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MICROBIAL PRODUCTION OF SURFACTANTS: SCREENING AND IDENTIFICATION OF TWO PROMISING ISOLATES AND THEIR BIOSURFACTANTS

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ABSTRACT

Microbial production of surfactants was tested in 1945 bacterial isolates. The tested isolates were recovered from 30 soil samples either contaminated with oil products, oil products and iron or uncontaminated. Biosurfactant production was tested using 3-phases screening protocol. ***Primary screening*** *was conducted by measurement of surface tension using the qualitative drop-collapse test (DCT) which resulted in the discovery of 28 high biosurfactant producing isolates. These isolates were subjected to* ***secondary screening*** *using a semi-quantitative microassay method for surfactants (Oil spreading test, OST) which resulted in the selection of 16 isolates (out of 28 high biosurfactant producers) that recorded highest scores.* ***Tertiary screening*** *was carried out on the 16 isolates using a du Nouy ring tensiometer for more sensitive quantitative measurement of surface tension. Of the 16 isolates tested in tertiary screening; a Gram positive isolate BS5, identified as Bacillus subtilis, and a Gram negative isolate BS20, identified as Pseudomonas aeruginosa, each showed the highest biosurfactant productivity compared to other members of its Gram group. The biosurfactant produced by B. subtilis isolate BS5 in mineral salts medium (MSM) was identified as surfactin, while, that produced by P. aeruginosa isolate BS20 was identified as rhamnolipid. TLC analysis revealed that surfactin showed one separated spot with an* R_f *value of 0.8, while, rhamnolipid biosurfactant showed two separated spots having* R_f *values of 0.4 and 0.68.*

INTRODUCTION

Biosurfactants constitute a diverse group of surface active molecules synthesized by microorganisms. They have been shown to have a variety of potential applications including remediation of organics and metals, enhanced oil recovery, as cosmetic additive, in biological control of plants, and many other biological activities as antibacterial and antifungal substances (Desai and Banat, 1997; Youssef *et al.*, 2004). These amphiphilic compounds present a wide structural diversity, and they can be classified into four groups: (i) glycolipids; (ii) lipoaminoacids and lipopeptides; (iii) polymers; and (iv) phospholipids, mono- and diacylglycerols and fatty acids. Because of their low toxicity, biodegradable character, and effectiveness at extreme temperature and pH values, there is an increasing interest in considering biosurfactants as a potential alternative to chemically synthesized surfactants (Sanchez *et al.*, 2006). The two well studied biosurfactant-producing organisms are *Pseudomonas* sp. and *Bacillus* sp. producing rhamnolipids and surfactin biosurfactants respectively.

Pseudomonas aeruginosa produces rhamnose containing glycolipids also called rhamnolipids when grown on a number of water miscible & immiscible substrates (Ortiz *et al.*, 2006). Rhamnolipids have gained considerable interest due to their low toxicity, biodegradable nature and diversity. Their range of potential industrial applications includes enhanced oil recovery, crude oil drilling, lubricants and bioremediation of water insoluble pollutants (Banat, 1995). Besides the environmental and industrial use of rhamnolipids, significant potential application is emerging for them as fine chemicals (Ortiz *et al.*, 2006). In this regard, the use of rhamnolipids as emulsifiers, penetrating agents and drug delivery systems in cosmetics and pharmaceutics is a great developing area of research (Ortiz *et al.*, 2006). Rhamnolipids show a great variety of biological activities as they have been shown to have antimicrobial action (Benincasa *et al.*, 2004), antiphytoviral effect and zoosporicidal activity (Ortiz *et al.*, 2006).

Surfactin is one of the most efficient biosurfactants so far known which belongs to the lipopeptide family excreted by *Bacillus subtilis*. Its structure is characterized by a heptapeptidic moiety linked to a beta hydroxyl-fatty acid. A natural diversity occurs, giving rise to homologues, differing from each other by the length (13 to 15 atoms of carbon) and the ramification of the fatty acid chain; and to isoforms, characterized by some differences in the peptidic sequence (Dufoura *et al.*, 2005). The increasing interest for these molecules is due to their excellent surface-active properties as it reduces the surface tension of water from 72 to 27 *mN/m* at a concentration as low as 0.005% (Arima *et al.*, 1968). In addition, surfactins exhibit diverse biological activities such as antiviral and antimycoplasma (Vollenbroich *et al.*, 1997a & b), antitumoral (Nitschke *et al.*, 2004), inhibition of fibrin clot and antibacterial properties (Arima *et al.*, 1968).

Biosurfactant production can be detected by measuring their properties. Of the measured properties is emulsification (Makkar and Cameotra, 1997), hemolytic activity (Banat, 1993), or cell surface hydrophobicity (Neu and Poralla, 1990; Pruthi and Cameotra, 1997). Another detection method is the colorimetric assay developed by Siegmund and Wagner (1991) which is based on the formation of insoluble ion pair between anionic surfactants, cationic cetyl trimethyl ammonium bromide (CTAB), and methylene blue (Siegmund and Wagner, 1991). Since this approach is specific for anionic surfactants, it cannot be used as a general method of screening for biosurfactant producers (Youssef *et al.*, 2004). There are a number of approaches that measure directly the surface activity of biosurfactants. These include surface and/or interfacial tension measurement (Mercad *et al.*, 1993), axisymmetric drop shape analysis profile (ADSA-P) (Van der Vegt *et al.*, 1991), drop collapse method (Bodour and Miller-Maier, 1998; Jain *et al.*, 1991) and the oil spreading technique (Morikawa *et* *al.*, 1993 & 2000; Youssef *et al.*, 2004). The measurement of surface tension has been used as a standard method to detect biosurfactant production. The most widely used method for the measurement of surface and interfacial tension is the *du* Nouy ring method, which measures the force required to pull a platinum wire ring through the liquid–air or liquid-liquid interface. This method is accurate and easy to use; however, it requires a specialized equipment (Harkins and Alexander, 1959) and a large volume of sample is required for analysis. In addition, measurement of surface tension using this method is time-consuming, which makes it inconvenient to use for screening of a large number of isolates (Youssef *et al.*, 2004). Therefore, a simple protocol to screen and quantify biosurfactant production in large numbers of microorganisms was developed (Youssef *et al.*, 2004). In this protocol, the cultures are first analyzed by using the drop collapse method. Positive results obtained by this method would constitute cultures that produce either moderate or high amounts of biosurfactants (above 60 mg/l). Second, the concentrations of biosurfactant produced can then be determined using the oil spreading technique for the purpose of reaching to the highest biosurfactant producer. Third, surface tension can then be used to confirm the results if required (Youssef *et al.*, 2004).

In the present study, a large number of bacterial isolates were recovered from the Egyptian soil and screened for their capability to produce biosurfactants using a screening protocol of three consecutive phases. Two promising biosurfactant-producing isolates, one Gram-positive and the other Gram- negative were identified and the nature of the biosurfactants produced by them were determined.

MATERIALS AND METHODS

Culture media

Mineral salts medium (MSM) containing 2% glucose as the sole carbon and energy source consisted of a mixture of two solutions (A and B). Solution A contained (g/L) $NaNO_3$ (2.5), $MgSO_4.7H_2O$ (0.4), NaCl (1.0), KCl (1.0), $CaCl_2.2H_2O$ (0.05), and 10 ml phosphoric acid (85%). This solution was adjusted to pH 7.2 with KOH pellets. Solution B contained (g/L) $FeSO_4.7H_2O$ (0.5), $ZnSO_4.7H_2O$ (1.5), $MnSO_4.H_2O$ (1.5), K_3BO_3 (0.3), $CuSO_4.5H_2O$ (0.15), and $Na_2MoO_4.2H_2O$ (0.1). One milliliter of solution B was added to 1,000 ml of solution A to form the MSM, then glucose was added and the complete medium was sterilized by autoclaving (Bodour *et al.*, 2003).

R2A agar (Becton Dickinson Company, Cockeysville, Md.) was used for isolation and enumeration of bacteria.

Chemicals

Unless otherwise indicated, all chemicals were of high quality available grades, supplied by *El-Nasr* chemicals Co. (Adwic), Egypt.

Collection of soil samples and recovery of isolates

Thirty soil samples were collected and stored at 4°C. The samples were taken from a depth of 10 cm below the ground surface. This precaution was taken into consideration to enhance the recovery of bacterial isolates as the surface microbial flora is largely affected by the UV rays of sunlight. Soil samples were classified as uncontaminated, contaminated with petroleum oil products only or cocontaminated with petroleum oil products and iron.

Isolates were recovered from the soil samples using the method developed by Bodour *et al.* (2003). A 5 g amount of each sample was placed into a 250 ml Erlenmeyer flask containing 50 ml of tap water and incubated at 23°C on a shaker (Newbrunswick) at 200 rpm for 21 days. On days 3, 7, 14, and 21, a sample from each soil slurry was serially diluted, plated onto R2A agar, and incubated for 1 week at 28ºC. After incubation, resultant colonies were enumerated, and at each sampling time, morphologically different colonies (approximately 12 to 20) were selected and cultured onto nutrient agar slants. The recovered isolates were stored onto nutrient agar slants at 4°C till screening them for biosurfactant production (Bodour *et al.*, 2003) and the resultant biosurfactant-producing isolates were routinely subcultured every month.

Screening for biosurfactant producing isolates

The collected isolates were tested for their capability to produce surfactants using primary, secondary and tertiary screening phases as recommended by Youssef *et al.*, (2004).

For each isolate, a loopful from a fresh slant was inoculated into 50 ml flask containing 5 ml MSM. The flasks were then incubated in an orbital shaking incubator at 28°C and 200 rpm for 7 – 9 days. Aliquots (1.5 ml each) of the produced culture were centrifuged (Hietech® Biofuge) at 10,000 *xg* for 5 min to prepare the cell free supernatant (CFS) (Bodour *et al.*, 2003). The CFSs prepared by this method were used for primary and secondary screening.

Primary screening was conducted by measurement of surface tension using the qualitative drop-collapse test (DCT) (Bodour *et al.*, 2003; Bodour and Miller-Maier, 1998). DCT was performed in wells (8 mm internal diameter) of the polystyrene lid of a 96-microwell (12.7×8.5 cm) plate (Nunclon, Denmark). A thin coat of 10W-40 oil (Pennzoil, Oil City, Pa.; 1.8 µl/well) was applied to each well. The coated wells were equilibrated for 24 h at 23°C, and then a 5µl aliquot of the respective CFS was delivered into the center of the well. If the drop remained beaded, the result was scored as negative, while if it spread and collapsed, the result was scored as positive for the presence of biosurfactant. The CFSs of the tested isolates were tested in triplicate. The MSM alone had a negative drop-collapse test.

Secondary screening was performed on the isolates obtained from the primary screening using a convenient method for microassay of surfactants called oil spreading technique (OST) that was developed by Morikawa *et al.* (1993, 2000); Youssef *et al.* (2004). This method is based on the feature of the biosurfactant to change the contact angle at the oil-water interface. The surface pressure of the oil displaces the oil. An aliquot of 20 µl crude oil was put onto the surface of 40 ml of distilled water in a Petri dish (140 mm in diameter). A thin membrane of oil is formed immediately. Then, 10 µl of the CFS was gently put on the center of the oil membrane. A visually detectable clear halo was produced and its diameter was measured. The sensitivity of this method was high enough to detect minute amount of biosurfactants (Morikawa *et al.*, 1993). In addition, DCT was also carried out as described previously but with monitoring the time required for complete drop collapse to occur.

Tertiary screening was carried out on the isolates obtained from the secondary screening and was performed by quantitative measurement of surface tension using a more sensitive method called the *du* Nouy ring method (Bodour and Miller-Maier, 1998). Seed cultures were prepared by inoculating 25 ml MSM contained in 250 ml flasks with a loopful from a fresh slant. The flasks were incubated at 28°C and 200 rpm using an orbital shaker for 24 h. Erlenmeyer flasks (250 ml) containing 50 ml MSM were then inoculated with the seed culture at 2% v/v and incubated at 28°C and 200 rpm using an orbital shaker for 7 days. Cultures obtained were centrifuged (Hietech Biofuge) at 10,000 *xg* for 5 min to prepare the CFSs. The cell-free supernatant of the tested isolate was placed into a specific clean glass beaker (50 ml, sample cup) of a Surface Tensiomat (Kruss) for measurement of the surface tension. Before conducting the experiment and between each pair of measurements, the sample cup was washed three times with distilled water and acetone in series and then allowed to dry. The platinum ring was similarly treated then it was flamed till redness and left to cool. In parallel, OST was also measured in this phase and the results of both methods were used for comparison of biosurfactant productivities of the selected isolates. The tested isolates were subjected to Gram stain using cells grown onto nutrient agar slants at 37°C for 20 h. Two isolates, one from the Gram positive group (isolate BS5) and the other from the Gram negative group (isolate BS20) were selected for further study.

Identification of the selected biosurfactant producing isolates

The selected Gram positive isolate BS5 was identified according to Claus and Berkeley, (1986) in Bergey's Manual of Systematic Bacteriology (1986), while, identification of the Gram negative isolate was done according to ERIC^{TM} Electronic RapID Compendium kits (Version: 1.0.75, Remel Inc. Lenexa, Kansas).

Identification of the biosurfactants produced by the two selected isolates (Bacillus subtilis BS5 and Pseudomonas aeruginosa BS20)

For Bacillus isolate:

Tentative identification of the nature of the biosurfactant produced by this *Bacillus* isolate BS5 was initially made. This was based on the literature accumulated on biosurfactants produced by *Bacillus subtilis* which entailed that BS-producing *Bacillus subtilis* strains commonly produce lipopeptide-type BS called surfactin. Accordingly, in this study an extraction method for surfactin was applied and surfactin was detected using TLC techniques against a surfactin reference standard (Fluka, obtained from Sigma-Aldrich, Germany).

Extraction of surfactin was accomplished as follows: Biosurfactant production was carried out in 1L Erlenmeyer flasks each containing 250 ml of MSM. The flasks were inoculated with the seed culture at 2% v/v and incubated under shaking conditions (250 rpm) and at 30°C for 4 days. The seed culture was prepared as described before in tertiary screening. The flasks were incubated at 250 rpm and 30°C for 36 h. At the end of the production period, the broth culture was centrifuged at 6000 rpm for 15 min to obtain the cell free supernatant. The crude biosurfactant extract was prepared by applying the extraction procedures described by Hsieh *et al.* (2004) and Vater *et al.* (2002). The cell free supernatant was acidified with 1N HCl to pH 2, left overnight at 4°C and then centrifuged at 6000 rpm for 15 min. The produced off-white to buff cakes in the centrifuge tubes was dried in a hot air oven at 70°C. The dried materials were transferred to 50 ml methylene chloride contained in 250 ml conical flask and left covered overnight at room temperature with intermittent shaking. The organic extract was filtered, then, the residue on the filter paper was re-extracted with another 50 ml fresh methylene chloride and re-filtered again. The pooled filtrate was evaporated under vacuum at 40°C. The residue obtained was dissolved in 20 ml dH_2O with a pH adjusted to 8.1 using 1 N NaOH. This solution represented the crude biosurfactant extract.

TLC experiments. This was carried out by eluting the crude biosurfactant extract against standard surfactin from *Bacillus subtilis* ($\geq$ 98%, Fluka, obtained from Sigma-Aldrich, Germany) onto TLC plates as described by Vater *et al.* (2002). An aliquot from each of the crude biosurfactant extract and standard surfactin solution (0.455 mg/ml) was loaded onto a TLC plate (5 X 10 cm). The plate was developed using a mobile phase consisting of chloroform:methanol:water (65:25:4 v/v/v) in an appropriate screw capped jar, also another mobile phase was tested as well consisting of chloroform: methanol: acetic acid (65:15:2 v/v/v). The mobile phase migration distance was 8 cm. The developed plate was air dried, sprayed with dH_2O and dried in a hot air oven at 120°C for a specified period that result in a contrast between the developed biosurfactant spots and the stationary phase background. This contrast occurs due to the difference in the evaporation rate of the sprayed water over the biosurfactant spots (evaporation occurs earlier) and that absorbed by the stationary phase (evaporation occurs later). The TLC plates were scanned at the time of maximum contrast and the separated spots were outlined and their R_f values were measured and compared.

For Pseudomonas isolate:

Preliminary identification of the biosurfactant produced by this *Pseudomonas* isolate BS20 was carried out using Siegmund-Wagner (SW) plates. SW agar is a medium previously developed for the detection of anionic extracellular rhamnolipid produced by *Pseudomonas* sp. SW plates were prepared and spot inoculated with a loopful from a fresh growth of isolate BS20 onto nutrient agar slant. The plates were then incubated at 28°C for 5-7 days. Rhamnolipid production was detected by the formation of dark blue halos around the grown spots against a light blue background (Siegmund and Wagner, 1991).

Extraction of rhamnolipids was accomplished as follows: The growth conditions & the preparation of the cell free supernatant (CFS) was carried out as described previously in extraction of surfactin**.** The CFS was acidified with 1N HCl to pH 2 and left overnight at 4°C. The cloudy CFS obtained was twice extracted with an equal volume of ethyl acetate in a separating funnel. The pooled organic phase was evaporated under vacuum at 40°C. The obtained brownish oily residue was dissolved in 20 ml dH_2O with pH adjusted to 7.1 using 1N $NaHCO_3$.

This solution represented the crude biosurfactant extract (Wu and Ju, 1998).

TLC experiments were performed on extracted rhamnolipid by eluting the crude biosurfactant extract against standard rhamnolipids (AgSciTech Inc, Logan, Utah, USA) using TLC as described by Matsufuji *et al.*, (1997). An aliquot of each of the crude biosurfactant extract and standard rhamnolipid solution (12.5 mg/ml) was loaded on a TLC plate (5 X 10 cm). The plate was developed using a mobile phase consisting of chloroform:methanol:water (65:25:4 v/v/v) in an appropriate screw capped jar, also another mobile phase was tested as well consisting of chloroform:methanol:acetic acid (65:15:2 v/v/v). The mobile phase migration distance in each case was 8 cm. The developed TLC plate was air-dried and sprayed with orcinol reagent (0.19% orcinol in 53% H_2SO_4). Then, the plate was put in a hot-air oven at 120°C for 15 min. The plate was photographed and the R_f values of the separated colored spots were measured and compared.

RESULTS

Recovery of bacterial isolates from soil samples

A total of 1945 bacterial isolates were recovered from 30 soil samples collected from different localities throughout Cairo, Egypt. Of the soil samples, 19 were contaminated with oil products (HC), 9 were contaminated with iron as well as oil products (HC) and 2 were uncontaminated (Table 1).

Screening of isolates for biosurfactant production

Primary screening of the isolates for biosurfactant production. The bacterial isolates were tested for their ability to produce biosurfactants after 7-9 days culture in MSM under shaking incubation. The culture supernatants were tested for the presence of biosurfactants using drop-collapse test (DCT) as a qualitative test for detection of biosurfactant-induced lowering in surface tension. Only 28 isolates were biosurfactant-producers as their CFSs showed complete drop-collapse on the hydrophobic oil surface in DCT (Table 1). These biosurfactant producing isolates were further subjected to secondary screening.

Secondary screening of the biosurfactant-producing isolates. The biosurfactant-producing isolates obtained from primary screening were further subjected to secondary screening using oil-spreading test (OST). Although OST is more sensitive than DCT in detecting and semiquantitating biosurfactant production, DCT was also conducted in parallel where the time required for the drop to collapse was determined. The biosurfactant-producing isolates were assigned codes and their scores in DCT and OST were recorded according to arbitrary scales defined in the legend of Table (2). The results of secondary screening (Table, 2) showed that; 12 isolates out of 28 recorded the highest score (++++) in both DCT and OST, 7 isolates recorded the highest score in DCT and variable scores in OST (+++, ++, + & ±) and the remaining 9 isolates recorded low scores (+++, ++, + & ±) with both tests.

Table (1): Soil sample characteristics and primary screening results for microbial production of surfactants.

Soil Sample No.	Nature of contaminant	CFU/g of soil[1]	No. of recovered isolates[2]	Biosurfactant producing isolates[3]
Uncontaminated				
1	None	9.3×10^{8}	82	0
2	None	3.4×10^{8}	80	2
HC contaminated				
3	Motor oil	2.0×10^{10}	52	0
4	Solar + Motor oil	7.2×10^{10}	79	0
5	Motor oil	9.2×10^{10}	74	0
6	Motor oil	7.4×10^{9}	67	0
7	Motor oil	7.4×10^{11}	41	3
8	Motor oil	7.2×10^{10}	61	1
9	Motor oil	8.7×10^{9}	69	0
10	Motor oil	1.6×10^{10}	38	0
11	Motor oil	1.0×10^{9}	49	0
12	Kerosene	1.1×10^{11}	50	0
13	Solar	1.8×10^{10}	42	0
14	Solar	1.6×10^{9}	51	0
15	Solar oil	1.4×10^{9}	48	0
16	Solar oil	1.0×10^{10}	56	2
17	Mazott	8.6×10^{9}	62	0
18	Mazott	1.8×10^{9}	88	3
19	Kerosene	2.5×10^{8}	43	0
20	Kerosene	8.8×10^{10}	63	1
21	Kerosene	5.6×10^{9}	78	1
HC & iron contaminated				
22	Break oil & iron	5.3×10^{10}	70	2
23	Break oil & iron	1.2×10^{11}	61	0
24	Gasoline 80 & iron	7.2×10^{8}	81	2
25	Gasoline 90 & iron	1.3×10^{10}	116	1
26	Gasoline 90 & iron	1.2×10^{9}	54	1
27	Gasoline 90 & iron	6.9×10^{8}	62	3
28	Gasoline 90 & iron	1.5×10^{9}	88	1
29	Gasoline 80 & iron	4.3×10^{9}	67	1
30	Gasoline 80 & iron	9.3×10^{9}	73	4
			$\Sigma = 1945$	$\Sigma = 28$

[1] The average viable count at the different sampling times (3rd, 7th, 14th, and 21st day).

[2] Total no of isolates picked up at the different sampling times (3rd, 7th, 14th, and 21st day).

[3] Total number of biosurfactant producing isolates among the recovered isolates.

Table (2): Results of secondary screening and Gram reaction of high-biosurfactant producing isolates obtained from primary screening[a].

Isolate code	Gram reaction	Score[b]		Isolate code	Gram R_x	Score[b]	
		DCT	OST			DCT	OST
BS1	positive	+ + + +	+ + +	BS16	negative	+ + + +	+ + + +
BS2	negative	+ + + +	+ + + +	BS17	positive	+ + + +	+ +
BS3	positive	+ + + +	+ + +	BS18	positive	+	+
BS4	positive	+ + + +	+ +	BS19	negative	+ + + +	+ + + +
BS5	positive	+ + + +	+ + + +	BS20	negative	+ + + +	+ + + +
BS6	negative	+ + + +	+ + + +	BS21	negative	+ +	±
BS7	negative	+ + + +	+ + + +	BS22	negative	+ +	±
BS8	negative	+ + + +	+ + + +	BS23	negative	+	±
BS9	negative	+ + + +	+ + + +	BS24	negative	+	±
BS10	positive	+ + + +	+ + +	BS25	positive	+ +	+
BS11	positive	+ + + +	+ +	BS26	positive	+	+ +
BS12	positive	+ + +	+	BS27	positive	+ + + +	+ + + +
BS13	positive	+ + + +	±	BS28	positive	+ + + +	+ + + +
BS14	negative	+	±				
BS15	negative	+ + + +	+ + + +				

[a] The isolates were grown in MSM under shaking incubation for 6 days.

[b] Score: **"+ + + +"** means that the drop collapses within 30 sec & the diameter of the clear zone is > 6 cm.

Score: **"+ + +"** means that the drop collapses in 0.5-1 min & the diameter of the clear zone is 3 – 6 cm.

Score: **"++"** means that the drop collapses in 1-2 min & the diameter of the clear zone is 2 – 3 cm.

Score: **"+"** means that the drop collapses in > 2 min & the diameter of the clear zone is 1 – 2 cm.

Score: **"±"** means that the diameter of the clear zone is < 1 cm.

No isolate recorded the highest score with OST and at the same time lower ones with DCT but the opposite was true. According to Gram reaction, out of the 28 isolates tested in secondary screening, 14 were Gram positive and 14 were Gram negative. From table (2), it was found that the Gram positive isolates BS 1, 3 – 5, 10, 27, 28 recorded the highest DCT scores "++++" while their OST scores were "++++" for isolates BS5, 27 & 28, "+++" for isolates BS1, 3 & 10 and "++" for isolate BS4. However, the Gram negative isolates BS2, 6, 7 – 9, 15, 16, 19 & 20 recorded the highest scores "++++" in both DCT & OST. These 16 Gram positive and negative isolates were subjected to tertiary screening.

Tertiary screening for the highest biosurfactant producing isolates obtained from secondary screening. The productivities of the selected 16 Gram positive and negative isolates were tertiary screened based on OST and surface tension (ST) measurement. High biosurfactant production and/or high biosurfactant activity causes a large clear zone diameter in OST and at the same time a high reduction in surface tension measurements. The surface tension was measured using appropriately diluted culture supernatants, this dilution is important from a discriminative point of view; since the ST lowering values reaches a plateau when the concentration of the biosurfactant exceeds the critical micelle concentration (CMC) (Youssef *et al.*, 2004). OST was additionally used because, although less accurate than ST measurement, has a larger dynamic range, i.e. it doesn't suffer from the plateau phenomenon demonstrated during ST measurements. The results of tertiary screening are shown in Table (3).

Table (3): Results of tertiary screening of the selected isolates based on OST and surface tension measurement.

Gram positive isolates			Gram negative isolates		
Isolate code	Clear zone diameter (cm) using OST	ST (mN/m) of 100-fold diluted supernatant	Isolate code	Clear zone diameter (cm) using OST	ST (mN/m) of 100-fold diluted supernatant
BS1	5.7	45	BS2	11.7	42
BS3	6	46	BS6	11.7	45
BS4	3.5	50	BS7	11.4	45
BS5	6.5	43	BS8	9.4	47
BS10	4.8	46	BS9	12	44
BS27	3.8	45	BS15	12	41.7
BS28	6	45	BS16	10.7	42
			BS19	12	42.9
			BS20	12	41

Isolate BS5 showed to be the highest biosurfactant producer among the Gram positive isolates (Table 3). This isolate showed the largest clear zone diameter in OST (6.5 cm) and the lowest surface tension value (43 mN/m). However, isolate BS20 showed to be the highest biosurfactant producer among the Gram negative isolates. This isolate showed the largest clear zone diameter in OST (12 cm) and the lowest surface tension value (41 mN/m).

Identification of the biosurfactant producing isolates BS5 and BS20

By microscopical examination (1000X) of a Gram-stained smear, isolate BS5 could be described as a Gram positive *Bacillus* species with terminal, sometimes central spores. According to Claus and Berkeley, (1986) and biochemical characteristics shown in Table (4), this isolate was identified as *Bacillus subtilis*.

Microscopical examination (1000X) of a Gram-stained smear of isolate BS20 revealed that it is a Gram negative organism with small, single, scattered, rod-shaped cells of variable lengths. This isolate was identified as *Pseudomonas aeruginosa* using ERIC^{TM} Electronic RapID Compendium kits.

Table (4): Biochemical characteristics of *Bacillus* isolate BS5.

Characteristics	Results[a]
Catalase production	+
Anaerobic Growth	-
Voges-Proskauer (V-P) test	+
pH of V-P broth after incubation: < 6	+
pH of V-P broth after incubation: > 7	-
Acid from D-Glucose/Gas	+/-
Acid from L-Arabinose	+
Acid from D-Xylose	+
Acid from D-Mannitol	+
Hydrolysis of Casein	+
Hydrolysis of Gelatin	+
Hydrolysis of Starch	+
Utilization of Citrate	+
Degradation of Tyrosine	-
Deamination of phenylalanine	-
Egg-yolk lecithinase	-
Nitrate reduced to nitrite (Gas/Reaction)	-/+
Formation of Indole	-
Formation of Dihydroxyacetone	+
Growth at pH 6.8, nutrient broth	+
Growth at pH 5.7	+
Growth in NaCl (2 – 10%)	+
Growth at 5°C	-
Growth at 10 – 40°C	+
Growth at ≥ 50°C	-
Growth in the presence of lysozyme.	-

[a] (+) means a positive result
(–) means a negative result

Determination of the nature of the produced biosurfactants

Surfactin in the crude biosurfactant extract of *B. subtilis* isolate BS5 was detected using TLC techniques against a surfactin reference standard (Fluka, obtained from Sigma-Aldrich, Germany). The developed TLC plate revealed one test surfactin spot which had exactly the same R_f value (0.8) as that of standard surfactin obtained from Sigam-Aldrich, Germany (Figure 1). When the mobile phase ($CHCl_3$:CH_3OH:H_2O at 65:25:4

v/v/v) was replaced with another one ($CHCl_3$:CH_3OH:CH_3COOH at 65:15:2 v/v/v), both test and standard surfactin gave also similar R_f values (data not shown). This finding confirms the identity of the biosurfactant produced by *Bacillus subtilis* isolate BS5 to be surfactin.

Fig. (1): A scanned image of TLC plate of the developed crude surfactin extract produced by *Bacillus subtilis* isolate BS5. The sample (Test) was developed against a standard (Std.) surfactin (Fluka, obtained from Sigma-Aldrich, Germany) using $CHCl_3$:CH_3OH:H_2O at 65:25:4 v/v/v as a mobile phase.

For the biosurfactants produced by the *Pseudomonas* isolate BS20, preliminary identification was performed using Siegmund-Wagner (SW) agar plates. When the tested isolate was inoculated and incubated onto SW agar plates, *Pseudomonas* colonies developed dark blue halos against the light blue SW agar plates indicating the production of rhamnolipids.

Thin layer chromatography was used to confirm the identity of the biosurfactant produced. This was performed by eluting the crude biosurfactant extract of *P. aeruginosa* BS20 against rhamnolipid reference standard onto TLC plates. The results in Figure (2) show two main spots in the test lane comparable to those found in the standard rhamnolipid lane. The lower spots of the test and standard rhamnolipids have R_f values of 0.4 and 0.45 respectively, while the upper ones have R_f values of 0.68 and 0.67 respectively. This similarity in R_f values indicates that the biosurfactant of *Pseudomonas aeruginosa* isolate BS20 is a rhamnolipid biosurfactant. Moreover, when the mobile phase ($CHCl_3$:CH_3OH:H_2O at 65:25:4 v/v/v) was replaced with another one ($CHCl_3$:CH_3OH:CH_3COOH at 65:15:2 v/v/v), test and standard rhamnolipids gave also similar R_f values (data not shown). This finding confirms the identity of the biosurfactant produced by *Pseudomonas aeruginosa* isolate BS20 to be rhamnolipid. The results also showed that both test and standard rhamnolipids exist in different homologues as proved by the two spots appearing in their lanes. These two spots were interpreted based on the chemical profile provided with the standard rhamnolipid to be L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RLL) for the more mobile spot and 2-o-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RRLL) for the less mobile spot. The close similarity between the R_f values of the two test rhamnolipid homologues and the RRLL & RLL homologues of standard rhamnolipid confirms the identity of these two test homologues to be RRLL and RLL. This means that each of RLL and RRLL has two lipid β-hydroxydecanoyl moieties (symbolized as L for the single lipid chain), RLL and RRLL differ however in the number of rhamnosyl moieties (symbolized as R for the single rhamnose moeity) being one in RLL and two in RRLL.

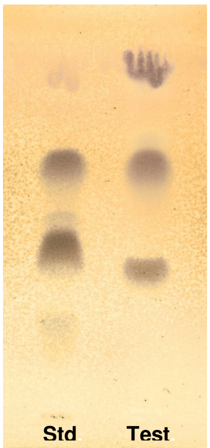

Fig. (2): A scanned image of the TLC plate of the developed crude rhamnolipid extract produced by *Pseudomonas aeruginosa* isolate BS20. The sample (Test) was developed against the rhamnolipid reference standard (Std.) (AgSciTech Inc, Logan, Utah, USA) using $CHCl_3$:CH_3OH:H_2O at 65:25:4 v/v/v as a mobile phase. The developed plate was sprayed with orcinol reagent and heated in an oven at 120°C for 15 min.

DISCUSSION

Different soil samples were collected from different localities in Cairo, Egypt for the purpose of isolating biosurfactants producing isolates. Of the soil samples, 19 were contaminated with oil products (HC), 9 were contaminated with iron as well as oil products (HC) and 2 were uncontaminated. Soil samples contaminated with hydrocarbons and/or iron were collected because microbial communities in these contaminated samples are expected to produce biosurfactants since it is hypothesized that biosurfactants are produced by microorganisms in order to facilitate the utilization of insoluble matters like hydrocarbons and essential metals (Lin, 1996).

For isolation of biosurfactants producing isolates, the method developed by Bodour *et al.* (2003) was used. Part of the soil sample was suspended in sterile tap water and incubated under shaking for 21 days. On days 3, 7, 14, and 21, a sample was plated on R2A agar and incubated for up to 1 week. These different sampling times allowed the recovery of rapid as well as slow grower bacteria. R2A agar was specifically used for plating because, on such medium bacterial colonies develop more slowly, are large enough to be counted easily, and there is little or no tendency towards spreading. Moreover, pigment production is enhanced on R2A medium and is readily observed after 3 to 5 days of incubation (Reasoner and Geldreich, 1985). Thus this medium is excellent for enumeration and isolation of bacteria based on morphological differences.

A total of 1945 isolates were recovered, they were enriched in MSM medium supplemented with glucose as the sole carbon source for testing their BS productivities. The use of glucose as the sole carbon source seems to be contradicting to the earlier hypothesis; that BS are produced by microorganisms in order to facilitate their growth on insoluble hydrocarbon (Lin, 1996). Although this hypothesis may be true, some biosurfactants have been reported to be produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol. However, in the screening process applied in this study, glucose rather than hydrocarbons was selected because, it is reported that glucose support the production of a variety of biosurfactants by the majority of microorganisms (Cooper and Goldenberg, 1987). The production of biosurfactants from carbohydrate substrates offers some advantages as compared with hydrocarbons; from an engineering point of view. This is because, hydrocarbon substrates require more sophisticated equipment and more power input to achieve an adequate dispersion of the insoluble hydrocarbons (Guerra-Santos *et al.*, 1984).

The screening for BS producing isolates was carried out according to the protocol suggested by Youssef *et al.* (2004). This protocol is suitable to screen and quantify biosurfactant production in large numbers of microorganisms. For primary screening, the culture supernatants of different isolates were analyzed by using the drop collapse test. Out of 1945 isolates collected, only 28 isolates gave positive results with DCT i.e. were able to lower the surface tension of the culture broth to a degree that cause a collapse of the applied CFS drop over the hydrophobic surface used in DCT. Positive results obtained by this method would constitute cultures that produce either moderate or high amounts of biosurfactants, meaning that only good BS producers will pass the primary screening phase (Youssef *et al.*, 2004). This may explain the low number of the BS producing isolates recovered in the present study. These good BS producers were further screened using the more sensitive oil spreading test (OST) (Morikawa *et al.*, 1993; Morikawa *et al.*, 2000; Youssef *et al.*, 2004), in which the amount of BS produced is assessed in terms of diameter of clear zone (the larger the clear zone the higher the BS concentration). In addition, DCT was also used in secondary screening phase and performed as in the primary screening but with monitoring the time required for complete drop collapse (the more rapid is the collapse the higher is the BS concentration). Of the 28 good producing isolates, 16 isolates were considered to produce promising levels of BS; 7 of them were Gram positive, and 9 were Gram negative (Table 3). These Gram-positive and negative isolates were subjected to tertiary screening using OST and direct measurement of surface tension of their culture supernatants. Since biosurfactants produced from Gram positive bacteria may differ from those produced from Gram negative bacteria, the best two biosurfactant producers, one from each group, were selected for further study. Isolates BS5 and BS20 were selected from the Gram-positive and negative groups respectively, since they showed the largest clear zone diameters in OST and the lowest surface tension values compared to other isolates of the respective Gram group.

The Gram positive isolate could be identified as *Bacillus subtilis* as described by Claus and Berkeley (1986), (in Bergey's Manual), while, the Gram negative isolate was identified as *Pseudomonas aeruginosa* using ERIC^{TM} Electronic RapID Compendium kits.

The nature of the biosurfactants produced by both isolates was determined. On reviewing the literature accumulated on biosurfactants produced by *Bacillus subtilis*, it was found that BS-producing *Bacillus subtilis* strains commonly produce lipopetide type BS called surfactin (Schallmey *et al.*, 2004). Accordingly, for *Bacillus* isolate BS5, an extraction method for surfactin was applied. Published data showed that most surfactin extraction methods were based on organic solvent extraction of acidified cell free supernatant. It has been proven that dichloromethane is the most suitable organic solvent since it could extract all of the surface activity present in the culture broth (Cooper *et al.*, 1981; Hsieh *et al.*, 2004; Sen and Swaminathan, 1997; Vater *et al.*, 2002). Therefore, extraction of acidified cell free supernatant with dichloromethane was applied and the crude biosurfactant extract was tested for the presence of surfactin using TLC technique against standard surfactin (Fluka, obtained from Sigma-Aldrich, Germany). It was found that, the test *Bacillus* biosurfactant spot had exactly the same R_f value as that of standard surfactin (Fig. 1). Moreover, when the mobile phase ($CHCl_3$:CH_3OH:H_2O at 65:25:4 v/v/v) was replaced with another one ($CHCl_3$:CH_3OH:CH_3COOH at 65:15:2 v/v/v), both test and standard surfactin gave also similar R_f values. These findings confirmed the identity of the biosurfactant produced by *Bacillus subtilis* isolate BS5 to be surfactin.

For *Pseudomonas* isolate BS20, preliminary identification of the biosurfactant produced by *Pseudomonas aeruginosa* isolate BS20 was carried out using Siegmund-Wagner (SW) agar plates, a medium previously developed for the detection of anionic extracellular rhamnolipid produced by *Pseudomonas* spp. (Siegmund and Wagner, 1991). The origin of the blue zone formed around rhamnolipid producing isolates growing on S.W. agar plates, is the formation of insoluble ion pairs between the secreted extracellular anionic substances with the cationic cetrimide and the basic dye methylene blue which are included in SW medium (Youssef *et al.*, 2004). The test *Pseudomonas* isolate BS20 developed a large obvious blue zone on SW agar, meaning that the produced extracellular surfactant is anionic in nature and is most probably rhamnolipid type biosurfactant.

Therefore, an extraction method for rhamnolipids was applied for the biosurfactant produced by the *Pseudomonas* isolate BS20. The BS produced by this isolate was detected using TLC technique against a rhamnolipid standard (AgSciTech Inc, Logan, Utah, USA). Published data showed that most rhamnolipid extraction methods were based on organic solvent extraction of acidified cell free supernatant. Ethyl acetate was selected as the organic solvent based on the previous experiments published in literature. Ethyl acetate was found to be the most efficacious since it resulted in the highest yields of crude rhamnolipids extracts when compared with other organic solvents (Schenk *et al.*, 1995). Therefore, in the present study, extraction of acidified cell free supernatant with ethylacetate was applied and the crude biosurfactant extract was tested for the presence of rhamnolipids using TLC technique against standard rhamnolipid obtained from AgSciTech Inc, Logan, Utah, USA. It was found that the developed test *Pseudomonas* biosurfactant contained two spots comparable to the main spots found in the standard rhamnolipid lane (Fig. 2). The two spots of the test biosurfactant had very similar R_f values to the main ones in the standard rhamnolipid lane. Moreover, when the mobile phase ($CHCl_3$:CH_3OH:H_2O at 65:25:4 v/v/v) was replaced with another one ($CHCl_3$:CH_3OH:CH_3COOH at 65:15:2 v/v/v), both test and standard rhamnolipids gave also similar R_f values. These findings confirmed the identity of the biosurfactant produced by *Pseudomonas aeruginosa* isolate BS20 to be rhamnolipid. The different spots separated in test BS lane are different homologues of rhamnolipid. They may be identified as 2-O-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RRLL) for the less mobile spot and L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RLL) for the more mobile spot based on the chemical profile provided with the standard rhamnolipid. This means that each of RLL and RRLL rhamnolipid homologues has two lipid β-hydroxydecanoyl moieties (symbolized as LL). The two homologues differ however in the number of rhamnosyl moieties (symbolized as R) being one in RLL and two in RRLL.

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ARABIC SUMMARY

الإنتاج الميكروبي للمواد النشطة سطحياً: الكشف عن الإنتاج والتعرف على عزلتين واعدتين وعلى المواد النشطة سطحياً المنتجة منهما.

للسادة الدكاترة

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من

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تم اختبار ألف و تسعمائة و خمس و أربعون عزلة بكتيرية، مفصولة من 30 عينة تربة ملوثة (بمنتجات الزيوت البترولية وحدها أو بتلك المنتجات والحديد) وغير ملوثة، لإنتاجيتها للمواد النشطة سطحياً باستخدام برنامج ثلاثي المراحل. و في مرحلة المسح الأولي ، تم قياس التوتر السطحي كيفياً باستخدام اختبار انهيار القطرة المتكورة على السطح الزيتي والذى أدى الى اكتشاف ثماني وعشرين عزلة منتجة للمواد النشطة سطحياً . وقد سجلت 16 عزلة من بين الثماني والعشرين عزلة أعلى قيم وذلك باجراء المسح الثانوى باستخدام طريقة دقيقة شبه كمية للكشف على المواد النشطة سطحيا (طريقة إزاحة بقعة الزيت) . واختبرت هذه 16 عزلة في مرحلة المسح الثالثة عن طريق قياس التوتر السطحي كمياً ياستخدام جهازقياس التوتر السطحي المعتمد على حلقة ال DuNouy و أظهرت عزلتان (إحداهما موجبة الجرام من جنس الباسيلس ساتيلس و الأخرى سالبة الجرام من جنس السودوموناس ايروجينوزا) أعلى إنتاجية للمواد النشطة سطحياً إذا ما قورن ذلك بالعزلات الاخرى المنتجة والمنتمية لنفس مجموعة الجرام لكلتيهما. وتم التعرف على طبيعة المواد النشطة سطحياً المنتجة بواسطة كل من العزلتين في وسط الأملاح المعدنية (MSM) ووجد أن عزلة الباسيلس ساتيلس BS5 منتجة للسرفاكتين، أما عزلة السودوموناس ايروجينوزا BS20 فوجد أنها منتجة للرامنوليبيد. وأظهرت تجارب كروماتوجرافيا الطبقات الرقيقة أن السرفاكتين بدا كمادة واحدة مفصولة لها معامل تأخير قيمته 0.8، بينما بدا الرامنوليبيد على هيئة مادتين مفصولتين ذواتى معامل تأخير 0.4 و 0.68.