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Comparative approach for detection of biosurfactant-producing bacteria isolated from Ahvaz petroleum excavation areas in south of Iran

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Abstract- The current study was undertaken to compare four analytical methods including drop collapse, oil spreading, surface tension (SFT) measurements, and blood agar lysis tests for detection of biosurfactant-producing bacteria. Among 32 biosurfactant-producing bacteria isolated from Ahvaz oil fields, in south of Iran, 16 isolates (50%) exhibited highest biosurfactant production. Eleven isolates (MASH.1 to MASH.11) demonstrated a reduction in surface tension from 65 mN/m to less than 41 mN/m. The results showed that about 91% of these highly biosurfactant producers had the same response levels of "++++" and "+++" in the case of both SFT and oil spreading methods. Among these, seven isolates had the haemolysis diameter less than 1 cm or between 1 and 2 cm on blood agar. As 64% of the best biosurfactant producers did not completely lyses blood, the ability of biosurfactant-producers for haemolysis may not always be trustworthy. According to our data, there is a good consistency between oil spreading technique and surface tension. As a conclusion, oil spreading method is the fastest, simplest and most consistent analytical method to be suggested for accurate measurements of biosurfactant producers.

Key words: biosurfactant, oil spreading, drop collapse, surface tension, blood lysis.

INTRODUCTION

Bacteria produce a wide range of biosurfactants with diverse and unlike chemical structures (Kosaric, 1992; Christofi and Ivshina, 2002; Ron and Rosenberg, 2002; Das and Mukherjee, 2005). Interest in microbial surfactants has progressively been increasing during recent years since they have higher biodegradability, higher specific activity at extreme temperatures, salinity, and pH levels and considered environmentally compatible with limited toxicity (Desai and Banat, 1997; Haba *et al.,* 2000; Maier and Soberón-Chávez, 2000; Gautam and Tyagi, 2006). Biosurfactants are interesting molecules with various biological functions. From a biotechnology prospective, the production of biosurfactants is important because of their numerous applications in environment, agriculture and industry (Banat, 1995; Benincasa *et al.,* 2004; Singh and Cameotra, 2004). These kind of natural microbial compounds are biologically surface-active agents and have many potential advantages over their synthetic relatives. Microorganisms were considered to be detrimental to the petroleum industry in the past. It is now well known that they can also be beneficial in terms of oil recovery. The low water solubility of many hydrocarbons, especially the polycyclic aromatic hydrocarbons (PAHs), is believed to limit their availability to microorganisms, which is a potential problem for bacteria. It has been assumed that surfactants would enhance the bioavailability of hydrophobic compounds (Ron and Rosenberg, 2002). Because of the potential applications, many biotechnological studies have been focused on the mass and commercial production of biosurfactants. Due to the unique properties and vast array of applications, the need for selection a simple and rapid as well as reliable analytical approach to detect the biosurfactant-producers with minimum number of false positives and/or negatives can not be ignored (Mukherjee *et al.,* 2006). Several analytical methods have so far been employed for directly measurement of surface activity of biosurfactant. These approaches include surface and/or interfacial tension measurement, drop shape analysis profile, glass slide test, method and the oil spreading technique (Harkins, 1959; Mulligan *et al.,* 1984; Banat, 1993; Van Dyke *et al.,* 1993; Carrillo *et al.,* 1996; Makkar and Cameotra, 1997, 1998; Menezes Bento *et al.,* 2005). Each type of these aforesaid methods has their own advantages and disadvantages. Although enormous efforts have been made to develop the analytical methods for accurate measurement of biosurfactant concentration and its activity, currently available reports on the comparison of these methods with each others to select the most reliable one is limited to a few reports (Bodour and Miller-Maier, 1998; Piaza *et al.,* 2006). The current study was undertaken to compare different analytical methods for measurement the activity of biosurfactant produced by bacterial strains with unknown taxonomic affiliations isolated from oil fields in south of Iran.

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MATERIALS AND METHODS

Media, enrichment and isolation of bacteria. The bacterial strains were isolated by the enrichment culture technique from the crude oil obtained from Ahvaz oil fields. For this purpose, 10 ml crude viscous oil samples were inoculated into 90 ml of supplemented nutrient broth (SNB medium) of the same composition as described by Francy *et al.* (1991). The medium incubation was performed at 30 °C on a rotary shaker incubator at 200 rpm for 7 days. Then, an aliquot of culture (0.1 ml) was spread on Bushnell-Haas agar(Difco, Germany) and incubated at 30 °C for 48 h. Diluted microbial suspensions in SNB were prepared and cultivated on Brain Heart agar (Merck, Germany) so as to obtain separate colonies. The procedure was repeated three times.

Single colonies of each bacterial isolate growing well on Brain Heart agar (Merck, Germany) were designated according to their morphological and biochemical characteristics. Furthermore all the isolates were examined by using optic microscope (Olympus Vanox AHBT3). Standard morphological and biochemical tests were used for their preliminary characterization. Among 32 bacterial strains, sixteen biosurfactant producer isolates were selected according to following method and maintained on nutrient agar (Merck, Germany) and Brain Heart agar slants at 4 °C. To assess the biosurfactant production efficiency by different bacterial isolates, a 100 ml nutrient broth medium was inoculated with a single colony of each bacterial isolates and incubated at 30 °C, 150 rpm for 24 h as seed culture. Then 2% (V/V) seed culture was transferred to a 500 ml Erlenmeyer flask containing 250 ml of E medium (Javaheri *et al.,* 1985) with the following components (g/l): KH_2PO_4, 2.7; K_2HPO_4, 13.9; NaCl, 50; yeast extract, 1; $NaNO_3$, 1; $(NH4)_2SO_4$, 1; and 10 ml of a trace metal solution; 10 g/l glucose or crude oil were added according to the experiment, to study the effect of carbon source on the biosurfactant production and activity. The metal solution was a modification of Wolins metal solution (Wolin *et al.,* 1963) and had the following composition (g/l): EDTA, 1; $MnSO_4{\cdot}H_2O$, 3; $FeSO_4{\cdot}7H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.1; $CoCl_2{\cdot}2H_2O$, 0.1; $ZnSO_4{\cdot}2H_2O$, 0.1; $CuSO_4{\cdot}5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_4, 0.01; $Na_2MoO_4{\cdot}7H_2O$, 0.01; and $MgSO_4$, 25.

Biosurfactant production assay. Bacterial isolates were grown in E medium with either glucose or crude oil as interchangeable carbon sources. The culture media were incubated at 30 °C in a rotary shaker (150 rpm) for 7 days. After that, cell-free supernatant was obtained using shear force of centrifugation (8000 *x g*, 20 min) and collected in centrifuge tube. In addition, samples from culture medium containing whole cells were obtained for measurement of surface-active properties.

The surface tension of the samples was measured by the Ring method using a KRUSS du Nouy Tensiometer K6 at room temperature (McInerney *et al.,* 1990).

Drop collapse measurement method was carried out according to Bodour and Miller-Maier (1998). Each bacterial isolate was streaked on blood agar and incubated at 37 °C for 48 h. The plates were visually checked for the existence of clearing zone around the colonies as a criterion for biosurfactant production. With this goal, the clear zones were measured and scored according to the diameter. Oil spreading measurement was done by addition of 20 ml of distilled water to a Petri dish (10 cm diameter). Then 8 µl of crude oil was transferred to the surface of the water using a HPLC syringe and left to stand for a while. After that, 10 µl of each bacterial isolate culture media were separately added to the surface of oil; this technique was a modification of the Morikawa *et al.* (2000). The existence of clear zones on the oil surface was inspected visually and its diameter was measured as an indicative of biosurfactant concentration; however, biosurfactant concentration was also measured, by using a calibration curve based on a commercially available biosurfactant, surfactin (Sigma, St. Louis, MO).

Determination of emulsifying activities. Bacterial isolates which were able to produce biosurfactant were evaluated for emulsifying activity according to the method reported by Das *et al.* (1998). For this purpose, cell-free supernatant was collected by centrifugation (8000 *x g*, 20 min) of the culture broth. Then, 2 ml of supernatant were mixed with 2 ml n-hexane or crude oil separately in a test tube. The mixture was vigorously stirred for 2 min and then allowed to stand for 48 h. Relative emulsion volume (EV, %) was measured according to the following equation:

$$EV\ \% = \frac{\text{Emulsion height (mm)} \times \text{cross-section area (mm}^2\text{)}}{\text{Total liquid volume (mm}^3\text{)}}$$

RESULTS AND DISCUSSION

Table 1 shows the results of four methods that were used to detect biosurfactant production by different isolates. Over sixteen isolates, seven (44%) could reduce the surface tension to 40 mN/m or less; four (25%) isolates had a SFT between 40 and 42 mN/m and five isolates (31%) exhibited a SFT of 42 mN/m or higher. Oil spreading method resulted in the same response level as for SFT for eleven of sixteen isolates (69%).

Results in Table 2 show a good consistency between oil spreading technique and surface tension with a Pearson rank correlation coefficient, $r_s = 0.456$, and Spearman correlation coefficient, $\rho = 0.459$, ranged between -1 (strong negative correlation) and 1 (strong positive correlation) (Table 3). Figure 1 show the relationship between the diameter (mm) of clear zone obtained by the oil spreading method and surface tension (mN/m) of the culture obtained by using glucose or crude oil as carbon source is consistent with the fact that crude oil, as a non-soluble carbon source, is better than glucose (soluble carbon source) for biosurfactant production.

In the case of blood agar lysis test, as a simple and easy method, all the sixteen biosurfactant-producing strains were able to lyse the blood agar (Table 2). However, a consistent correlation between this test and other analytical techniques were not achieved. Complete haemolysis with a diameter > 3 cm did not occur necessarily in the higher biosurfactant-producing isolates. For instance; strains MASH.12 and MASH.14 could be able to lyse blood agar completely (++++), with a diameter of lysis > 3 cm, but the results obtained from oil spreading technique or surface tension for these strains showed low activity. In contrast a weaker haemolysis, with a response level of "+" or "++", occurred for 64% of the eleven strains which showed a good capability in producing a considerable amount of biosurfactant as checked by oil spreading method (Table 2); 60% of isolates that did not exhibit a good SFT (++) could lyse blood agar with response levels of "++++" and "+++". Different response levels were found for haemolysis and oil spreading for strains MASH.12 to MASH.16. It was previously reported that blood agar lysis test could be used as a simple and quick method for preliminary screening of biosurfactant producers (Mulligan *et al.,* 1984; Banat, 1993; Carrillo *et al.,* 1996). This is a marked contrast with our finding. Among sixteen isolates, just five strains (31%) could lyse blood agar completely (++++). Blood agar lysis method had a high percentage of either false-negatives or false-positives. Therefore, blood agar lysis test is not suggested as a reliable method for screening biosurfactant producers.

TABLE 1 - Efficacy of the four analytical methods in predicting biosurfactant production

Strain	Surface tension (mN/m)	Clear zone diameter by oil spreading (mm)	Blood agar lysis diameter (cm)	EV % n-Hexane	EV % Crude Oil
MASH.1	38.5 ± 0.0	3.0 ± 0.1	8.0 ± 1.0	0.0	11.45 ± 0.45
MASH.2	38.3 ± 0.3	2.5 ± 0.1	1.1 ± 0.1	0.0	0.0
MASH.3	38.8 ± 0.3	3.0 ± 0.2	5.5 ± 0.5	0.0	1.3 ± 0.2
MASH.4	38.8 ± 0.3	2.5 ± 0.2	1.5 ± 0.1	0.0	19 ± 2
MASH.5	39.5 ± 0.0	3.0 ± 0.1	2.0 ± 0.3	6.7 ± 0.2	7.6 ± 0.2
MASH.6	39.5 ± 0.0	2.5 ± 0.3	0.9 ± 0.0	0.0	10.0 ± 1
MASH.7	40.0 ± 0.0	2.5 ± 0.1	4.0 ± 0.5	0.0	5.1 ± 0.4
MASH.8	40.5 ± 0.0	1.5 ± 0.1	0.9 ± 0.1	0.0	0.7 ± 0.04
MASH.9	40.7 ± 0.6	2.0 ± 0.0	0.9± 0.0	13.3 ± 0.5	7.5 ± 1.0
MASH.10	40.8 ± 0.3	2.5 ± 0.1	0.8 ± 0.0	0.0	7.6 ± 0.2
MASH.11	40.8 ± 0.3	1.5 ± 0.0	0.9 ± 0.0	0.0	1.3 ± 0.1
MASH.12	42.0 ± 0.0	1.0 ± 0.1	6.0 ± 1.0	0.0	7.6 ± 0.4
MASH.13	42.8 ± 0.3	3.0 ± 0.1	2.5 ± 0.5	15.0 ± 0.9	1.3 ± 0.2
MASH.14	43.8 ± 0.3	1.5 ± 0.2	5.0 ± 0.8	20.0 ± 1.3	1.3 ± 0.15
MASH.15	45.8 ± 0.3	3.0 ± 0.2	1.0 ± 0.0	0.0	0.71 ± 0.08
MASH.16	46.8 ± 0.3	2.5 ± 0.1	1.0 ± 0.1	6.7 ± 0.4	7.6 ± 0.3

TABLE 2 - Comparison of response levels of the analytical methods applied for the measurement of biosurfactant activity by sixteen biosurfactant producer isolates

Strain	Surface tension[a]	Oil spreading[b]	Blood agar lysis[c]
MASH.1	++++	++++	++++
MASH.2	++++	++++	++
MASH.3	++++	++++	++++
MASH.4	++++	++++	++
MASH.5	++++	++++	+++
MASH.6	++++	++++	+
MASH.7	++++	++++	++++
MASH.8	+++	+++	+
MASH.9	+++	+++	+
MASH.10	+++	++++	+
MASH.11	+++	+++	+
MASH.12	++	++	++++
MASH.13	++	++++	+++
MASH.14	++	+++	++++
MASH.15	++	++++	++
MASH.16	++	++++	++

[a] ++: SFT ≥ 42 mN/m, +++: 40 mN/m ≤ SFT ≤ 42 mN/m, ++++: SFT ≤ 40 mN/m.
[b] diameter. ++: D < 1.5 mm, +++:1.5 mm ≤ D < 2.5 mm, ++++: D ≥ 2.5 mm.
[c] hemolysis, with diameter of lysis. +: incomplete hemolysis, D < 1 cm, ++: complete hemolysis, with 1 cm ≤ D < 2 cm, +++: complete hemolysis, with 2 cm ≤ D < 3 cm, ++++: complete hemolysis, with D ≥ 3 cm.

TABLE 3 - Statistical correlations between analytical methods

	Pearson correlation coefficient (r_s)			Spearman correlation coefficient (ρ)		
	Surface tension	Oil spreading	Blood agar lysis	Surface tension	Oil spreading	Blood agar lysis
Surface tension	1	0.456	0.007	1.000	0.459	0.036
Oil spreading	0.456	1	-0.032	0.459	1.000	0.103
Blood agar lysis	0.007	-0.032	1	0.036	0.103	1.000

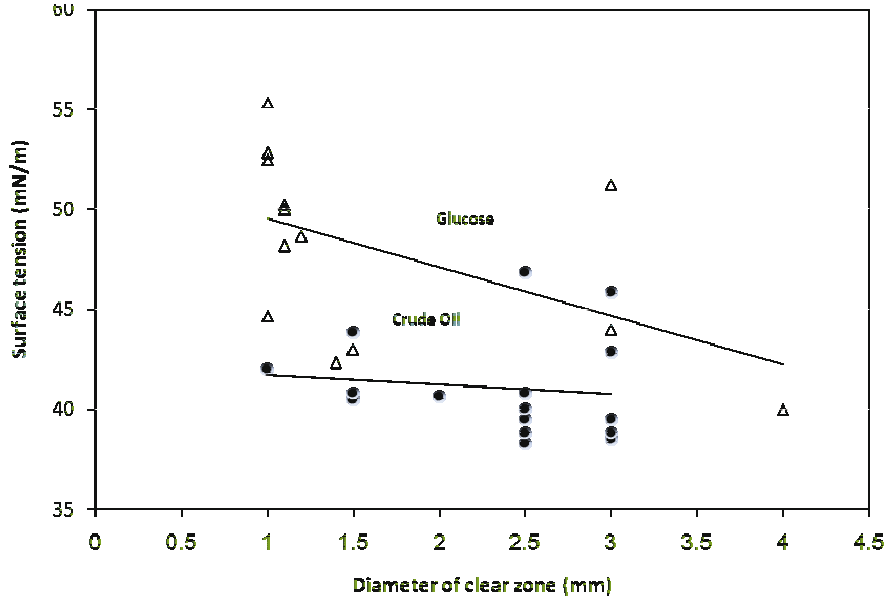

FIG. 1 - Relationship between the diameter (mm) of the clear zone obtained by the oil spreading method and surface tension (mN/m) of the culture, with two different carbon sources (glucose: open triangles; crude oil: closed circles).

Utilisation of the emulsification activity assay as an analytical method for screening biosurfactant producers revealed that five strains (MASH.5, MASH.9, MASH.13, MASH.14 and MASH.16) over sixteen had some emulsification capacity with n-hexane (Table 1). Using crude oil instead of n-hexane, the majority (94%) of bacterial isolates exhibited some emulsification activity with a maximum EV % of 19%; however, a poor correlation was found with results obtained from surface tension (SFT) experiments as the large euclidean distances are 153.076 and 142.476 between SFT and EV % for n-hexane and crude oil respectively, calculated by proximity-dissimilarity matrix. Thus the emulsification activity assay should not be considered as a consistent method for the screening of biosurfactant producers as well.

One-way analysis of variance (F-test with $p < 0.05$ and error level $\alpha = 0.05$) was performed to estimate and compare the mean of biosurfactant activity obtained by using different methods. Statistical tests were used calculate Pearson and Spearman correlation coefficients; both Pearson rank correlation coefficient, r_s, and Spearman correlation coefficient, ρ, can range between -1 (strong negative correlation) and 1 (strong positive correlation). All statistical analyses were performed using SPSS (version 12.0) software package. Table 3 shows the results obtained. Surface tension and oil spreading technique were correlated with a Pearson correlation coefficient $r_s = 0.456$ and a Spearman correlation coefficient $\rho = 0.459$. A weak negative Pearson ($r_s = -0.032$) and a weak positive Spearman ($\rho = 0.103$) correlation coefficient between oil spreading technique and blood agar lysis were detected. Surface tension and blood agar lysis showed very weak Pearson ($r_s = 0.007$) and Spearman ($\rho = 0.036$) correlation coefficients. These analyses indicate that blood agar lysis is linearly uncorrelated to the other two methods, whereas a linear correlation between surface tension and oil spreading technique exists.

Taking into account all results, an excellent consistency between oil spreading technique and SFT methods was obtained. Accordingly, we conclude that oil spreading method is the fastest, simplest, time-saving and most reliable technique, to be suggested as an analytical method for screening biosurfactant producers.

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