

## Biosurfactan Production by *Bacillus* sp. Isolated from Petroleum Contaminated Soils of Sirri Island

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**Abstract: Problem statement:** Biosurfactants are active surface components produced by some bacteria and fungi. These molecules reduce surface and interfacial tension in aqueous solutions and hydrocarbon mixtures. The most important application of biosurfactants is in oil industry to enhance oil quality and facilitate oil extraction. The aim of this study was to isolate biosurfactant producing bacteria and optimize the conditions like temperature and pH for maximum biosurfactant production.

**Approach:** Samples were collected from 8 selected points of oil contaminated soils in Sirri Island-Iran. Primary screening tests including hemolytic activity, Drop collapse technique and Oil Spreading method were preformed and species with the best results were picked for complementary screening tests like emulsification activity, foaming and surface tension measurement. **Results:** Totally, 160 bacteria species were isolated. During primary and complementary screening tests, 59 species showed hemolytic activity, 46 had drop collapsing ability and 18 species showed positive results in emulsification, foaming and surface tension reduction. Finally, two *Bacillus* sp. were found to be able to reduce surface tension more than 30 mNm<sup>-1</sup>. **Conclusion:** Two strains with a high amount of biosurfactant production and emulsification ability were resulted from the present study. According to the high potential of *Bacillus* sp. especially for Microbial Enhanced Oil Recovery (MEOR) and Bioremediation of oil contamination we can hope that further study of the isolates characteristics and looking for new local strains can play an important role in their application in oil industry.

**Key words:** Biosurfactant, oil contaminated soils, *Bacillus* sp., Microbial Enhanced Oil Recovery (MEOR), drop collapse, hemolytic activity, emulsification activity

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### INTRODUCTION

Biosurfactants are biological amphipathic compounds produced by various Bacteria, fungi and molds (Noudeh *et al.*, 2007; Dehghan-Noudeh *et al.*, 2009). They are generally lipid compounds whose features are related to two ends present in the molecule, one end is hydrocarbon part which is less soluble in water (hydrophobic end). The hydrophobic part of the molecules is a long- chain of fatty-acids, hydroxyl fatty acid or  $\alpha$ -alcy hydroxy-fatty acids. The other end is hydrophilic, more soluble in water and consists of carbohydrate, amino acid, cyclic peptide, phosphate and carboxylic acid or alcohol (Chayabutra *et al.*, 2001; Volchenko *et al.*, 2007; Chen *et al.*, 2007; Mata-Sandoval *et al.*, 1999; Maier and Soberon-Chavez,

2000). Due to their advantages such as lower toxicity, high biodegradability, higher foaming, better environmental compatibility, the ability to act in high temperatures, low pH and different salinity levels and low production costs, biosurfactants are preferred to synthetic and chemical surfactants (Dehghan-Noudeh *et al.*, 2009; Deleu and Paquot, 2004). These components have extended applications in petrochemical and oil industries, pharmacy, medical, cosmetics, food and pharmaceutical (Makkar and Cameotra, 2002; Van Ginkel, 1996; Babu *et al.*, 1996). Thus among all Oil industry is the greatest market of these compounds (Dyke *et al.*, 1993). One of the major problems facing the modern industrial development is the power resources. From the economical point of view; pumping power losses during the flow of transported liquids in

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pipelines are one of the major problems facing many industrial applications (especially petroleum). The most famous drag reducing agents are: Polymers, fibers, soaps or surfactants (Haider *et al.*, 2011). Biological treatment most commonly involves the breakdown of contamination into nontoxic forms using microbiological processes. Thus, bioremediation may be defined as the use of living organisms to remove environmental pollutants from soil, water and gases (Mukred *et al.*, 2008). Biosurfactant producing microorganisms are able to be used in bioremediation and oil leak clearance in Soil and water environments (Head and Swannell, 1999; Mulligan, 2005; Urum *et al.*, 2006). Microbial Enhanced Oil Recovery (MEOR) is one of the most recent and practical aspects of biosurfactants application. Industrial and semi-industrial production of biosurfactants would be possible by isolation of appropriate species and evaluation of physiological and metabolic features beside Substrate utilization of crude materials (Okerentugba and Ezerony, 2003; Fiechter, 1992; Finnerty, 1994; Lazar *et al.*, 2007). The aim of this study was to isolate local biosurfactant producing bacteria and optimize the conditions like temperature and pH for maximum biosurfactant production.

## MATERIALS AND METHODS

**Sampling:** Samples were collected from 8 different areas of oil contaminated soils, extracted oil reservoirs, oil pipeline leakages and oil sewage and sludge dumps in Sirri Island, Persian Gulf, Iran.

**Cultivation media:** Culture media were synthesized in the lab by Banat method (Rahman *et al.*, 2002a; Rasooli *et al.*, 2008) for microorganism enrichment and Robert method (Robert *et al.*, 1989; Dehghan-Noudeh *et al.*, 2009; De Lima *et al.*, 2009) for biosurfactant production (Table 1 and 2).

**Primary isolation:** Samples were aseptically transferred to laboratory and enriched in shaker incubator (n-BioTek, Korea) at 200 rpm for 21 days in 30°C (Rahman *et al.*, 2002b; 2002c; Cunha *et al.*, 2004; Bicca *et al.*, 1999). After enrichment serial dilutions in saline normal solution were prepared, cultivated on mineral salts agar media and incubated (Memmert and Germany) in 30°C for 48 h. After purification, morphology of colonies was studied. Pure colonies were inoculated into mineral salts media and incubated in 30°C in shaker incubator (200 rpm) for 96 h (Rahman *et al.*, 2003). The same culture was used for further study of biosurfactant producing strains. To obtain cell free media when necessary, cultures were

centrifuged (Boeco and Germany) at (4000 rpm) for 30 min in 4°C and supernatant was collected to perform the tests.

### Primary tests for screening of biosurfactant producing bacteria:

**Hemolytic activity analysis:** Isolates were screened on blood agar plates (Merck) containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected by presence of a clear zone around bacterial colonies (Plaza *et al.*, 2006; Youssef *et al.*, 2004).

**Drop collapse method:** In this test, according to Bodour and Miller-Maier method (Youssef *et al.*, 2004), a modified oil collapse test was carried out using 96 well microtiter-plates containing 100 µL mineral oil which was equilibrated for an hour at room temperature. About 10 µL of the culture was added to the surface of the oil. After 1 min, the shape of the drop on the surface of the oil was observed. The results were interpreted as (+) to (+++) corresponding partial to complete spreading on the oil surface. Those cultures that gave rounded drops were scored as negative (-) indicating the lack of biosurfactant production.

Table 1: Mineral salts medium for enrichment and biosurfactant production

Compounds (g L <sup>-1</sup> )	Mineral salts medium for enrichment (g L <sup>-1</sup> )	Mineral salts medium for biosurfactant production (g L <sup>-1</sup> )
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.00	-
KH <sub>2</sub> PO <sub>4</sub>	2.40	0.50
K <sub>2</sub> HPO <sub>4</sub>	4.80	1.00
MgCl <sub>2</sub>	0.08	-
(NH <sub>4</sub> )MO <sub>7</sub> O <sub>24</sub> .4H <sub>2</sub> O	0.01	-
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.03	-
Citric acid	0.40	-
NaNO <sub>3</sub>	-	4.00
MgSO <sub>4</sub> .7H <sub>2</sub> O	-	0.50
KCl	-	0.10
FeSO <sub>4</sub> .7H <sub>2</sub> O	-	0.01
Yeast extract	-	0.01
Trace element	2 (mL)	0.05 (mL)
Carbon source	(2%) crude oil	(2%) crude oil

Table 2: Trace elements, mineral salts medium for enrichment and biosurfactant production

Compounds (g L <sup>-1</sup> )	Mineral salts medium for enrichment (g L <sup>-1</sup> )	Mineral salts medium for biosurfactant production (g L <sup>-1</sup> )
FeSO <sub>4</sub> .7H <sub>2</sub> O	1.00	-
NaMoO <sub>4</sub>	2.00	0.10
MnCl <sub>2</sub>	1.00	1.75
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.03	3.10
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.25	2.00
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.25	-
H <sub>3</sub> BO <sub>3</sub>	-	1.50

**Oil spreading method:** Oil spreading technique was carried out according to the method described previously by Youssef *et al.* (2004) and Plaza *et al.* (2006). Briefly, 50 mL of distilled water was added to the petri plate followed by addition of 100  $\mu$ L of crude oil to the surface of water. Then 10  $\mu$ L of cell free culture broth was dropped on the crude oil surface. The diameter of clear zone on the oil surface was measured and compared to 10  $\mu$ L of distilled water as negative control.

**Complementary tests for screening biosurfactant producing bacteria:** Prominent strains with the best positive test results were selected for complementary tests.

**Surface tension measurements:** This method is the most common and assured method to identify biosurfactant production. Since surface tension reduction is depended on environment temperature, surface tensions of all samples were determined in 24°C. Surface tension reduction was measured using Tensiometer (Kruss GmbH Hamburg and Germany) and by submerging the platinum ring in the cell free culture broth and recording the force required to pull it through the air-liquid interface (Batista *et al.*, 2006; Volchenko *et al.*, 2007; Cha *et al.*, 2008). The results were compared to distilled water and medium composition (as negative control) and 20 (as a positive control).

**Emulsification activity ( $E_{24}$ ):** The emulsification activity was measured using the method described by Rahman *et al.* (2003) (Dehghan-Noude *et al.*, 2005) and Desai and Banat (1997) (Chayabutra *et al.*, 2001). About 5 mL of hydrocarbon (Crude oil) and 5 mL of cell free medium (supernatant) were inoculated to a test tube and homogenized by vortexing (Genius and Germany) at high speed for 2 min. After 24 h, the emulsification activity was calculated using following formula (Lai *et al.*, 2009):

$$E_{24} (\%) = \frac{\text{total height of the emulsified layer}}{\text{total height of the liquid layer}}$$

**Foam formation activity:** All isolates with positive results in primary screening tests were grown separately in 250 mL Erlenmeyer flasks, each containing 50 mL of nutrient broth (Merck at pH 7.2) medium. The flasks were incubated at 37°C on a shaker incubator (200 rpm) for 96 h. Foam activity was detected as duration of foam stability, foam height and foam shape in the graduated cylinder (Chayabutra *et al.*, 2001; Dehghan-Noudeh *et al.*, 2003).

## RESULTS

A total number of 160 strains were isolated in the present study including 110 Gram positive and 20 Gram

negative Rods. Also 15 Gram positive and 15 Gram negative spherical bacteria were isolated. Among these, 59 strains had beta-hemolytic activity, 46 were able to collapse oil and 20 could spread oil. Finally, 18 isolates were selected for complementary techniques after performing primary differential tests. These isolates showed different amounts of surface tension decrease from 29-56  $\text{mN m}^{-1}$  and emulsification activity ( $E_{24}$ ) from 51.54-80.36% (Table 3, Fig. 1). Also 11 strains had foam formation activity of 30-135 min (Table 4, Fig. 2). According to the results and data analysis, strains  $S_7$ , NO7 and  $S_5$ , NO6 showed the best biosurfactant production rate.

**Biosurfactant Production Enhancement by pH and Temperature Optimization:** Changing media pH from 6.5 to 8 with a 0.5 shift showed that the two final isolates produced maximum biosurfactant levels in pH = 7 (Fig 3). Also the optimized temperature was determined (Fig 4) (Rasooli *et al.*, 2008; Priya and Usharani, 2009).

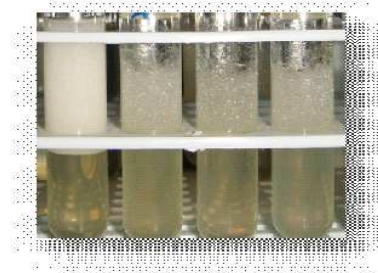


Fig. 1: Emulsification test for strain  $S_5$ , No 6



Fig. 2: Foam forming activity for strain  $S_7$ , No7

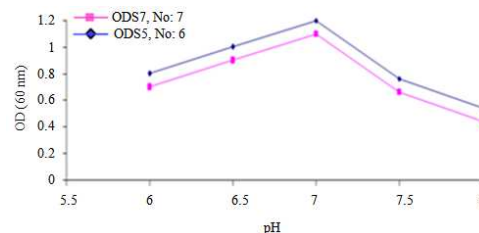


Fig. 3: Optimazation of pH

Table 3: Detection of biosurfactant producing isolates by preliminary and complementary screening methods

Isolate	Hemolytic activity*	Oil spreading*	Oil collapse**	Surface tension (mN m <sup>-1</sup> ) ± SD	E <sub>24</sub> In presence of crude oil
S <sub>1</sub> , NO3	++	+	+	42	69/12%
S <sub>1</sub> , NO4	++	++	+	55	57/14%
S <sub>2</sub> , NO12	++	++	+	46	59/96%
S <sub>2</sub> , NO5	+	+	+	47	58/01%
S <sub>2</sub> , NO16	++	++	+	55	55/16%
S <sub>3</sub> , NO19	+	+	+	50	61/12%
S <sub>3</sub> , NO13	-	+	++	53	57/05%
S <sub>3</sub> , NO1	++	+	+	49	65/08%
S <sub>4</sub> , NO8	++	-	++	45	69/05%
S <sub>4</sub> , NO4	++	+	++	49	71/26%
S <sub>5</sub> , NO6	++	++	-	40	79/98%
S <sub>6</sub> , NO3	+	++	++	49	66/73%
S <sub>6</sub> , NO12	-	++	+	51	80/06%
S <sub>7</sub> , NO7	+++	+	++	29	80/36%
S <sub>7</sub> , NO2	++	+	++	45	51/54%
S <sub>8</sub> , NO14	+	-	++	50	59/51%
S <sub>8</sub> , NO5	-	+	+	56	72/85%
S <sub>8</sub> , NO12	+	+	+	51	67/53%
Water	-	-	-	70	-
Culture medium	-	-	-	65	-
Tween 20	-	-	-	35	-

(+)\*: For areas with less than 1 cm in diameter, (++)\*: For areas with a diameter of 1 to 3 cm, (+++)\*: For areas with more than 3 cm in diameter, (+)\*\*: Drops with diameters less than 1/5 cm, (++)\*\*: Drops with diameters between 1 and 1/5 cm, (+++)\*\*: Drops with diameters more than 1/5 cm

Table 4: Foam properties at different Strains

Isolate	Foam stability		Foam properties
	course (min)	Foam height (mM)	
S <sub>1</sub> , NO4(A)	95	53	++
S <sub>2</sub> , NO12	55	8	+
S <sub>2</sub> , NO5	45	8	+
S <sub>3</sub> , NO1	75	7	+
S <sub>4</sub> , NO8	75	11	++
S <sub>4</sub> , NO4	35	7	+
S <sub>5</sub> , NO6	105	16	++
S <sub>6</sub> , NO3	30	7	+
S <sub>7</sub> , NO7	135	28	+++
S <sub>7</sub> , NO2	60	9	+

(+): Bubbles with coarse sizes, very disperse and low stability, (++): Bubbles with medium sizes, concentrated and medium stability, (+++): Bubbles with fine sizes, concentrated and high stability

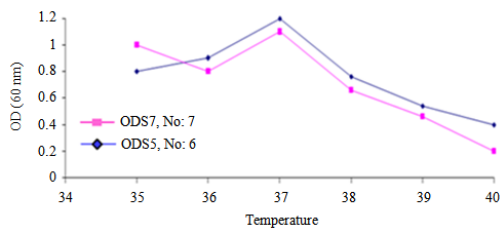


Fig. 4: Optimazation of temperature

### DISCUSSION

As expected, isolated strains from oil contaminated soils of Sirri Island had a great ability in biosurfactant production. This was in accordance with the result of Firenraz and colleagues work in (1991) that showed a

doubled number of biosurfactant producing bacteria in oil contaminated soils (Francy *et al.*, 1991). According to Banat (1995) study, an effective biosurfactant is the one that reduces water surface tension from 72-27 mN m<sup>-1</sup>. Our two final isolates showed this ability in accordance with (Okerentugba and Eneronye (2003) (Benincasa *et al.*, 2004) studies which both proved that strains isolated from areas with permanent contamination had better emulsification activity (Okerentugba and Ezerony, 2003; Francy *et al.*, 1991).

### CONCLUSION

MEOR represents a truly eco-friendly petroleum recovery process employing biotechnological resources and techniques that can be used to replace and augment the traditional EOR processes and flooding chemicals. Many countries have envisaged that one-third of their oil recovery programs will utilize MEOR techniques by the year 2010 (Sen, 2008; Schneider and Tabeling, 2011). Surface active compounds or biosurfactants production has become more important in recent years. These compounds play an important role in increasing oil recovery (Banat, 1995; Sen, 2008). Isolation of two strains with high emulsification activity and biosurfactant production that have the potential to be industrially used both for MEOR and oil contamination bioremediation, proves that it is necessary to perform such researches in future to obtain novel results and localize bioscience application in oil industry of oil producing countries like Iran.

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