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Assessment of different screening methods for selecting biosurfactant producing marine bacteria

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Different screening methods namely, hemolytic assay (HA), modified drop collapse (MDC), tilted glass slide (TGST), oil spread method (OSM), blue agar plate (BAP), hydrocarbon overlaid agar (HOA) plate, emulsification index (EI), emulsification assay (EA) were assessed for their efficiency to detect biosurfactant producing marine bacteria. Forty-five strains of bacteria, comprising 18 *Acinetobacter* and 27 other bacteria along with positive MTCC reference strains were examined. HA, MDC, TGST efficiently detected 15, 17 and 14 biosurfactant producers respectively. Five hemolytic cultures did not show any biosurfactant production in MDC, TGST, and/or OSM. The emulsification of kerosene was also poorer. These results suggest that HA is not totally reliable. Six bacterial isolates produced biosurfactant in OSM, and MDC as well as TGST. MDC and TGS tests demonstrated good activity for nine isolates and proved to be the essential methods. None of the bacteria produced glycolipid on BAP. Cultures showing >30% of emulsification with kerosene were found to be positive in at least one of the above mentioned screening methods. The reference strains, Gram negative bacterium MM73b produced 68% the highest emulsification and demonstrated biosurfactant production in modified drop collapse, tilted glass slide test with highest emulsification units of 213.8 (EU/ml) for petrol. In case of xylene, *Acinetobacter* spp. MM74 demonstrated 187.5, *Acinetobacter* spp. WB42 demonstrated 170.4 emulsification units. HOA plate identified 31 and 22 bacteria for diesel and crude oil degradation respectively. Thus, this method proved to be significant one. We suggest that single method is not suitable to identify all type of biosurfactants, and recommend that drop collapse, tilted glass slide test, oil spread method followed by emulsification assay are more suitable for primary screening.

[**Keywords:** *Acinetobacter*, biosurfactants, bioemulsifiers, hydrocarbon, screening methods, bacteria, microbial cultures]

Introduction

Oil spills, effluents of petrochemical industries, refineries and their hazardous substances lead to pollution of marine ecosystem[1]. The hydrocarbon moieties from such spills persist for long time in the marine ecosystem. Under such conditions, many marine microorganisms produce surface active substances like biosurfactants/bioemulsifiers to solubilize and assimilate hydrophobic compounds[2]. These amphiphilic moieties bind to water insoluble hydrocarbons[3]. Thus, due to the constant exposure, marine microorganisms develop biodegrading machinery and degrade various toxic compounds[4]. Realizing such activity has led to number of screening methods such as hemolysis of erythrocytes[5], oil spread[6], modified drop collapse[7], tilted glass slide[8], blue agar plate method[9], emulsification index[10], emulsification assay[11-12] and hydrocarbon degradation on agar plates[13] for detection of biosurfactant/ bioemulsifier producers.

Although different screening methods are available, it is however, difficult to detect the types of biosurfactant/bioemulsifier produced by the microbes using a single method owing to the chemical and functional properties. In view of this, it appears that several screening methods are needed to understand the ability of a single hydrocarbonclastic microbe in producing biosurfactant. Hence, for efficient detection of potential biosurfactant producers, combination of various screening methods are required which was evaluated during this study.

Materials and Methods

Seawater, sediment, shells samples were collected at high and low tides from eastern (West Bengal off Fraserganj), western (off Mumbai, Shivree) and

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southern (off Chennai and Calicut) Indian coasts during October 2003 to September 2004. In addition to this, samples were also procured from Iran, Caspian Sea (Anzali Port). Samples were enriched in 500 ml Erlenmeyer flasks containing 100 ml of Baumann's[14] medium with 3.5% NaCl (w/v), incubated under 200 rpm at 28^{O}C up to one week. After observation of turbidity in medium, samples were plated on various media viz., Zobell marine medium (ZMM, Himedia), *Acinetobacter* minimal medium (AMM)[15], cysteine lactose electrolyte deficient (CLED Himedia) and Holton's media. These media were prepared in artificial seawater (ASW) containing NaCl, 35.0; NH_4NO_3, 4.0; KH_2PO4, 6.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.01; $FeSO_4.7H_2O$, 0.01 and also natural sea water (NSW collected from Mumbai coastal area). Plating of enriched samples was done at regular interval of 24 hrs up to four to five days in order not to miss slow growers. About 112 bacterial isolates were obtained and maintained on ZMM at 4^{o}C until taken up for further studies. Among the 112 isolates 45 isolates were non-motile, encapsulated coccobacilli that were oxidase negative, catalase positive were assigned to genus *Acinetobacter*. Identification of genus *Acinetobacter* was confirmed by chromosomal DNA trans-formation assay[15]. About 18 such confirmed strains along with 20 Gram negative and seven Gram positive marine isolates were selected for further work.

Biosurfactant producing reference cultures viz., *Bacillus subtilis* MTCC1427, *Bacillus pumilus* MTCC1456, *Bacillus subtilis* MTCC2422, *Bacillus subtilis* MTCC2423; *Bacillus sphaericus* MTCC2473 and *Pseudomonas aeruginosa* MTCC2297 were also included in this study. These cultures were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Cultures were maintained on nutrient agar (NA) containing (g/L) beef extract: 1.0, yeast extract: 2.0, peptone: 5.0, NaCl: 5.0, agar: 15, *p*H 7.2. Cultures were grown aerobically at respective temperatures suggested by the supplier.

The following screening assays were carried out for detecting biosurfactant production by *Acinetobacter* spp. and other marine bacteria. ZMM and Zobell marine broth (ZMB), nutrient agar and minimal medium with 3.5% NaCl (w/v) were used as the basal media. Biosurfactant production by both marine isolates and reference cultures from MTCC was examined.

Hemolytic activity (HA)

Bacterial cultures were streaked on ZMM supplemented with 5% fresh human blood[5] and incubated at 28^{o}C for 48-72 hrs. Observation was made for ∝, β and ϒ hemolysis. Hemolytic activity was correlated with the production of biosurfactant[5].

Oil spread method (OSM)

Twenty-four hour old inoculum grown in ZMB was used. Petriplate base was filled with 50 ml of distilled water. On this water, twenty microlitre of crude oil was layered uniformly. Further, ten microlitre of culture was added at different spots on the crude oil which is coated on water surface. Occurrence of clear zone was an indication of a biosurfactant producer[6].

Modified drop collapse (MDC) method

Wells of microtitre plate were thinly coated with Pennzoil ZDX (SAE 20W-40, India). The microtitre plate was left undisturbed for 15 minutes for forming the uniform thin coating of Pennzoil in the well. Five microlitre bacterial culture grown in ZMB at 28^{o}C under 200 rpm for 24 hrs were added individually to the centre of the well. The biosurfactant producers were detected from the drop collapsing within a minute from the oil coated well[7].

Tilted glass slide (TGS) test

A colony each of marine bacterial cultures was grown for 24 hrs on ZMA mixed with a drop of 0.9% NaCl at one end of the glass slide which was not coated with any oil. The slide was tilted and the biosurfactant production confirmed when the drop began dipping down[8].

Blue agar plate (BAP) method

Anionic biosurfactant, specifically rhamnolipids were detected by this technique. Mineral salts agar medium (MSA)[9] supplemented with carbon sources (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/ml)-methylene blue (MB: 0.2 mg/ml) were prepared. Carbon sources tested were mannitol, glycerol, sodium citrate, sodium acetate, peptone and glucose. For marine bacteria 3.5% (w/v) NaCl was added in the above MSA preparation. A dark blue halo around the culture was considered as positive for biosurfactant production.

Emulsification index (EI)

Emulsification activity was measured by vortexing 1 ml of culture supernatant grown in ZMB at 28°C for 24 hrs. Further, 4 ml of water and 6 ml of kerosene for 2 minutes to obtain maximum emulsification. After 48 hrs emulsification index[10] was calculated by measurement of the height of the emulsion layer (a), divided by the total height (b), multiplied by 100 (EI = a/b × 100). This assay was performed in same size glass test tubes.

Hydrocarbon overlay agar (HOA) method

ZMA plates were coated individually with 40 microlitre of kerosene, hexadecane, benzene, toluene, diesel or crude oil. Pure bacterial isolates were spotted on these coated plates. Plates were incubated for 7-10 days at 28°C. Colony surrounded by an emulsified halo was considered positive for biosurfactant production[13]. As petrol and xylene were evaporating very fast from the plates, their emulsification was checked out by an assay described by Patil & Chopade[11].

Emulsification assay (EA)

Culture inocula were prepared by growing them overnight in 10 ml ZMB in 100 ml Erlenmeyer flasks at 28°C and 200 rpm. Cultures were centrifuged at 10,000 rpm for 15 min at room temperature. Three mililitre of supernatant was mixed with 0.5 ml of petrol. This mixture was mixed vigorously for 2 min. This mixture was left undisturbed for one hour at 28°C to separate aqueous and oil phase. Aqueous phase was removed carefully with the help of 1 ml micropipette and absorption was measured. Same procedure was followed for xylene also. Uninoculated broth was taken as a blank. Absorbance of aqueous phase was measured by using spectrophotometer (UV1601 Shimadzu Corporation, Japan) at wavelength of 400 nm. Emulsification activity per ml (EU/ml) was calculated[11] by using the formula, 1 Emulsification unit = 0.01 × Dilution factor.

Results

All 45 strains comprising *Acinetobacter* and other marine bacterial isolates were examined for a variety of biosurfactant producing and hydrocarbonclastic activities. Results from various screening protocols are listed in Tables 1 and Table 2. Fifteen isolates, comprising six of *Acinetobacter* spp. (Table 1) and nine others (Table 2) were positive for biosurfactant production in the hemolytic assay. Among 15 hemolytic isolates, 12 showed α hemolytic activity. Five hemolytic cultures did not show biosurfactant production in the remaining seven screening methods. Low emulsification activity was also observed in these cultures suggesting that the assay is not totally reliable. Results from other tests including OSM, DCM, TGST, BAP are also shown in Table 1 and Table 2. The MDC demonstrated eight *Acinetobacter* spp. and nine other bacteria as biosurfactant producers. Only six isolates showed biosurfactant activity in the OSM. Same cultures were positive in MDC and TGST. The MDC and TGST were good equally indicating biosurfactant production by nine isolates. The TGST detected four *Acinetobacter* spp. and ten other bacterial isolates as biosurfactant producers. From the reference cultures, *Bacillus* spp. did not show hemolytic activity. However, biosurfactant producing activity of these strains could be confirmed by MDC, OSM and TGST. *Pseudomonas aeruginosa* MTCC 2297, on contrary, demonstrated α hemolytic activity, positive for MDC and BAP method. However, it showed negative test in OSM and TGST. Thus the hemolytic activity must be considered as an unreliable criterion for the detection of biosurfactant activity of a bacterial culture. Comparatively, MDC, OSM and TGST are consistent. The BAP method is a highly special technique for detection of glycolipid producing microorganisms. Glycolipid production was not detected in any of the tested strains in BAP method. High EI were observed for *Acinetobacter* spp. MM74 (Table 1) and other bacterial isolates was WB64 and MM3b7 (Table 2). The culture showing good emulsification activity (>30%) was seen to be positive in at least one of the above mentioned screening methods. The lowest emulsification activity (5%) was observed in MSS96. Most of the isolates demonstrated 20-35% of emulsification.

The HOA plate method identified hydrocarbon-clastic bacteria efficiently. Detailed qualitative assessment for biosurfactant/bioemulsifier for all bacteria studied are given in Tables 3 and 4. Diesel and crude oil were respectively utilized by 31 and 22 marine isolates. This number is significant as compared with other hydrocarbons. Kerosene was utilized by four *Acinetobacter* spp. and four Gram negative bacteria. Three species of *Acinetobacter* and two others degraded hexadecane. More number of reference cultures than marine isolates utilized

hexadecane. Only a few isolates could utilize benzene and toluene. Among the reference strains only *Pseudomonas aeruginosa* MTCC 2297 degraded benzene. Whereas *Bacillus* spp. MTCC 2422 and MTCC 2423 utilized toluene and diesel. The EA identified maximum numbers of isolates that emulsifies xylene and petrol efficiently. The emulsification units of 213.8 (EU/ml), the highest, by MM73b. For xylene, the EU of 187.5 by was followed by WB42 (170.4 EU/ml). *Bacillus Subtilis* MTCC 2422 produced high emulsification of 400.0 and 291.4 EU/ml for xylene and petrol respectively. *Pseudomonas aeruginosa* MTCC 2297 utilized all six tested hydrocarbons however, showed least emulsification units for petrol and xylene.

Discussion

Biosurfactants/bioemulsifiers play a key role in emulsifying hydrocarbons. Biosurfactants and bioemulsifiers are thought to be very suitable alternatives to chemical surfactants due to their properties like eco-friendly, less/no toxicity, biodegradability, high specificity, selectivity at temperature, *p*H, salinity and synthesis from cheaper renewable substrates[16]. The functional properties such as emulsification, wetting, foaming, cleansing, phase separation, surface activity, and reduction in viscosity of crude oil for transportation[17] are interesting. Therefore, search of biosurfactant producing microorganisms is an important area of research is particular for bioremediation.

In this study, eight different screening methods were assessed for selecting biosurfactant producing marine bacteria. Through confirmation of hemolytic activity is a commonly preferred method to screen biosurfactant producing culture, it was seen in this study that it is not a very useful test for marine bacterial cultures. Further same reference cultures negative for hemolytic activity did show biosurfactant production in MDC, OSM and TGST. Similar observations were true with *Acinetobacter* spp. MM74 and Gram negative bacteria viz., MM73b and

Table 1 — Biosurfactant production by marine *Acinetobacter* spp.

Organism	Hemolytic activity	Modified drop collapse	Oil spread	Tilted glass slide	Blue agar plate*	Emulsification# (%)
Acinetobacter spp.						
CS 1	Nil	Nil	+	Nil	Nil	20
CS 3	∝	Nil	Nil	Nil	Nil	29
CS 5	∝	+	Nil	Nil	Nil	Nil
CS 6	Nil	Nil	+	+	Nil	**32**
CS 9	Nil	+	Nil	Nil	Nil	20
CS 11	∝	+	Nil	Nil	Nil	Nil
CS 20	β	+	++	Nil	Nil	25
CS 23	Nil	Nil	Nil	Nil	Nil	**39**
WB 40	β	Nil	Nil	Nil	Nil	**44**
WB 42	Nil	++	Nil	+	Nil	Nil
WB 45	Nil	+	Nil	+	Nil	Nil
WB 55	Nil	+	Nil	+	Nil	Nil
MM 74	Nil	+	Nil	Nil	Nil	**68**
MSS 96	∝	Nil	Nil	Nil	Nil	5
Reference strains positive for biosurfactant production						
Bacillus subtilis MTCC1427	Nil	Nil	+	+	Nil	**40**
Bacillus pumilus MTCC 1456	Nil	Nil	Nil	+	Nil	24
Bacillus subtilis MTCC 2422	Nil	++	++	+	Nil	**75**
Bacillus subtilis MTCC 2423	Nil	++	++	+	Nil	**78**
Bacillus sphaericus MTCC 2473	Nil	++	++	Nil	Nil	**36**
Pseudomonas aeruginosa MTCC 2297	∝	+	Nil	Nil	+	**42**

CS = Caspian Sea; WB = West Bengal; MM = Mumbai mussel; MSS = Mumbai, Shivree sediment; ∝ = Reduction of hemoglobin to met = hemoglobin (medium becomes greenish); β = Lysis of RBC, medium around the colony becomes colourless; Nil = Negative test; + = Positive test; ++ = Good activity; * = CTAB, blue agar plates = Marine bacteria were checked with and without NaCl (3.5% w/v); # = Values are the mean of three readings. Emulsification >30% is indicated **in bold** to denote high emulsification activity.

Table 2 — Biosurfactant production by marine Gram negative and Gram positive bacterial isolates

Organism	Hemolytic activity	Modified drop collapse	Oil spread	Tilted glass slide	Blue agar plate*	Emulsification# (%)
Gram negative bacteria						
CS 2	Nil	Nil	Nil	Nil	Nil	27
CS 7	Nil	Nil	Nil	Nil	Nil	20
CS 13	Nil	+	+	Nil	Nil	20
WB 64	Nil	+	Nil	+	Nil	**57**
WB 68	Nil	Nil	Nil	+	Nil	**34**
MM 76	∝	Nil	Nil	+	Nil	Nil
WB 69	β	Nil	Nil	Nil	Nil	**40**
MSS 86	∝	Nil	Nil	Nil	Nil	**37**
MSS 89	∝	Nil	Nil	Nil	Nil	19
IW 106	∝	Nil	Nil	+	Nil	Nil
IW 108	Nil	+	Nil	+	Nil	Nil
IW 112	Nil	Nil	Nil	Nil	Nil	Nil
CS 2a	Nil	Nil	Nil	Nil	Nil	Nil
WB 28a	∝	+	+	+	Nil	Nil
WB 28b	∝	Nil	Nil	+	Nil	Nil
CS 3b	∝	+	Nil	Nil	Nil	18
WB 68a	Nil	Nil	+	Nil	Nil	**42**
WB 68b	Nil	Nil	Nil	Nil	Nil	Nil
MM 73b	Nil	+	Nil	+	Nil	**68**
Gram positive bacteria						
WB 41	Nil	+	Nil	+	Nil	Nil
WB 63	Nil	Nil	Nil	Nil	Nil	Nil
MG 77	∝	++	Nil	+	Nil	Nil
MS 85	Nil	+	Nil	Nil	Nil	**50**
MSS 90	Nil	Nil	Nil	Nil	Nil	22

IW = Iran water; IOW = Iran oily water; Emulsification >30% is indicated **in bold** to denote high emulsification activity.
Other abbreviations as defined in Table 1 legend.

WB64. As also noticed by other investigators[18-20]. Confirmation of biosurfactant production through other screening methods becomes essential to select potent biosurfactant producers as proven in this study. However, none of the tested marine bacteria produced glycolipids.

Emulsification activity is one of the criteria to support the selection of potential biosurfactant producers. Emulsifying activities (E_{24}) determine productivity of bioemulsifier[21]. Ellaiah *et al.*[10] screened 68 bacterial isolates from soil and found only 6% of isolates with good emulsification activity of up to 61%. During this study, emulsification of kerosene by MM73b and MM74 was up to 68%. This observation is important to suggest that potent biosurfactant producing cultures can be detected through such assays. The cultures showing >30% emulsification activity were also positive for biosurfactant production in two or three other methods. It is also possible to detect biosurfactant producing and hydrocarbon degrading activity simultaneously on agar plate by overlaying with hydrocarbon[12]. Maximum number of isolates positive for kerosene, hexadecane, benzene, toluene and crude oil degradation were also positive for diesel utilization. Measurement of emulsification units help to choose the carbon and energy source for biosurfactant/bioemulsifier production. Patil and

Table 3 — Qualitative assessment of biosurfactant/bioemulsifier production by marine *Acinetobacter* spp.

Marine isolates	Hydrocarbon overlaid agar plate method#				Emulsification activity* (EU/ml)			
	Kerosene	Hexadecane	Benzene	Toluene	Diesel	Crude oil	Petrol	Xylene
Acinetobacter spp.								
CS 1	Nil	Nil	Nil	Nil	++	Nil	102.4	109.4
CS 3	+	Nil	Nil	Nil	++	Nil	58.1	79.2
CS 5	Nil	Nil	Nil	Nil	+	Nil	52.5	138.4
CS 6	Nil	Nil	Nil	Nil	+++	Nil	88.0	64.2
CS 9	Nil	+++	Nil	Nil	Nil	+++	77.1	15.0
CS 11	Nil	Nil	Nil	Nil	Nil	++	62.4	90.6
CS 12	Nil	Nil	Nil	+	Nil	++	62.6	79.3
CS 15	Nil	Nil	Nil	Nil	++	+++	146.7	90.6
CS 20	Nil	Nil	Nil	+	+	Nil	75.9	134.6
CS 23	Nil	Nil	Nil	+	++	++	102.7	108.6
WB 40	++	++++	Nil	Nil	++	+++	67.3	102.6
WB 42	Nil	+++	Nil	Nil	+	+++	37.0	170.4
WB 45	Nil	Nil	Nil	Nil	Nil	+++	21.9	148.7
WB 55	Nil	Nil	Nil	Nil	Nil	Nil	73.0	90.3
MM 74	Nil	Nil	Nil	Nil	++	Nil	168.1	**187.5**
MSS 96	Nil	Nil	Nil	Nil	+++	Nil	2.5	28.0
CS 2b	+	Nil	Nil	Nil	++	+++	38.8	20.3
MM 73a	++	Nil	Nil	Nil	+	Nil	17.2	31.5
Reference strains positive for biosurfactant production								
Bacillus subtilis MTCC1427	+++	++	Nil	Nil	Nil	+	161.2	158.5
Bacillus pumilus MTCC 1456	Nil	+++	Nil	Nil	Nil	+++	125.2	156.5
Bacillus subtilis MTCC 2422	Nil	+++	Nil	+++	+++	++	**291.4**	**400.0**
Bacillus subtilis MTCC 2423	Nil	+++	Nil	+++	+++	++	**245.1**	**361.2**
Bacillus sphaericus MTCC 2473	Nil	Nil	Nil	Nil	++	Nil	31.2	133.2
Pseudomonas aeruginosa MTCC 2297	+++	++	+++	+++	+	+++	59.0	68.8

CS = Caspian Sea; WB = West Bengal; MM = Mumbai Mussel; MSS = Mumbai Shivree Sediment; Nil = Negative; + = Positive; ++ = Weak positive; +++; ++++ = Good activity; # Hydrocarbons used were AR grade and smeared on to Zobell marine agar plates; positive signs indicate the cultures with emulsified halos for various hydrocarbons; *Emulsification units (EU/ml) mean of three experiments; Figures **in bold** indicate high emulsification units. Emulsification activity (EU/ml) = 1 Emulsification unit = 0.01 O. D. multiplied by dilution factor of absorbance at 400 nm.

Chopade[11] introduced emulsification assay based on emulsification units of the tested oils. They selected *Acinetobacter junii* SC14 for bioemulsifier production. Thus, by examining emulsification units, it is possible to select a potent biosurfactant/ bioemulsifier producer. We recommend these assays as one of the important and effective assay for screening the biosurfactant/bioemulsifier producers.

It is important to note that most of the researchers have used maximum two or three screening methods for selection of biosurfactant producers[19]. We suggest a single method is not suitable to identify all type of biosurfactants. Therefore, a combination of various methods is required for effective screening. To the best of our knowledge, this is the first report assessing eight different screening methods for selecting biosurfactant producing marine bacteria. In conclusion, we recommend that DCM, TGST, OSM followed by EA are more suitable for primary screening.

Table 4 — Qualitative assessment of biosurfactant/bioemulsifier by marine bacterial isolates

Marine isolates	Hydrocarbon overlaid agar plate method #						Emulsification activity* (EU/ml)	
	Kerosene	Hexadecane	Benzene	Toluene	Diesel	Crude oil	Petrol	Xylene
Gram negative bacteria								
CS 2	Nil	Nil	Nil	Nil	+	Nil	58.1	79.2
CS 4	Nil	Nil	Nil	Nil	Nil	Nil	91.7	71.2
CS 7	Nil	Nil	Nil	Nil	+++	Nil	84.5	6.7
CS 13	Nil	Nil	Nil	Nil	++	Nil	65.2	99.0
WB 64	Nil	Nil	Nil	Nil	+++	Nil	46.8	153.1
WB 68	Nil	Nil	Nil	Nil	++	+	51.2	35.4
WB 69	Nil	Nil	Nil	Nil	Nil	Nil	23.7	45.9
MM 76	++	Nil	Nil	Nil	++	+++	45.6	5.2
MSS 86	Nil	+++	Nil	Nil	Nil	+++	21.1	45.6
MSS 89	Nil	Nil	+	+	+	Nil	6.2	32.8
IW 106	Nil	+++	+	+	++	++++	19.6	14.1
IW 108	Nil	Nil	+	+	++	++++	18.8	63.5
IW 112	Nil	Nil	Nil	Nil	Nil	Nil	8.0	0.1
CS 2a	Nil	Nil	Nil	Nil	+++	+++	41.6	8.7
WB 28a	+	Nil	Nil	Nil	+	+++	10.1	176.2
CS 3b	+	Nil	Nil	Nil	++	+++	92.7	93.4
WB 28b	Nil	Nil	Nil	Nil	Nil	Nil	56.2	20.8
WB 68a	Nil	Nil	Nil	Nil	Nil	Nil	31.1	40.4
WB 68b	Nil	Nil	Nil	Nil	Nil	Nil	46.2	114.3
MM 73b	+++	Nil	Nil	Nil	Nil	+++	**213.8**	110.8
Gram positive bacteria								
WB 41	Nil	Nil	Nil	Nil	+	+++	69.4	9.6
WB 63	Nil	Nil	Nil	Nil	+++	++	**183.8**	34.8
MG 77	Nil	Nil	Nil	Nil	+++	+++	80.6	15.4
MS 85	Nil	Nil	Nil	Nil	Nil	Nil	22.2	73.9
MSS 90	Nil	Nil	+	+	++	Nil	3.7	56.1
MSS 99	Nil	Nil	Nil	Nil	+++	Nil	34.9	69.4
IOW 101	Nil	Nil	+	+	+++	+++	1.4	32.2

IW = Iran water; IOW = Iran oily water; Nil = Negative; + = Positive; ++ = Weak positive; +++; ++++ = Good activity; #Hydrocarbons used were AR grade and smeared on to Zobell marine agar plates; positive signs indicate the cultures with emulsified halos for various hydrocarbons; *Emulsification units (EU/ml) mean of three experiments; Figures **in bold** indicate high emulsification units. Emulsification activity (EU/ml) = 1 Emulsification unit = 0.01 O. D. multiplied by dilution factor of absorbance at 400 nm.

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