gives an indication of presence of biosurfactants [34]. Finally, drop-collapse test is another easy to be performed method to test for biosurfactant production. However, a significant concentration of surface active compounds must be present in order to cause a collapse or drop to the petroleum crude droplets; since the stability of the droplets is dependent on biosurfactant concentration and correlates with surface and interfacial tension [34].

Our study showed that there were variations in the results of screening for different methods used. All isolates scored positive results for oil displacement test; since this method can detect biosurfactant even at low activity or concentration. The drop-collapse test and emulsification assay observed as vice versa; with all isolates scored negative results. Therefore it was suspected that the concentration of biosurfactant present in the cell-free supernatant of the 6 isolates were quite low to be detected by qualitative screening. Furthermore, emulsification index could not be calculated due to negative results of emulsification activity. Only BSP 6 was found to have scored positive results for more than one qualitative screening test which were oil displacement test and microplate assay. Hence, other screening methods which based on surface activity measurement should be conducted in the future to support these qualitative screening results. Conclusively, BSP 6 which was found to be closely related to *Comamonas aquatica* was found to be the best potential biosurfactant producer in from this study.

5. CONCLUSION

The 6 bacteria were successfully isolated from petroleum contaminated

sites and identified. There were isolates from the genera of *Pseudomonas*, *Comamonas* and *Stenotrophomonas* based on molecular identification of their 16S rRNA gene. They were found to be potential hydrocarbon degraders based on their ability to grow on minimal media supplemented with petroleum crude oil as sole carbon source. BSP 6 which was found to be closely related to *Comamonas aquatica*was the most promising biosurfactant producer based on all the screening methods performed. Further tests particularly based on surface activity measurement should be conducted in the future to support these qualitative screening results.

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