Production and Characterization of Biosurfactants Produced by *Bacillus* spp and *Pseudomonas* spp Isolated from the Rhizosphere Soil of an Egyptian Salt Marsh Plant

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Abstract: Seventeen bacterial strains were isolated from the rhizosphere soil of an Egyptian salt marsh plant and screened for biosurfactant production. 76.5 % of the bacterial strains were found to produce biosurfactants, they were identified as *Bacillus* spp (4 strains) and *Pseudomonas* spp (9 strains), of which *P. aeruginosa* was represented by 6 strains. From the preliminary experiment, (*Bacillus* SH 20, SH 26 and *Pseudomonas aeruginosa* SH 29, SH 30) were the most active biosurfactant producers. The four main active biosurfactant producers were selected and studied. The results showed that *P. aeruginosa* SH 29 represents a good candidate for the production of the biosurfactants when grown on both nutrient broth (NB) and inorganic salt media (ISM) supplemented with waste frying oil. On the other hand *Bacillus* spp (SH 20 and SH26) were active biosurfactant producers when grown on molasses. Waste frying oil and molasses represent good, cheap and easily available substrates which have the advantage of reducing the production cost and help economic production of biosurfactants. The results of using different vegetable oils varied with the variation of media and bacterial strains. Olive oil was promising followed by sunflower oil and soybean oil. All of the four bacterial strains were able to emulsify the studied hydrocarbon oils and vegetable oils but with different E24 values. *Bacillus* spp SH 20, SH 26 produced the highest E24 values for petroleum oil (84.4 ± 5.2 and 75.0 ± 5.6 % respectively). This was followed by *P. aeruginosa* SH 30 (66.7 ± 3.8 %) and *P. aeruginosa* SH 29 (62.0 ± 3.4 %). The results also showed that the produced biosurfactants in the present study were stable at 0-121 °C, pH 1-14 values and at different concentrations of NaCl. An attempt was made to isolate the biosurfactant produced by *P. aeruginosa* SH 29 when grown in waste frying oil (2% w/v). The production yield of this crude product was estimated as 2.8 g/L. This crude material was selected and kept for further purification and studies. Accordingly, the four bacterial strains may be useful in petroleum industry (e.g petroleum recovery, cleaning of oil storage tanks and recovery of oil from oily sludge) and they may help in bioremediation of oil contaminated sites.

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1. Introduction

Surfactants are surface active compounds that can be chemically synthesized or biologically formed (biosurfactants). Chemically synthesized surfactants are toxic, non-degradable and may be accumulated in living tissues leading to the development of cancer diseases **(Seghal *et al*, 2009; Lakshmipathy *et al*, 2010)**. Biosurfactants are preferable to chemical surfactants due to the following characteristics: low or no toxicity, biodegradability, better environmental compatibility, ability to act at wide range of temperature, pH values and salinity levels. Furthermore, they may be produced from industrial wastes and agriculture products which represent cheap substrates **(Deleu and Paquot, 2004; Dehgan-Noudeh *et al*, 2009; Ghayyomi-Jazeh *et al*, 2012)**.

Biosurfactants are amphiphilic compounds and they of two parts, a hydrophobic part (non-polar part) and a hydrophilic part (polar part). The hydrophobic part consists of a long chain fatty acids (saturated or unsaturated). The hydrophilic part is more soluble in water and consists of carbohydrates, amino acids, cyclic peptides, phosphates, carboxylic acids or alcohols (**Chayabutra *et al*, 2001; Chen *et al*, 2007; Volchenko *et al*, 2007**).

Biosurfactants are able to reduce the surface and interfacial tensions and have the capacity to mix two immiscible solutions. These properties give to the biosurfactants important characteristics such as emulsification, wetting, detergency, phase separation and decreasing the viscosity of petroleum oils **(Satpute *et al*, 2010; Jamal *et al*, 2012).** Accordingly, interest towards the biosurfactants increased in recent years as they are potential candidates for many commercial applications such as petroleum, pharmaceutical, biomedical and food processing industries**.**

Xu *et al* (2011) reported that although biosurfactants may be low cost and produced from cheaper renewable resources, their use in industry is limited due to lack of public acceptance of

biosurfactant-producing microorganisms, and the high purity necessary for food, cosmetics and pharmaceutical applications. For these reasons they are mainly used for the environmental treatments.

Most microbial surfactants are complex molecules, comprising different structures that include peptides, glycolipids, glycopeptides, fatty acids and phospholipids **(Desai and Banat, 1997; Comeotra and Makkar, 1998).**

In order to reduce the production costs of biosurfactants, the yield and product accumulation must be increased through the development of economical engineering processes and the use of cost effective substrates for the growth of microorganisms as biosurfactant production. The choice of inexpensive raw materials is important to the economy of the process (**Mercade and Manresa, 1994; Nitschke *et al*, 2004**).

The cost of the substrates will greatly influence the economical use of the biosurfactants. The use of vegetable oils and their wastes as source of biosurfactants is promising but required more efforts and research to full realization (**Makkar *et al*, 2011**). Waste edible oils such as waste frying oil, contribute to the environmental pollution. These wastes are mostly not utilized efficiently, and sometimes are recovered improperly and sold illegally as waste edible oils (WEOs) to damage the people's health (**Guo and Chen, 2009; Yao and Min, 2010; Zhang *et al*, 2012**). There are few reports which utilized the vast potential of these frying oils for biosurfactant production **(Nitschke *et al*, 2004; Fleurackers, 2006; Shah *et al*, 2007; Zhu *et al*, 2007; Zhang *et al*, 2012**).

Another inexpensive and easily available good substrate for the production of biosurfactants is molasses (**Maneerat, 2005 a,b; Raza *et al*, 2007; Onbasli and Aslim, 2009**). The search for biosurfactant-producing microorganisms is still an important area of research because of the diversity of their molecules and the wide variety of their applications.

To the best of our knowledge, no studies have been performed on the biosurfactant-producing bacteria in the Egyptian habitats. Accordingly, the present study was carried out in order to search out potent biosurfactant produced by microorganisms found naturally in an Egyptian soil collected from the rhizosphere of a desert-salt marsh plant.

2. Materials and methods

1. Isolation and detection of biosurfactant-producing bacteria from the rhizosphere soil.

Soil samples were collected from the rhizoshere soil of *Urospermum picroides* plant grown in a salt marsh locality at Borg Al-Arab 30 km from Alexandria-Egypt.

One gram of the soil was serially diluted in sterile saline solution. One ml of the proper dilutions was used to inoculate a nutrient agar plate. Three inoculated plates were incubated at 30 °C for a period of 2-3 days after which colonies from a plate showing higher counts were isolated and purified by using the streaking method over nutrient agar plates.

Seventeen purified bacterial isolates were selected and tested for biosurfactant production by using the plug assay method (**Diab and Shereen-unpublished data**) as follows:

- Each bacterial strain was streaked over a nutrient agar plate and incubated at 30 °C for two days. Agar discs (10 mm) from the bacterial growth were cut off by a cork borer and transferred to the surface of nutrient agar plate covered with a thin layer of petroleum oil. The plate was left for 1-2 hours at room temperature after which the development of clear zones around the discs were observed and their diameters were measured.

The most active bacterial strains (showing clear zones of 25-30 mm) were selected and studied for production and characterization of biosurfactants.

2. Production of biosurfactants by the selected most active bacterial strains using different resources.

Two types of culture media were used, nutrient broth (NB) and inorganic salt media (ISM). The composition of the ISM medium was described by **Balogum and Fagade (2010).** Each of the above media was supplemented by the following resources (2% w/v): molasses, orange skin, waste frying oil, sunflower oil, corn oil, olive oil and soybean oil.

Each of the most active purified bacterial strains was grown in two separate 250 ml flask, one containing 50 ml NB and the other containing 50 ml ISM supplemented with different resources. The cultures were incubated at 30 °C on rotary shaker operated at 150 rpm for a period of 48 hours (for NB) and seven days (for ISM medium). At the end of the incubation periods the cultures were centrifuged at 6000 rpm for 30 minutes for the removal of cells. The cell free broth cultures (supernatant) were tested for the production and activities of biosurfactants by using the following tests:

a) Oil spread test (oil displacement area ODA)

This test was carried out as described by **Techaoei *et al* (2011)** and **Priya** and **Usharani (2009) as follows:**

40 ml sea water was introduced into a petri dish (9 mm diameter), 20 µl of crude oil was spread on the

surface of the sea water. 10 µl of supernatant was placed on the center of the oil film. The diameter of the clear zone formed was measured and then the area of clear zone circle was calculated as oil displacement area (ODA) using the following equation

$$ODA = 3.14 \times r^2$$

b) Emulsification activity (emulsification index E24)

This test was carried out according to **Tabatabaee *et al* (2005),** and **Techaoei *et al* (2011)** as follows:

In a screw caped tube 4 ml of the supernatant culture medium was added to 4 ml of each of the following oils: petroleum oil, paraffin oil, kerosene, corn oil, soybean oil and sunflower oil.

The tubes were vortexed at high speed for 2 minutes. The mixture then was allowed to settle for 24 hours and the emulsification index was measured as follows:

$$E24 = \frac{\text{The hieght of the emulsion layer}}{\text{The total hieght of the mixture}} \times 100$$

3. Stability of the biosurfactants

a) Thermo-stability test

This test was carried out according to **Techaoei *et al* (2011)** and **Haddad *et al* (2009)** as follows:

Ten ml portions of cell free culture medium were exposed to various temperatures (0-121 °C) for 30 minutes and they were allowed to cool at room temperature. The activity of the biosurfactants was measured by using the ODA test.

b) Effect of different pH values

Cell free supernatant was adjusted at different pH values (1-14). The activity of the biosurfactant in each pH was measured by using the ODA test (**Haddad *et al* ,2009)**.

c) Effect of salinity

The effect of NaCl concentration on the biosurfactants activity was tested by adding different concentrations (0-20 % w/v) of NaCl to the cell free supernatant. They were allowed to stand for 20 minutes after which the activity of the biosurfactant was measured by using the ODA method.

4. Extraction of the biosurfactant produced by Pseudomonas aeruginosa SH 29.

During the screening for the production of biosurfactants by different bacterial strains isolated from the rhizosphere soil, it was found that *Pseudomonas aeruginosa* SH 29 was able to produce biosurfactant of higher ODA activity when grown on both NB and ISM media supplemented with waste frying oil (2% w/v). the waste frying oil is a cost effective and easily available substrate that has a lot of advantages. This strain was selected and grown in NB medium with waste frying oil. The culture was incubated at 30 °C for 48 hours on a rotary shaker operated at 150 rpm. At the end of the incubation period the culture broth was centrifuged at 6000 rpm for 20 minutes to remove bacterial cells. The biosurfactant in the cell free supernatant was extracted two times with methylene chloride-methanol (2:1) at room temperature.

The organic phase was collected and evaporated to dryness on a water bath, leaving oily yellowish brown material. The production yield of this crude product was 2.5 g/L, it was selected and kept for further purification and studies.

5. Identification of the bacterial strains

The isolated bacterial strains were identified according to Bergey's Manual of Determinative Bacteriology **(Holt *et al*, 1994).**

3. Result and discussion

Seventeen bacterial strains were isolated from the rhizosphere soil of the desert plant ‘’*Urospermum picroides*” which is a dominant plant covering an area of salt marsh soil located at Borg Al-Arab 30 km from Alexandria. The 17 isolated bacteria were preliminary screened for the production of biosurfactants by using the agar plug method (Diab and Shereen unpublished data). The results showed that out of the 17 isolates, 13 (76.5 %) were biosurfactant producers, as indicated from the development of clear zones (Plate 1). Of these 13, four (30.8 %) were gram positive spore-forming rods and were identified as belonging to the genus *Bacillus*. The other nine isolates (69.2 %) were gram negative rods and were identified as *Pseudomonas* strains, six of these isolates were *Pseudomonas aeruginosa* strains.

These results demonstrate that the unpolluted rhizosphere soil of *Urospermum picroides* plant supported high level of biosurfactant-producing bacteria which were dominated by members of *Pseudomonas* and *Bacillus* genera.

It is well known that the root exudates of plants may encourage the growth and activities of the rhizosphere microorganisms including the biosurfactant-producers, which may improve the bioavailability of the organic compounds in the rhizosphere and help in the biodegradation process. **Oleszezuk *et al* (2007)** suggested that the rhizosphere effect on pollutants may be similar to the influence of biosurfactants on these compounds. An interesting report by **Jenning and Tanner (2000)** suggest to search not for biosurfactant-producers, but for biosurfactants themselves with the soil matrix.

From the screened bacterial isolates, four bacterial strains (showing 25-30 mm diameter clear zones) were selected and studied for their ability to produce biosurfactant when grown in presence of different substrates. The four bacterial strains were *Bacillus* spp. (SH 20 and SH 26) and *Pseudomonas aeruginosa* (SH 29 and SH 30). The produced biosurfactants were then tested for their stability under extreme condition. The biosurfactants activity in the present work was measured by using the ODA method. This method is a good indicator for biosurfactant production for the following reasons:

- It is very sensitive and requires small sample volume **(Techaoei *et al*, 2011)**.
- It is rapid, easy to be carried out and doesn't require any specialized equipments **(Plaza *et al*, 2006).**
- It depends on the decrease in water-oil-interfacial tension caused by the biosurfactant regardless its structure **(Morikawa *et al*, 2000).**

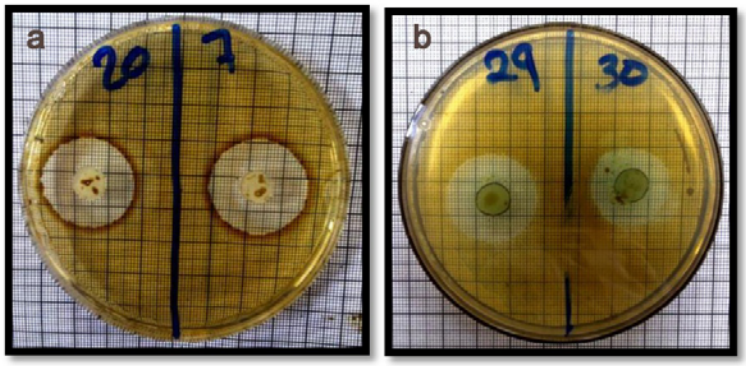

Plate 1. Photographs showing active biosurfactant producers:
a. Two *Bacillus* strains (SH 20 and SH 7)
b. Two *Pseudomonas aeruginosa* strains (SH 29 and SH 30).

The results of the production of biosurfactants by the four bacterial strains *Bacillus* spp. (SH 20 and SH 26) and *Pseudomonas aeruginosa* (SH 29 and SH 30) when grown on different culture media and resources are shown in Table (1). The results obtained could be summarized in the following points:

- When *Bacillus* spp (SH 20 and SH 26) were grown in ISM medium supplemented with vegetable oils, they failed to produce active biosurfactants, while in the presence of molasses they were able to produce surfactants of 51.2 ±3.5 and 32.1 ±2.7 cm^2 ODA respectively. On the other hand when this ISM medium was supplemented with waste frying oil, only *P. aeruginosa* (SH 29) was able to produce biosurfactant of 45.3 ±2.8 cm^2 ODA.

- When NB was supplemented with molasses, *Bacillus* sp SH 20 was able to produce very active biosurfactants of 63.6 ±3.4 cm^2 ODA, while in the presence of waste frying oil the four bacterial strains produced biosurfactants of 28.3 ±1.3 cm2 (for *Bacillus* sp SH 26) to 48.2 ±2.8 cm2 ODA (for *P.aeruginosa* SH 29).

The above results demonstrate that *P.aeruginosa* SH 29 represents a good candidate for the production of biosurfactants when grown in both NB and ISM media supplemented by waste frying oil, a cheap and available substrate. The use of these substrates have the advantage of reducing the production cost and of helping the economic production of biosurfactants**. Makkar *et al* (2011)** reported that residual cooking and/or waste frying oils are a major source of nutrient rich low cost fermentative waste, and there are few reports which utilize these wastes for biosurfactant production. **Shah *et al* (2007)** reported that large quantities of waste cooking oils are generated in restaurants worldwide and It has been estimated that an average 100 billion L of oil waste/week is produced in the United State alone. **Fleurackers (2006)** studied the production of biosurfactants by *Candida bombicola* TCC22214 when grown on waste frying oil. According to his conclusion, this was a successful feasibility study for using waste frying oil as substrate. **Haba *et al* (2000)** examined nine *Pseudomonas* strains for the production of biosurfactants from waste frying oil (sunflower oil). They found that *Pseudomonas aeruginosa* 4712 was able to produce 2.7 g/L rhamnolipid.

The present study shows also that molasses is another good, cheap and easily available substrate for the production of biosurfactants. **Onbasli and Aslim (2009)** examined the production of rhamnolipids by *Pseudomonas luteola* B17 and *Pseudomonas putida* B12 when grown on different sugar beet molasses concentrations. They achieved a maximum yield of rhamnolipid when 5 % (w/w) molasses was used.

Joshi *et al* (2008) studied biosurfactant production by *Bacillus licheniforms* K51, *Bacillus subtilis* 20 B and *Bacillus subtilis* HS3 using molasses as source of nutrients under thermophilic conditions. They obtained higher yield with 5-7 % (w/v) molasses. **Abdel-Mawgoud *et al* (2008)** reported that in an effort to economize surfactin production by *Bacillus subtilis* BS5, they optimized the environmental and nutritional production conditions for economizing of the production process. They used 16% molasses, 5 g/L $NaNO_3$ and mixture of trace elements. They achieved a biosurfactant yield of 1.12 g/L.

Bioconversion of waste materials is considered to be of prime importance for the near future because of its favorable economics, low capital and energy cost and ease of operation **(Savarino *et al*, 2007; Ferreira, 2008; Montoneri *et al*, 2009 a,b).**

As for the production of biosurfactants in NB medium supplemented by different vegetable oils, it can be seen that the most active biosurfactants (giving more than 42 ODA cm^2) were produced by :

- *Bacillus* sp SH 20 when grown on olive oil (52.8 ±3.4 ODA cm^2);
- *Bacillus* sp SH 26 when grown on sunflower oil and olive oil (55.4 ± 2.3 and 50.2 ± 2.7 ODA cm^2 respectively);
- *Pseudomonas aeruginosa* SH 29 when grown on soybean oil and sunflower oil (63.0 ± 3.2 and 50.2 ± 1.3 cm^2 ODA respectively); and
- *Pseudomonas aeruginosa* SH 30 when grown on olive oil (50.2 ± 2.8 cm^2 ODA).

Monteiro *et al* (2009) studied the production of biosurfactant by *Trichosporon montevideens* when grown on mineral medium supplemented with sunflower oil. They found that the produced glycolipid biosurfactant has a good surface activity and emulsification stability. **Rahman *et al* (2002)** studied the production of biosurfactant by *Pseudomonas aeruginosa* when grown on sunflower oil compared to soybean oil. They achieved 2.98 g/L of the rhamnolipid in presence of sunflower and 4.31 g/L in presence of soybean oil. **Aboulseoud *et al* (2008)** studied the production of rhamnolipid by *Pseudomonas flourescens* when grown on olive oil. The produced biosurfactant exhibited good surface activity and emulsification stability. **Thaniyavarn *et al* (2006)** examined *Pseudomonas aeruginosa* A41 for the production of biosurfactants when grown on olive oil and palm oil. They obtained 6.58 g/L biosurfactant with olive oil and 2.99 g/L with palm oil. These authors demonstrated the possibility of having a low-cost-large scale production of biosurfactant using palm oil. **Daverey and Pakshirajan (2010)** studied the production of sophorolipid by the yeast *Candida bombicola* when grown on soybean oil and molasses. They obtained higher yield under the optimized conditions. **Coimbra *et al* (2009)** examined six *Candida* strains for the production of biosurfactants when grown on soybean oil, fish oil and glycerol. Maximum production yield was obtained when soybean oil was used. **Thaniyavarn *et al* (2008)** found that sophorolipid produced by *Pichia anomala* was able to develop 69.43 cm^2 ODA when grown on 4 % soyean oil. **Rahman *et al* (2002)** obtained high yield of rhamnolipid by *Pseudomonas aeruginosa* when grown on soybean oil (4.31 g/L) as compared to sunflower oil (2.98 g/L).

Apart from studies using vegetable oils or other carbon sources for the production of biosurfactant, researchers are looking at more economic processes of using wastes related to these oils **(Makkar et al, 2011).**

The present work demonstrates the potential application of waste frying oils, molasses and certain vegetable oils in economic production of biosurfactants. The use of these substrates is promising but needs more investigations to reach full realization.

Economic production of biosurfactants is governed by different factors such as the cheap and abundant availability of carbon sources, the types of culture media (organic or inorganic), optimization of culture conditions, the environmental factors and finally the suitable active microorganisms **(Mukherjee *et al*, 2008; Mutalik *et al*, 2008; Mukherjee *et al*, 2006).**

Table 1. Activities of the biosurfactants produced by using the four bacterial strains (as measured by the ODA method) when grown on molasses (M), orange skin (OS), waste frying oil (WFO), sunflower oil (SUN), corn oil (CO), olive oil (OL) and soybean oil (SB).

Bacterial Strains	Media	ODA cm^2						
		M	OS	WFO	SUN	CO	OL	SB
***Bacillus* sp. SH 20**	ISM	51.4 ± 3.5	0.8 ± 0	-	-	3.1 ± 0.3	-	-
	NB	63.6 ± 3.4	3.1 ± 0.4	26.1 ± 2.7	32.2 ± 2.5	19.6 ± 0.7	52.8 ± 3.4	38.5 ± 2.1
***Bacillus* sp. SH 26**	ISM	32.1 ± 2.7	-	-	-	-	-	-

	NB	13.1 ± 0.9	-	28.3 ± 1.3	55.4 ± 2.3	34.2 ± 2.5	50.2 ± 2.7	41.8 ± 3.2
***P.aeruginosa* SH 29**	ISM	-	-	45.0 ± 2.8	32.3 ± 1.3	33.2 ± 1.3	-	28.0 ± 1.0
	NB	-	1.0 ± 0	48.2 ± 2.8	50.2 ± 1.3	33.2 ± 2.5	24.6 ± 0.3	63.0 ± 2.8
***P.aeruginosa* SH 30**	ISM	28.0 ± 1.0	3.2 ± 0.4	-	32.3 ± 2.8	30.2 ± 4.2	35.6 ± 3.4	1.1 ± 0.3
	NB	-	-	44.0 ± 2.6	21.2 ± 1.0	33.2 ± 3.0	50.2 ± 2.8	15.0 ± 1.0

Results of the emulsification activities (E24) of each of the four bacterial strains when grown on NB medium supplemented with vegetable and hydrocarbon oils are illustrated in Table (2). The results show that all the four bacterial strains were able to emulsify the six studied oils but with different emulsification activities (E24). The findings indicate that *Bacillus* spp SH 20 and SH 26 produced higher E24 values for petroleum oil (84.4 ± 5.2 and 75.0 ± 5.6 % respectively), followed by *Pseudomonas aeruginosa* SH 30 (66.7 ± 3.8 %) and *Pseudomonas aeruginosa* SH 29 (62 ± 3.4 %). High E24 values (54.4 ± 1.4 , 60.0 ± 2.8 %) were also measured for the bacterial strains when kerosene was used. **Techaoei *et al* (2011)** reported that one of the desirable characteristics of biosurfactants is the emulsification properties. Some strains showed positive ODA values, but at the same time they failed to show emulsification characters. The above authors found that two of the 25 bacterial isolates tested were able to give high E24 values of 57.8 %.

Balogum and Fagade (2010) studied seven bacterial strains for their emulsification properties with kerosene. They found that *Pseudomonas mallei* had the highest E24 value (65 %). On the other hand they measured the least E24 values (2.5 %) for *Pseudomonas syringae*, *Bacillus pasteuri* and *Klebseilla* sp. **Anyanwu *et al* (2011)** reported that the ability of biosurfactants to emulsify hydrocarbon-water mixture has been demonstrated to increase hydrocarbons biodegradation and is potentially useful in enhanced oil recovery. The same authors found that the emulsification index (E24) for *Serratia marcessens* NSK-1 with soybean oil and olive oil was 98% and 95 % respectively. On the other hand, they found that the least E24 values were for crude oil (64%) and kerosene (50 %). These authors suggested that the ability of bacterial strains to form stable emulsion with different hydrocarbon and vegetable oils gives to the produced biosurfactants many advantages in petroleum and pharmaceutical industries.

Based on the above discussion, the four bacterial strains used in the present study may be utilized for petroleum recovery, cleaning of oil storage tanks, recovery of oil from oil sludge and help in bioremediation of oil contaminated sites. At the same time the four bacterial strains were also able to form comparatively good E24 values (44.8 ± 4.8 – 58.8 ± 3.4 %) with vegetable oils suggesting that these bacterial strains may be also valuable in food and pharmaceutical industries.

Table 2. Emulsification activities of the different bacterial strains when vegetable oils and hydrocarbons were used.

Oils	ODA cm^2			
	***Bacillus* sp. SH 20**	***Bacillus* sp. SH 26**	***P.aeruginosa* SH 29**	***P.aeruginosa* SH 30**
Corn oil	58.8 ±3.4	47.9 ±2.0	48.0 ±2.8	52.4 ±2.5
Soybean oil	47.6 ±2.0	44.8 ±4.8	56.2 ±4.2	45.6 ±2.0
Sunflower oil	54.5 ±3.5	48.9 ±1.7	47.8 ±3.3	51.4 ±2.3
Kerosene	55.3 ±2.0	54.4 ±1.7	57.1 ±4.4	60.0 ±2.8
Paraffin oil	48.8 ±1.8	53.6 ±2.3	48.9 ±2.4	52.0 ±1.0
Petroleum oil	84.4 ±5.2	75.0 ±5.6	62.2 ±3.4	66.7 ±3.8

Results of the stability of the biosurfactants produced by the two *Bacillus* sp and the two *Pseudomonas aeruginosa* spp at different pH values (1-14) show that the biosurfactants produced by the bacterial strains were stable at wide range of pH values (1-14) but with different values of ODA (Table 3). The results obtained could be summarized as follows:

- Biosurfactant produced by *Bacillus* sp SH 20 showed the least activity at pH 1-3 (3.1 cm^2 ODA). At pH 6 the activity increased to reach ODA of 44.2 ± 2.5 cm^2. Optimum pH values (showing a maximum of 63.6cm^2 ODA) were pH 7 and pH8. At higher pH values the activity gradually decreased to reach 50.2 ±2.5 cm^2 ODA at pH14.
- Biosurfactant produced by *Bacillus* sp SH 26 showed its optimum activity (50.2cm^2 ODA) at wide range of pH (3-12). At pH 1 and 14 the

activity slightly decreased to reach 44.2 ± 3.1 and 47.8 ±3.1 cm^2 ODA respectively.

- Biosurfactant produced by *P. aeruginosa* SH 29 had optimum activity (63.3 ±3.4cm^2 ODA) at pH 7. Below and above this pH, the activity decreased to reach 44.8-50.2cm^2 ODA.
- *P. aeruginosa* SH 30 produced biosurfactant with optimum activity (58.4 ±3.4cm^2 ODA) at pH 7. Above this value the activity gradually decreased to reach 50.2 cm^2 at pH 10-14. Below the optimum pH the activity dramatically decreased to 15.9 ± 3.5 and 19.6 ± 2.0 at pH 1 and 3 respectively.

The above results show that out of the four bacterial strains studied, *Bacillus* sp SH 20 and *P. aeruginosa* SH 29 produced biosurfactants of good activities at both acidic and alkaline pH values. However, biosurfactants produced by the other two strains *Bacillus* sp SH 26 and *P. aeruginosa* SH 30 showed good activities only at neutral to alkaline pH values. Optimum activities of biosurfactant produced by the four bacterial strains were achieved at neutral to slightly alkaline pH (7-8). **Techaoei *et al* (2011)** found that the activity of the biosurfactant produced by *Pseudomonas aeruginosa* was stable at pH 4-11 values. At higher and lower pH, the activity decreased. **Rufino *et al* (2008)** found that the biosurfactant produced by *Candida lipolytica* was very stable at wide range of pH 2-12. Other researchers also reported that the biosurfactants produced by different microorganisms were stable at wide range of pH, salinity and temperature (**Monterio *et al*, 2009; Aboulseoud *et al*, 2008; Prieto *et al*, 2008; Reis *et al*, 2004**).

Table 3. Effects of different pH values on the activities of biosurfactants produced by *Bacillus* spp. and *Pseudomonas* spp. The activities of the biosurfactants were measured by using the ODA method.

Bacterial strains	*ODA cm^2*							
	pH 1	**pH 3**	**pH 6**	**pH 7**	**pH 8**	**pH 10**	**pH 12**	**pH 14**
***Bacillus sp.* SH 20**	3.1 ± 0.4	3.1 ± 0.3	44.2 ± 2.5	63.6 ± 3.7	63.6 ± 5.3	56.7 ± 1.2	50.2 ± 4.0	50.2 ± 2.5
***Bacillus sp.* SH 26**	44.2 ± 3.1	50.2 ±2.7	50.2 ± 4.5	50.2 ±1.0	50.2 ±1.7	50.2 ± 1.6	50.2 ± 1.1	47.8 ± 3.1
***P. aeruginosa* SH 29**	44.8 ± 2.3	47.8 ± 3.1	50.2 ± 3.1	63.6 ± 3.4	50.2 ± 1.1	50.2 ± 4.0	50.2 ± 2.5	50.2 ± 1.7
***P. aeruginosa* SH 30**	15.9 ± 3.5	19.6 ± 2.0	38.5 ± 2.1	58.4 ± 3.4	56.7 ± 3.3	50.2 ± 2.5	50.2 ± 4.5	50.2 ± 5.4

As for the stability of the produced biosurfactants in presence of different concentrations of NaCl, the results (Table 4) showed that the activities of the biosurfactants differ according to the different concentrations of NaCl (0-20 % w/v). Biosurfactant produced by *Bacillus* sp SH 26 was highly stable as compared to the other strains, while biosurfactant produced by *P.aeruginosa* SH 29 and *Bacillus* sp SH 20 showed the least stability. When the percentage activities (on the basis of the activity at 0% NaCl concentration) are considered (Figure 1), the results show that biosurfactant produced by *Bacillus* sp SH 26 was the most stable. It showed 95.3%, 88.4%, 83.9 % and 44.4% stability at 5%, 10 %, 15% and 20% NaCl respectively. On the other hand biosurfactant produced by *Pseudomonas aeruginosa* SH30 showed 76.7% and 47.1% stability at 5 and 10% NaCl respectively. At higher concentrations of NaCl the stability dropped to 25.1%. **Techaoei *et al* (2011)** found that the produced biosurfactant was stable at moderate concentration of NaCl (8%). **Rufino *et al* (2008)** found that the produced biosurfactant was active and very stable at 2-10% concentrations of NaCl.

All of the biosurfactant produced in the present work showed good stability at wide range of temperature (0-121 °C). **Rufino *et al* (2008)** found that the biosurfactant that they studied was stable at 0-120 °C, while **Sarubbo *et al* (2007)** reported loss of biosurfactant emulsification capacity after heating the biosurfactant for one hour at 70 °C.

The present results confirmed the stability of the produced biosurfactant at wide ranges of pH, temperature and salinity. These qualities enable these biosurfactants to be of potentially used in petroleum and other industries.

During the screening for the production of biosurfactants by the different bacterial strains, it was found that *Pseudomonas aeruginosa* SH 29 was able to produce biosurfactant of higher ODA activity when grown on both ISM and NB culture media supplemented by waste frying oil, a cost effective and easily available substrate. This strain was selected and cultivated in NB medium with waste frying oil (2 % w/v). The biosurfactant formed in the cell free supernatant broth culture was extracted with methylene chloride : (methanol (2:1)). The organic phase was dried leaving behind an oily yellowish

brown product. The production yield of this crude material was estimated as 2.8 g/L. The biosurfactant produced by *P. aeruginosa* SH 29 in culture broth was stable at high temperature reaching 121 °C (when exposed for 30 minutes), at wide ranges of pH (1-14) and showed 31.7-25.1 % stability in presence of 5-15 % NaCl. It showed E24 values of 51.1 % and 62.2 % with kerosene and petroleum oil respectively and 47.8-56.2 % with vegetable oils. These findings revealed that this biosurfactant could be very useful in situation when extreme conditions are presents such as enhancing oil recovery, bioremediation of soil and marine environment. The crude product was kept for further purification and studies.

The present study demonstrates that although the production yield was only 2.8 g/L, the produced biosurfactant showed better activity. it may be possible to increase the production yield at very low cost by improving the production procedures and technology and the optimization of culture broth composition.

Table 4. Effect f different concentrations of NaCL on the activities of the biosurfactants produced by the different bacterial strains as measured by using ODA method.
± (standard deviation), n= 2.

Bacterial strains	*ODA cm²*				
	NaCl %				
	Zero %	*5 %*	*10 %*	*15 %*	*20 %*
1. Bacillus sp. SH 20	*56.5 ± 3.5*	*23.7 ± 2.4*	*23.7 ± 2.3*	*9.6 ± 2.0*	*3.1 ± 0.7*
2. Bacillus sp. SH 26	*55.4 ± 3.1*	*52.8 ± 3.3*	*49.0 ± 2.8*	*46.5 ± 2.1*	*24.6 ± 2.3*
3. P. aeruginosa SH 29	*50.2 ± 2.5*	*15.9 ± 2.4*	*12.6 ± 2.3*	*12.6 ± 0.8*	*7.1 ± 0.6*
4. P. aeruginosa SH 30	*50.2 ± 4.5*	*38.5 ± 2.1*	*23.7 ± 2.4*	*12.6 ± 0.8*	*12.6 ± 2.0*

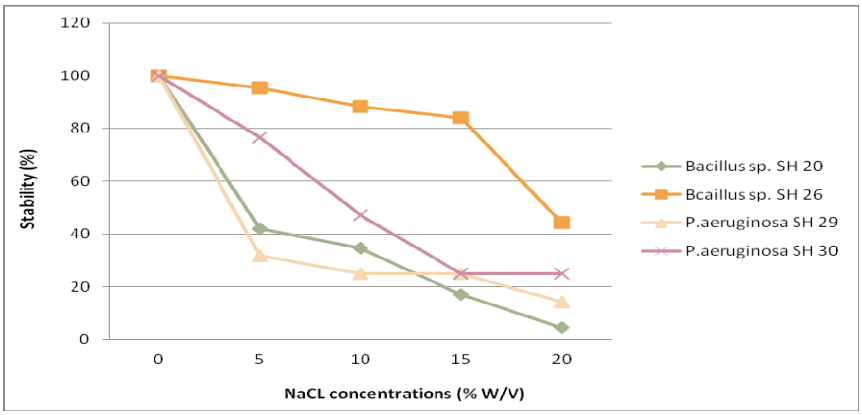

Figure 1. Stability (%) of the biosurfactants produced by the different bacterial strains in presence of different concentrations of NaCL on the basis of 0-NaCL stability.

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