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Isolation of Potential Biosurfactant Producer from Oil Contaminated Soil and Water

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Abstract

Biosurfactant are extracellular macromolecules produced by bacteria, yeast, and fungi when grown on different carbon sources. It has the ability to reduce surface tension of a liquid, interfacial tensions between two liquids and between a liquid and a solid. This study was conducted to isolate potential biosurfactant producer from oil contaminated soil and water. Soil and water samples were obtained from food court area in front of Universiti Malaysia Kelantan, Jeli Campus. Isolation of biosurfactant producing bacteria was carried out on minimal salt medium (MSM) supplemented with palm oil as sole carbon source. Five potential biosurfactant producers; WS2, WS4, WS5, SS2 and SS5 were successfully isolated and identified by 16S rRNA analysis. Isolate WS4, SS2 and SS5 showed highest similarity to *Klebsiella sp* and the other two isolates, WS2 and SS5 showed highest similarity to *Pseudomonas sp* and *Nanobacterium sp* respectively. While *Klebsiella sp* and *Pseudomonas sp* were reported as prevalent biosurfactant producer, no report is available on the production of biosurfactant by *Nanobacterium sp.* All isolates showed variation in biosurfactant characterization assays which are emulsification test, drop collapse test, oil spreading test, blood haemolysis and blue agar plate assay.

1. Introduction

Biosurfactants are amphiphilic molecules with the ability to reduce surface tension of a liquid, interfacial tension of polar and non-polar liquids or interfacial tension between a liquid and a solid. Biosurfactant consists of two portions of molecules which are hydrophobic and hydrophilic portions which mean they can dissolve in aqueous and non- aqueous solution. The nonpolar hydrophobic portion is based on long-chain fatty acids, hydroxy fatty acids or α-alkyl-a-hydroxy fatty acids, while a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol forms the polar part of hydrophilic portion. They are extracellular biological compounds that are secreted by microorganisms during late logarithmic and stationary growth phase and basically are classified based on the microbial origin and the chemical structure (Jaysree *et al*., 2011). Biosurfactant producers are ranging from fungi, yeast and bacteria. Production of biosurfactants such rhamnolipids, surfactin, and lipopeptide were reported from *Pseudomonas aeruginosa, Bacillus subtilis* and *Candida sp* (Diniz Rufino, Moura de Luna, de Campos Takaki, & Asfora Sarubbo, 2014; Ramírez *et al*., 2015; Soberón-Chávez, Lépine, & Déziel, 2005).

Due to their amphiphilic nature, biosurfactant has found wide applications in agriculture, food industries, detergent making, cosmetic formulations, pharmaceutical industries and bioremediation. This microbial compound has been used as emulsifier and antiadhesive in food industries, wetting agent in detergent formulation and antimicrobial agent in cosmestic and pharmaceutical industries (Md, 2012; Usman, Dadrasnia, Lim, & Fahim, 2016). Biosurfactant producing microorganism are also used in bioremediation due to their ability to mineralized polyaromatic hydrocarbons (PAHs) and also heavy metal (Bezza & Chirwa, 2016; Luna, Rufino, & Sarubbo, 2016). Chemically synthesized surfactant which has been used widely in agriculture, cosmetic, food and pharmaceutical industries pose a threat to environment and human health due to their recalcitrant nature and toxicity. Biosurfactant is an alternative to chemical surfactant because they are biodegradable and low in toxicity (Md, 2012).

2. Materials and Methods

2.1. Isolation of Potential Biosurfactant Producer

Isolation of potential biosurfactant producer was carried out on minimal salt media (MSM) supplemented with 2% (v/v) palm oil. The compositions of 1 L mineral salt media are 200 mL of 5X minimum salts, 0.1 M

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magnesium sulphate and 1.0 M sodium chloride. 5X minimal salt solution consist of 33.9 g/L disodium phosphate, 15g/L potassium phosphate, 2.5g/L sodium chloride and 5.0g/L ammonium chloride. The medium was autoclaved at 15lbs pressure (121ºC) for 15 minutes. For the minimal agar medium preparation, 200 mL of sterile 5X minimal salts and 15g/L agar was added to 750 mL sterile distilled water. Then, 2 mL sterile 0.1M magnesium sulphate solution, 0.1mL of 1.0M sodium chloride was aseptically added into the medium. The medium was mixed and final volume was adjusted to 1000 mL. 2% (v/v) of palm oil was added as the carbon source for the media. Soil and water samples were collected from drainage at food court area in front of UMK Jeli Campus, Kelantan. The samples were serially diluted with sterile dH_20 and spread on MSM supplemented with 2% (v/v) palm oil. Agar plates were incubated at 30°C until bacteria colonies were observed on the media. Colonies grown on MSM media supplemented with 2% (v/v) palm oil were streaked several times on MSM media to obtain pure culture. The colonies were then observed under microscope to ensure there is no mixed culture. Pure bacteria isolates were used for further characterization.

2.2. Emulsification Test

2 mL of palm oil was added in 2mL of supernatant of bacterial culture obtained after centrifugation. The mixture was vortexed for 1 minute and let to stand for 24 hours at room temperature. Using 10% Sodium dedocyl sulphate (SDS) as positive control, emulsification layer was observed and recorded. Test was repeated using kerosene oil.

2.3. Oil Spreading Assay

10 µL of engine oil was added to surface of 30 mL distilled water in a petri dish to form a thin layer of oil. 10 µL culture supernatant was gently placed on the centre of oil layer. Presence of biosurfactants is observed when the oil displaced and formed a clear zone. The displaced diameter were measured after 30 seconds

2.4. Drop Collapse Assay

A drop of palm oil was placed on a glass slide. Then, 10 µL of culture supernatant was dropped on the oil surface. Water is used as negative control.

2.5. Blue Plate Assay

This method was used to detect the anionic biosurfactants such as rhamnilipids (Siegmund & Wagner, 1991). 100 µL of culture supernatant was loaded into each well prepared in methylene blue agar plate. The plate then incubated for 48-72 hours at 37°C. Positive result showed a dark blue halo zone around the well which indicates the presence of anionic biosurfactant

2.6. Blood Hemolysis Assay

Pure culture of bacteria isolates was streaked on 5% horse blood agar. Then, it was incubated at 37°C for 48 – 72 hours. Clear zones around the colonies indicate the presence of biosurfactant production.

2.7. Genomic DNA Extraction

Bacteria genomic DNA were extracted using G-spin™ Total DNA Extraction Kit (INtRON Biotechnologies). Bacteria isolates were grown in Luria Bertani broth for 16 hours at 30°C prior to extraction. Genomic DNA from the isolates were used as template for further 16S rRNA amplification.

2.8. 16S rRNA Amplification

Amplification of 16S rRNA from bacteria isolates were carried out using primers 68F (5' TNA NAC ATG CAA GTC GAR 3') and 1392R (5' ACG GGC GGT GTG TRC 3'). PCR temperature profiles were set as follows; initial denaturation (95°C for 5 minutes), denaturation (95°C for 90 seconds), annealing (55°C for 30 seconds), extension (72°C for 90 seconds) and final extension (72°C for 10 minutes). PCR amplification was carried out for 30 cycles.

2.9. DNA Sequencing and Analysis

PCR product were purified using MEGAquick-spin™ Total Fragment DNA Purification Kit (INtRON Biotechnologies) prior to sequencing. Purified PCR products were sent to First BASE Laboratories Sdn Bhd (Selangor, Malaysia) for sequencing service. DNA sequences were analysed using BioEdit Sequence Alignment Editor and similarity search was done using BLAST tool at NCBI webpage (www.ncbi.nlm.nih.gov/BLAST).

3. Results and Discussion

Isolation of potential biosurfactant producers were carried out using samples collected from the area which heavily contaminated with oil. Biosurfactant producers were naturally found in soil and water heavily contaminated with aliphatic and aromatic hydrocarbon (Batista, Mounteer, Amorim, & Totola, 2006; Bento, de Oliveira Camargo, Okeke, & Frankenberger, 2005; Yadav et al., 2016). This group of microorganism capable of utilizing hydrocarbon as their carbon source and convert them into harmless product (Saxena & Singh, 2010). Five potential biosurfactant producers were successfully isolated from soil and water samples. The ability of the isolates to produce biosurfactants were investigated by several quantitative and qualitative assays (Table 1). Emulsification test was performed to determine the emulsion index (E24) which is the height of the emulsion layer (cm) divided by total height (cm) multiplied by 100. Microorganism produce bioemulsifier to increase the

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bioavailability of poorly soluble substrate thus makes it easier to be mineralized and utilized them as carbon source (Beal & Betts, 2000). It will reduce the surface tension between water and hydrophobic substance and forming emulsion thereby increase the growth surfaces for bacteria. All isolates except isolate SS5 show ability to emulsify palm oil with isolate WS2 shows the highest emulsification index. As for kerosene, only isolate SS2 shows the ability to form emulsion. According to Uzoigwe, C *et al.* (2015) biosurfactants and bioemulsifiers have different properties based on physical-chemicals properties and physiological roles. Biosurfactants have low molecular weight compounds composed of sugars, amino acids, fatty acids and some functional groups like carboxylic acids. While, biosemulsifiers are higher in molecular weight compounds which consist of complex mixture of heteropolysaccharides, lipopolysaccharides, lipoproteins and proteins. Bioemulsifiers play role in emulsifying two immiscible liquids and have less effective activity towards surface tension reduction (Uzoigwe, Burgess, Ennis, & Rahman, 2016). Isolate with low emulsification index was shown to be able to degrade more than 80% diesel oil in soil (Yadav *et al*., 2016).

Drop collapse test was conducted to investigate the ability of the bacteria isolates to reduce surface tension. Oil drop will remain stable and a polar water molecule will be repelled from hydrophobic surface if the culture supernatant does not contain surfactants. On the other hand, the oil drop will spread or collapse if the liquid contains surfactants because the interfacial tension between culture supernatant and the hydrophobic surface has been reduced. From the result, only one isolate, WS2 which shows positive result for drop collapse test (Figure 1). However, all bacteria isolates showed positive result for oil spreading assay with isolate WS5 showed the widest oil displacement area which is 3.5cm (Figure 2). Oil spreading test is more sensitive than drop collapse test (Ariech & Guechi, 2015) and the area of oil displacement is directly proportional to the concentration of biosurfactant in the culture broth (M, Y, & TA, 2000). However, none of the isolates showed positive result for blue plate assay suggesting that biosurfactant produced by the isolates are not from anionic group. As for blood hemolysis assay, none of the isolates show beta hemolysis activity. However, those isolates with gamma hemolysis showed positive result for other biosurfactant characterization assays. Some studies reported that hemolytic assay is not reliable and less sensitive to be used as screening method for biosurfactant production as many factors other than biosurfactants such as lytic enzymes, protease as well as virulence factors that are produced by bacteria can also cause the clearing zones. These factors can lead to a lot of false negative and false positive results (Płaza, Zjawiony, & Banat, 2006; Thavasi, Sharma, & Jayalakshmi, 2011).

Table 1: Quantitative and qualitative assays result for biosurfactant production

Assay \ Isolates	WS2	WS4	WS5	SS1	SS2	SS5
Emulsification test (E24)[a] using palm oil	32.43	21.62	21.62	29.73	21.62	-
Emulsification test (E24)[a] using kerosene	-	-	-	-	53.13	-
Oil spreading assay[b]	2.6	1.5	3.5	1.2	2.5	2.2
Drop collapse assay[c]	+	-	-	-	-	-
Blue plate assay[d]	-	-	-	-	-	-
Blood hemolysis assay[e]	Gamma	Gamma	Gamma	Gamma	Gamma	Gamma

[a]result expressed as emulsion index (%), [b]result expressed as oil displacement area in (cm), [c]result expressed as + (positive) – (negative), [d]result expressed as + (positive) – (negative), [e]result expressed as type of hemolysis

All the isolates were further identified by 16S rRNA analysis. The successfully amplified gene were sent for sequencing at 1st Base Laboratories (M) Sdn Bhd. Sequenced PCR product then was analysed using BLAST software at NCBI webpage (www.ncbi.nlm.nih.gov/BLAST). Result from BLAST analysis showed that isolate WS2 has 95% identity to *Pseudomonas otidis*, isolate WS4 has 83% identity to *Klebsiella pneumoniae*, isolate WS5 has 96% identity to *Nanobacterium sp*, isolate SS2 has 94% identity to *Klebsiella varicella* and isolate SS5 has 97% identity to *Klebsiella pneumonia*. Based on the result, most of the isolates are similar with *Klebsiella* genus and one isolate, WS2 showed 95% identity to *Pseudomonas otidis*. These two genera are most prevalent surfactant producers and hydrocarbon degrader. Other than these two groups of bacteria, other most prevalent producers are from genera *Bacillus, Micrococcus, Flavobacterium, Achromobacter, Arthrobacter, Acinetobacter, Corynebacterium, Aeromonas, Alkaligens, Moraxella* and *Streptococcus* (Mishra, Jyot, Kuhad, & Lal, 2001). One isolate WS5 showed 96% identity to *Nanobacterium persia. Nanobacterium sp* is commonly associated with urinary tract stone (Drancourt *et al*., 2003) and no report available on the production of biosurfactant from this bacteria species.

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Figure 1: Drop collapse test. A. Isolate WS2 B. Negative control (water)

Figure 2: Oil spreading assay

Isolation of biosurfactant producers were widely carried out by many research groups due to their promising applications in bioremediation, biotechnology and biopharmaceuticals industry. Similar studies were conducted locally and around the globe focusing on the ability of biosurfactant producers to degrade environmental pollutants such as polyaromatic hydrocarbons (PAHs) and heavy metals (Affandi, Suratman, Abdullah, Ahmad, & Zakaria, 2014; Yadav *et al*., 2016; Zafar, Shafiq, Nadeem, & Hakim, 2016). Other significant potential of biosurfactant is MEOR or 'Microbial Enhanced Oil Recovery'. Microbial surfactants are able to lower the interfacial tension at the oil-rock interface thus increase the yield of oil recovery. Microbial surfactants such as sophorolipids, surfactins and rhamnolipids were investigated for potential application in MEOR (Elshafie *et al*., 2015; Gudiña *et al*., 2015; Liu, Lin, Wang, Huang, & Li, 2015).

4. Conclusion

In this present study, five potential biosurfactant producers were successfully isolated from soil and water contaminated with oil. Quantitative and qualitative assays for biosurfactant production conducted on the isolates revealed nonuniformity in the result suggesting different types of biosurfactants were produced by the isolates. From 16S rRNA analysis, three of isolates showed highest similarity with *Klebsiella sp*, while the other two were similar with *Pseudomonas sp* and *Nanobacteria sp.* While *Klebsiella sp* and *Pseudomonas sp* were reported as prevalent biosurfactant producer, no report is available on the production of biosurfactant by *Nanobacterium sp.*

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