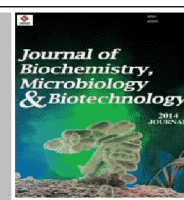




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## Isolation and screening of high efficiency biosurfactant-producing bacteria *Pseudomonas* sp.

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### ABSTRACT

Biosurfactants are becoming an important alternative to chemical surfactants in almost every sector in the modern industry because of their improved properties compared to their chemical counterparts. The aim of this study is to isolate and identify bacterial strain from different environment conditions with the capacity to produce rhamnolipid-biosurfactants when grown on blue agar plate (bap) selective medium. Five strains were screened out using the combination of modified drop-collapse test, oil spreading and emulsification index ( $e_{24}$ ) test. A strain with an outstanding performance and demonstrated good activity in all the above screening methods was successfully isolated and had shown comparable results against triton-x 100, a chemical surfactant. The emulsifying capacity of this strain was evaluated by the emulsification index ( $e_{24}$ ) and it ranged from 54.7% - 62.1%. Using the morphological and biogenesis iii microplate analysis, the strain was identified as *pseudomonas aeruginosa*. In this study, an efficient and simple protocol to screen out rhamnolipid-biosurfactants producing bacteria was used and this finding will also help to add novel members to the biosurfactants group as well as expanded its current knowledge regarding the diversity and productive capability biosurfactants from a single specific strain. Therefore, this strain might be useful as an alternative to chemical surfactants for a wide range of potential applications.

### INTRODUCTION

Biosurfactants attracted attention as a green alternative to chemical surfactants and their applications have been widely spread over the past five decades as they provide improved properties which chemical surfactant is lacking in. Biosurfactants are amphiphilic molecules containing hydrophilic head and hydrocarbon tail moieties that reduce surface and interfacial tension by accumulating at the interface between immiscible fluids. They are produced either on microbial cell surfaces or excreted extracellularly in growth or fermentation medium to facilitate the diffusion of a hydrocarbon substrate into the cell. Biosurfactants are generally grouped into glycolipids, lipopeptides, phospholipids, fatty acid salts and polymeric biosurfactants. Of all currently known biosurfactants, rhamnolipids (a glycolipid-type biosurfactants) have the highest potential of becoming the next generation of biosurfactants introduced to the market [11] owing to several characteristics such as low in minimum surface tension (30-32 mN/m), high emulsifying activity, ignoble critical micelle concentration (CMC) reaching 5 mg/l and higher affinity for hydrophobic organic molecules [2,3].

A wide range of microorganisms had been reported for the production of biosurfactants in literature [12]. Due to the high-yield biosurfactants production, most of the strains which reported to produce rhamnolipid had been identified as *Pseudomonas aeruginosa* and they are considered safe for the usage of food, cosmetic and pharmaceutical products [10]. In addition, the reason why *P. aeruginosa* is often employed as biosurfactants-producing or oil-degrading strain may be because of its natural ability to survive and grow in a wide range of environmental conditions especially in contaminated environments. Besides the ease of isolation and screening, its rapid growth as well as the high biosurfactants production also plays a major role [24]. Due to the increasing demand of microbial biosurfactants in the vast array of application, discovery and evaluation of new biosurfactant-producing strains as well as economical biosurfactants production are important because different strains varied from their biosurfactants natures. Based on the above matter, this present work deals with an isolation and screening of efficient biosurfactants-producing bacteria, and the production of biosurfactants using palm oil as the carbon source which is cheaper and abundantly available in Asia. Thus, this work can

provide excellent materials and resources to expand current knowledge on biosurfactants.

## MATERIALS AND METHODS

### Sampling area

For the isolation of biosurfactants-producing bacteria, the sample was collected from different environments such as compost of oil palm empty fruit bunch, lake, spoilt milk and fried rice, soil, as well as wastewater.

### Isolation of biosurfactant-producing *Pseudomonas* sp.

Samples obtained were subjected to isolation by serially diluted and plated on selective agar medium. Serial dilution was made by diluting the samples with sterile 0.9% NaCl solution until desired dilutions were performed. Diluted samples were spread onto *Pseudomonas* Agar plate (CM0559, OXOID, England) supplemented with CN and CFC selective supplement. The plates were incubated at 30 °C for 3 to 4 days. The appearance of bacterial colonies was observed daily and differentiated based on their color, shape and size. The bacterial colonies which grew on the agar plate were further screened for biosurfactants potential.

### Screening of biosurfactants-producing bacteria *Pseudomonas* sp.

#### Blue Agar Plate (BAP) method

Primary screening of biosurfactant-producing *Pseudomonas* sp. was performed by Blue agar plate method (BAP). The mineral salts agar media (MSA) supplemented with glucose as carbon sources (2%) and cationic surfactant cetyltrimethylammonium bromide (CTAB: 0.5 mg/ml) - the basic dye methylene blue (MB: 0.2 mg/ml) was prepared as reported previously [18]. The colonies grown from isolation process was then transferred onto freshly prepared BAP and incubated at 30 °C for the duration of 4 to 11 days. Formation of dark blue halo zone around the culture is considered as positive for biosurfactant producing *Pseudomonas* sp. Further screening of biosurfactants production was carried out using three different assays; Drop collapse test, Oil spreading test and Emulsification index test [18].

#### Drop Collapse Test

Drop collapse test was performed in order to screen the best surfactant producing *Pseudomonas*. A 2 µl of motor oil was spotted onto a piece of Parafilm sheet while 5 µl of the cell free culture broth was added and the drop size was observed 5 min later. Positive results were considered for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by deionized water (negative control). Triton X-100 (a chemical surfactant) (Sigma Aldrich) solution was used as a positive control. Cultures that give rounded drops were scored as (-) negative, indicative of the lack of biosurfactant production [22].

#### Oil Spreading Test

30 ml distilled water was put into Petri dishes. 50 µl diesel was dropped on to the water surface to form oil film, in the midst of which 5 µl fermentation broth (diluted by 10 times with water) was dropped. The diameter of the oil expelling circles formed was measured. Distilled water (without surfactant) was used as negative control and Triton X-100 was used as positive control [24].

**Qualitative Emulsification Index ( $E_{24}$ ) Test** The emulsifying activity of culture supernatants was estimated by adding 0.5 ml of sample fluid and 0.5 ml of hexadecane to 4.0 ml of distilled water. The tube was vortexed for 10s, holds stationary for 1min, and then visually examined for turbidity of a stable emulsion. This method is a qualitative method, thus it was used only in the screening purpose [15].

### Identification and characterization of isolated *Pseudomonas* by morphological and Biolog GEN III MicroPlate analysis

Isolated microorganism was identified through observation of growth on the Nutrient agar plate and gram-stained. Identification of species level was performed using Biolog GEN III MicroPlate (Biolog, Hayward, CA, USA) following the instruction of the manufacturer's and was sent to Focus Biotech Sdn. Bhd. later for analysis.

### Biosurfactants production in shake flasks

One loopful of culture was inoculated in 20 ml of seed media (Nutrient broth) in a 100 ml flask and incubated on a rotary shaker at 30 °C and 180 rpm for 10 h. 5% of inoculum was transferred to the production medium and incubated at 30 °C in an incubator shaker at 180 rpm for three days. The growth of the isolate was carried out according to basal salt medium of Zhang et al. [24]. The fermentation broth was composed of (g/L) NaNO<sub>3</sub> 2.5, K<sub>2</sub>HPO<sub>4</sub> 4.0, KH<sub>2</sub>PO<sub>4</sub> 4.0, CaCl<sub>2</sub> 0.1, MgSO<sub>4</sub> 0.2, NaCl 1.0, KCl 1.0, yeast extract 1.0 and 1% (v/v) palm oil (as the sole carbon source). Flask without carbon source was used as control. The pH of the media was adjusted to 7 using 1 N NaOH and autoclaved by 121 °C for 20 min. Time course samples of culture medium were drawn in appropriate time intervals and monitored for cell growth and biosurfactants production in terms of emulsification index test and biosurfactants weight [24].

### Biosurfactants Quantification

#### Extraction of biosurfactant

The extraction of biosurfactant produced from isolated *Pseudomonas* was performed according to the procedure described previously by Samadi et al. [17] and Apama et al. [3]. The cell was removed by centrifugation at 9000 rpm for 30 minutes at 4 °C and the biosurfactants were recovered by acidifying the supernatant to pH 2.0 using 1 M sulfuric acid, prior to biosurfactant extraction from the culture medium. The biosurfactant was extracted using equal volume of chloroform-methanol (2:1) mixture, shaken well and allowed to stand until phase separation. The organic phase was separated and the extraction was repeated twice. The pooled extract was concentrated in a rotary evaporator at 40 °C and then dried at 50 °C to constant weight under vacuum. The dried product was washed with absolute ethanol for three times to remove residual pigments. It was weight and expressed as g/l [17, 3].

#### Quantitative Emulsification index ( $E_{24}$ ) test

The ability of the biosurfactant to emulsify hydrophobic substrate, such as hydrocarbons and vegetable oil (sunflower oil) was determined. A 2 ml of cell-free broth containing biosurfactants was added into each test tube containing 2 ml hydrophobic substrate. The mixture was vortexed vigorously for 2 min and left undisturbed for 24 h. The emulsion index ( $E_{24}$ ) was determined as the percentage of height within the emulsion layer divided by the total height [1,3].

## RESULTS AND DISCUSSION

### Source of microorganism

Microorganisms are abundance in natural environments such as compost of oil palm empty fruit bunch, lake, spoiled milk and fried rice, soiland waste water. They occur almost always in a combined population composed of a multitude of different strains and species. For analyzing the properties of a defined organism out of such varied population, a pure culture is required.

### Isolation of biosurfactants-producing *Pseudomonas* sp.

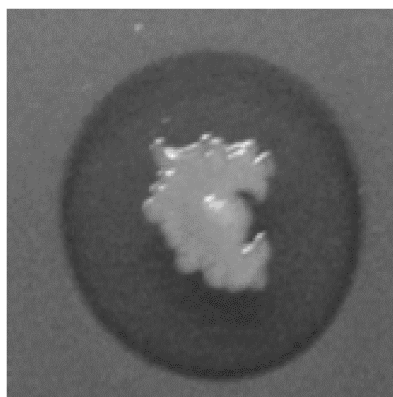
*Pseudomonas* sp. is a strain often reported throughout the literature for biosurfactant production and crude oil degradation, predominantly *Pseudomonas aeruginosa* due to their excellent performance [1,14,20]. Due to this fact, this study focused on the isolation of *Pseudomonas*-producing biosurfactant using the *Pseudomonas* selective agar plate. *Pseudomonas* Selective Agar was designed to be selective for *Pseudomonas* spp. or *Pseudomonas aeruginosa*. *Pseudomonas* CFC Supplement was used for the selective isolation of *Pseudomonas* spp. generally, while *Pseudomonas* CN supplement was used for the selective isolation of *Pseudomonas aeruginosa*. Approximately 2856 colonies were successfully isolated from different environment conditions after incubation at 30 °C for 3 to 4 days. The presence of blue-green pigmentation around the bacterial colonies in the

plate indicates that the colonies isolated are *Pseudomonas* spp. Even so, further tests must be carried out to confirm the identity of these microorganisms.

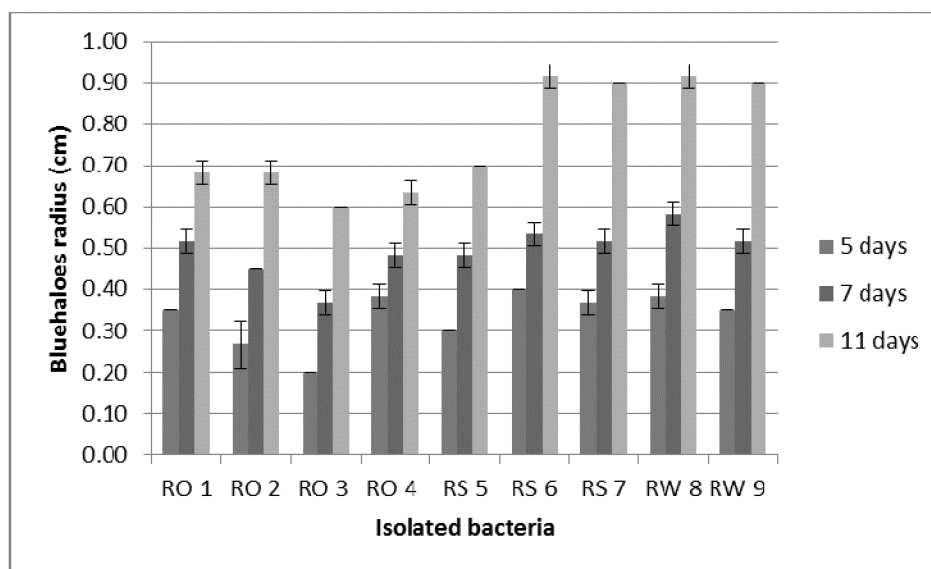
### Screening of biosurfactants-producing *Pseudomonas* sp.

#### Blue Agar Plate (BAP) method

Specific screening methods like the colorimetric CTAB agar plate or known as blue agar plate was employed as preliminary screening method. It explored the ability of strains to interfere with hydrophobic interfaces since it is suitable only to a limited group of biosurfactants like extra cellular glycolipids or anionic biosurfactant specifically rhamnolipid. This screening method can give qualitative and/or quantitative results. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue [8]. 741 out of 2856 colonies were picked up and cultured onto blue agar plates. After 4 to 11 days incubation at 30 °C, 263 colonies showed blue haloes around their colonies and were considered as positive for biosurfactant production (Figure 1). The highest blue halo radius were detected in 9 isolates isolated from oil spilled (RO1, RO2, RO3, RO4), Sri Serdang lake (RS5, RS6, RS7) and compost sludge (RW8, RW9) as shown in Figure 2.



**Figure 1:** Biosurfactant production after 7 days growth as indicated by blue halo surrounding colony of RS 6. Bars represent 0.5cm



**Figure 2:** Radius of clear zone formed by 9 isolates after 5,7 and 11 days of incubation at 30 °C

In total, 5 strains with big blue haloes ( $\geq 0.5$ cm radius) were screened out and incubated on slant nutrient agar media, cultured at 30 °C for 24h and stored at 4 °C for next evaluation. The strains were subjected to fermentation and biosurfactants producing capacities was confirmed through other screening methods through the analysis of drop collapse test, emulsification index test and oil spreading test. A combination of various screening methods are required for efficient detection of potential biosurfactants producer because a single screening method is difficult to use in order to detect the types of biosurfactants produced by the microorganism owing to the chemical and functional properties. Drop collapse test, oil spreading test and emulsification index test are reliable methods as it has been applied several times for screening purposes [18, 9].

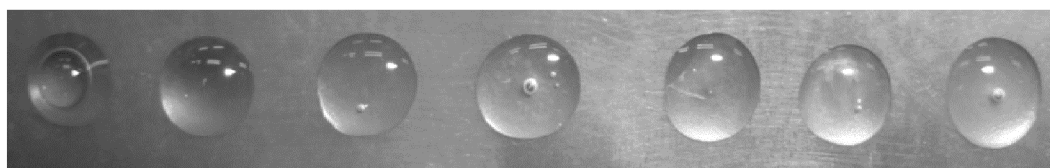
#### Drop Collapse Test

This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension.

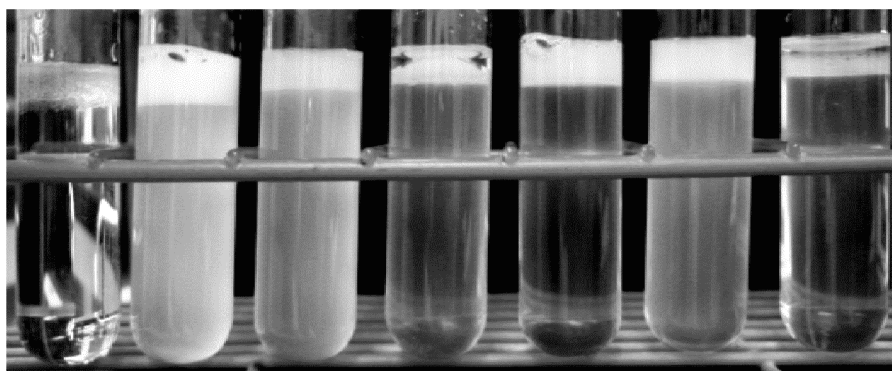
This drop collapse assay is rapid and easy to carry out, does not requires specialized equipment and only need a small volume of sample. Figure 3 shows all the isolate gave positive response for this test and these isolates can be potentially applied as a good biosurfactant producer. Belcher et al. [6] mentioned that this method only generates a positive response if there is a high concentration of surfactant present. This method is an efficient to be applied for screening purpose as it is a fast method and generates better visualization results. From this test, by using a cell-free medium from saturated cultures, all 5 isolates are positive for biosurfactants activity.

#### Oil spreading test

If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone of the oil surface correlates to surfactant activity, also called oil displacement activity. The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. Youssef et al. [23] and Zhang et al. [24] demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms. The results showed that even with 5 uL fermentation broth (diluted by 10 times with water), the strain could produce notable ( $>4$  cm diameter) oil expelling circles (ranging from 4.0 cm to 5.1 cm). RS6 strain produced the highest oil displacement activity with a diameter of 5.1 cm. This shows that it has the most biosurfactant activity.



**Figure 3.** Drop collapse test done on parafilm; From left to right: distilled water (negative control), 1% Triton-X 100 (positive control), RO1, RS6, RS7, RW8 and RW9



**Figure 4.** Emulsification index test, from left to right: negative control, positive control, RW9, RW8, RS7, RS6 and RO1.

**Qualitative Emulsification Index (E<sub>24</sub>) Test** This assay is based upon the emulsification capacity of biosurfactant was developed by Cooper and Goldenberg, [7]. The emulsification activity is one of the most important factors of a surfactant. Based on Figure 4, RS6 and RW9 strains shows the most similar properties to Triton-X 100 (positive control) as it produced better turbidity compared to RO1, RS7 and RW8.

Based on the results obtained from all the screening methods performed, bacteria that shown great potential for biosurfactants production are the bacteria with maximum oil displacement diameter and the best emulsification index activity. Thus strains RS6 is the best biosurfactants producer and was identified for further study.

**Identification and characterization of isolated *Pseudomonas* by morphological and Biolog Gen III MicroPlate analysis**

Morphological test indicated that this isolate was gram-negative, rod-shaped bacteria. From Biolog Gen III MicroPlate analysis, the result shows that this sequence gave the closest ID to *Pseudomonas aeruginosa* with 99.5% probability as shown in Figure 5.

**Biosurfactant production in shake flask**

Biosurfactant production depends on the composition of the growth medium used. Since biosurfactant production is highly variable, the fermentations of RS6 were first investigated by using palm oil as sole carbon source. Incubation temperature, 30 °C, was selected in this study because both *P. aeruginosa* MA01 isolated

from spoiled apples [1] and *P. aeruginosa* strains isolated from crude-oil contaminated environments [24] produced optimum biosurfactant when grown at 30 °C. Besides, study done by Apama et al. [3] showed that *P. aeruginosa* sp. 2B isolated from areas contaminated with petroleum compounds also produced maximum biosurfactants at 30 °C. During early stage of fermentation, oils appeared on the upper part of the aqueous medium or attached on glass wall. After 24 hours of incubation, the oils stretch gradually turned into dispersed oil droplets in the medium and finally disappeared even after left out without shaking. This phenomenon shows that there is a good production of biosurfactants in the medium that emulsify these hydrophobic carbon sources. In this study, pH of culture medium does not changed significantly and it was around 7.0 during incubation time.

Palm oil has a balanced fatty acid composition in which the level of saturated fatty acids is almost equal to that of the unsaturated fatty acids. The major component acids in palm oil are palmitic acid (44-45%) and oleic acid (39-40%), along with linoleic acid (10-11%). Palm oil has high content of disaturated (POP and PPO) and monounsaturated (POO and OPO) triglycerides (TGs) [19]. Thus, palm oil was chosen as carbon source to study biosurfactant production because it have considerably high composition of oleic acid, therefore contribute to high yield of biosurfactants and showed good properties in both emulsification activity and oil displacement activity besides being more economical and abundantly available in Malaysia.

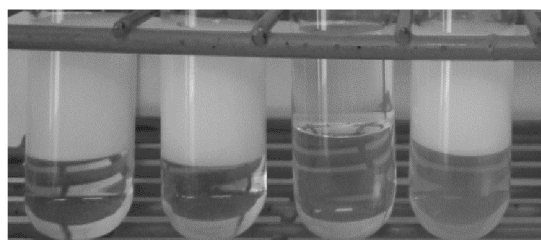
Date & Time of Read		May 09 2013			
Biolog ID DB		Biolog GEN III DB.I5G			
Result		Species ID: <i>Pseudomonas aeruginosa</i>			
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Rank	PROB	SIM	DIST	Organism Type	Species
1	0.995	0.657	4.890	GN-Nent	<i>Pseudomonas aeruginosa</i>
2	0.003	0.001	8.538	GN-Nent	<i>Pseudomonas fluorescens</i> biotype G
3	0.001	0.000	9.408	GN-Nent	<i>Pseudomonas citronellolis</i>
4	0.001	0.000	9.439	GN-Nent	<i>Pseudomonas maculicola</i>

**Figure 5:** The Biolog GEN III MicroPlate analysis result

**Table 1:** Substrate specificity and emulsification activity of biosurfactants produced by RS6 using 1% (v/v) palm oil

Carbon source	Emulsification index ( $E_{24}$ )			
	Sunflower oil	Hexadecane	Benzene	Kerosene
1% Triton-X 100	64.0 ± 0.6	61.8 ± 1.3	62.5 ± 3.0	62.5 ± 2.0
1% SDS	66.0 ± 0.6	64.5 ± 0.0	63.7 ± 0.7	63.7 ± 0.7
1.0% palm oil	62.1 ± 0.3	54.7 ± 0.0	57.1 ± 0.0	57.1 ± 0.0

**Table 1:** demonstrates the biosurfactant produced by RS6 that could emulsify vegetable oil (sunflower oil) and different hydrocarbons (hexadecane, benzene and kerosene). The cell-free culture broth shows appreciable emulsification indices against sunflower oil ( $62.1 \pm 0.3\%$ ) and hydrocarbons (hexadecane ( $54.7 \pm 0.0\%$ ), benzene ( $57.1 \pm 0.0\%$ ) and kerosene ( $57.1 \pm 0.0\%$ ). It showed comparable result against chemical surfactant 1% Triton-X 100 and 1% SDS used as positive control. Moreover, the results are also in accordance to those reported by Abbasi et al. [1] who reported *Pseudomonas aeruginosa* MA01 biosurfactant could emulsify corn and coconut oils more than 50% and it has a stable emulsifying activity for hexane, kerosene, brake oil, hexadecane, xylene and toluene ranging from 55.8 to 100%.

**Figure 6:** Emulsification index test, from left to right: 1% Triton X-100, 1% SDS, control and RS6 against hexadecane

### Quantification of Biosurfactants Production

#### Extraction of biosurfactant

The cell-free supernatant was acidified with 1 M  $H_2SO_4$  to pH 2.0 before extraction to enhance the precipitation of biosurfactant. Solvent-solvent extraction using the mixture of chloroform-methanol gave a final product of oily-yellow color. This result can be explained as biosurfactant is a lipid containing amphiphatic molecules. Due to the presence of hydrophobic end, the biosurfactants was dissolved in the organic solvent and extracted out. A 4.32 g/l biosurfactant had been produced using 1% (v/v) palm oil. Least biosurfactant production which is 0.52 g/l was observed in the medium without carbon source. Markus et al. [11] mentioned that biosurfactant production was affected by the type of carbon source used. High product titers have been exclusively described with vegetable oil as sole carbon source in combination with *Pseudomonas aeruginosa* strains. This statement is in agreement with those obtained by Abbasi et al. [1] which produced more than 12 g/l biosurfactant when provided with oil of vegetable origin, such as soybean, canola corn and olive but less efficient to consume soluble substrate. Besides there is also a wide range of biosurfactant production yield by *Pseudomonas* species using vegetable oils and/or their wastes as carbon source has been reported in which *P. aeruginosa* LBI produced biosurfactant when grows on water insoluble substrates sunflower oil (4.9 g/l), soybean oil (4.8 g/l) and olive oil (5.3 g/l) [5]. Different levels of

biosurfactant production using different types of vegetable oils maybe a result of the oils properties themselves. Although all oils are the esters of glycerin and fatty acids chemically, nevertheless the chemical and physical properties of natural oils vary widely because different proportions of fatty acids and structures of triacylglycerol that they contained [1].

#### Quantitative emulsification index ( $e_{24}$ ) test

Table 1 demonstrates the biosurfactant produced by RS6 that could emulsify vegetable oil (sunflower oil) and different hydrocarbons (hexadecane, benzene and kerosene). The cell-free culture broth shows appreciable emulsification indices against sunflower oil ( $62.1 \pm 0.3\%$ ) and hydrocarbons (hexadecane ( $54.7 \pm 0.0\%$ ), benzene ( $57.1 \pm 0.0\%$ ) and kerosene ( $57.1 \pm 0.0\%$ ). It showed comparable result against chemical surfactant 1% Triton-X 100 and 1% SDS used as positive control. Moreover, the results are also in accordance to those reported by Abbasi et al. [1] who reported *Pseudomonas aeruginosa* MA01 biosurfactant could emulsify corn and coconut oils more than 50% and it has a stable emulsifying activity for hexane, kerosene, brake oil, hexadecane, xylene and toluene ranging from 55.8 to 100%.

These results indicate that the emulsifying agents present in biosurfactant produced by all these substrate might have increased the viscosity of the aqueous phase and reduced the tendency of the dispersed oil globules to migrate and coalesce. These

biosurfactant can also stabilize emulsions by absorption at the interface and forming a coating around the dispersed particles. This high emulsification index activity results highlighted the possibility for it to be used in bioremediation of hydrocarbon-contaminated sites or in the petroleum industry and also suggest a potential application in food industry as cleaning and emulsifying agent.

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