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RESEARCH ARTICLE

"Assessment of different screening methods and identification of biosurfactant producing bacteria isolated from Egyptian oily polluted soil samples"

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Abstract

Different screening methods namely hemolytic assay, oil spread technique, drop collapse method, emulsification index (E_{24}), blue agar plate and measurement of surface tension were assessed for their efficiency to detect biosurfactant producing soil bacteria. The aim of the present study was to investigate biosurfactant-producing bacteria found in the oily polluted soil in Giza, Alexandria and El-Beheraa governorates, Egypt. Overall, 64 bacterial strains were Isolated, characterized, and purified from 12 soil samples. Screening for biosurfactant-producing bacterial strains was confirmed through 6 conventional screening tests. among this isolates 68.74% gram-positive, furthermore, 56.24% of the isolates showed hemolytic activity, 40.63 % exhibited positive results for oil-spreading test and E_{24}, 9.38% produced positive results for cetyltrimethyl ammonium bromide (CTAB) agar plate assay, and drop-collapse activity was found in 32.81% of the isolates, the most potent biosurfactant producing bacteria confirmed by measuring their ability to reduce surface tension by tensiometer. The dominant species were *Bacillus* and *Pseudomonas;* with an occurrence rate of 30% for each. Results indicated that these isolates have significant role for environmental applications such as bioremediation and industrial biotechnology.

INTRODUCTION

Surfactants are Amphiphilic molecules with both hydrophilic and hydrophobic moieties which have different characteristic properties such as reduce surface tensions, increase solubility, detergency power, wetting ability, and foaming capacity and it is applied as adhesives, flocculating, wetting and foaming agents, de-emulsifiers, and penetrants (**Makkar and Cameotra, 2002**; **Franzetti *et al.*, 2010; Perfumo *et al.*, 2010)**. Due to toxicity and high cost of chemical surfactants production now most of the researchers were focusing on biosurfactants production from biological sources. The surfactants produced by microorganisms are called as Biosurfactants which becoming important biotechnology products for industrial and medical applications due to their specific modes of action, low toxicity, relative ease of preparation and widespread applicability **(Smyth *et al*., 2010**).
Interest in these microbes has been steadily increasing owing to their need for bioremediation in our environment **(Porob, *et al.*, 2013).** Besides bioremediation, biosurfactants also have the potential to be used in enhanced oil recovery, herbicide and pesticide formulations, detergents, health care and cosmetics, pulp and paper, coal, textiles, ceramic processing and food industries, uranium ore-processing, and mechanical dewatering of peat (**Shoeb *et al*., 2013; Kaya *et al*., 2014).**

Bacteria are the main group of biosurfactant-producing microorganisms, although it is also produced by some yeast and filamentous fungi **(Ebrahimi *et al.*, 2011).** A number of studies have reported the potential of *Bacillus* species as biosurfactant producers and they produce lipopeptide type of biosurfactant; while rhamnose containing glycolipids are produced by Pseudomonas species **(Nitschke and Pastore, 2004).**

Although different screening methods are available, it is difficult to detect presence of biosurfactant by single method, due to their different in chemical structures such as mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein and phospholipid, so that application of various screening methods are recommended.

The objective of the present study is to investigate biosurfactant-producing bacteria inhabiting oily polluted soil in Egypt and qualify the different screening method.

MATERIALS AND METHODS:

Sampling

Twelve (12) oil polluted soil samples were collected from different localities (Giza, Alexandria and El-Beheraa governorates, Egypt) in sterile polythene bags, labeled appropriately and transfered to the laboratory for further study.

Isolation of biosurfactant producing bacteria

The collected samples were serially diluted (10^{-1} to 10^{-6}) and plated on basal mineral media containing (g/l): NH_4NO_3 (2.0), KCl (0.1), KH_2PO_4 (0.5), K_2HPO_4 (1.0), $CaCl_2$ (0.01), $MgSO_4.7H_2O$ (0.5), $FeSO_4.7H_2O$ (0.01), Yeast extract (0.1) and 10 ml of trace salts solution containing (g/l): 0.26 g H_3BO_3, 0.5 g $CuSO_4.5H_2O$, 0.5 g $MnSO_4.H_2O$, 0.06 g $(NH_4)_6Mo_7O_{24}.4\ H_2O$ and 0.7 g $ZnSO_4.7\ H_2O$), supplemented with 1 % petroleum oil **(Shoeb, 2006; Rashmi, *et al.*, 2012).** The pH of the medium was adjusted to 7.0 ± 0.2**.** The plates were then inverted and incubated at 35°C, for 48 h., Colony surrounded by an emulsified halo was considered being positive for biosurfactant production. The purified bacterial isolates were stored in nutrient agar slants and kept under refrigerated condition (4°C) for further experimentation.

Screening for biosurfactant producing bacteria

The purified bacterial isolates were tested for biosurfactant production by following methods.

1- Oil spreading technique

Fifty ml of distilled water were added to a large petridish, followed by spreading 1 ml of petroleum crude oil as layer on the water surface. Now add 20µl of the supernatant of the cultures isolated from the soil to the center. The biosurfactant producing organism can displace the oil and spread in the water **(Morikawa, *et al.*, 2000; Bodour and Miller, 2002).**

2- Blood hemolysis test

Bacterial strain was streaked on blood agar plate and incubated at 37°C for 48-72 h., observation was made for α, β, and γ hemolysis. Hemolytic activity was correlated with the production of biosurfactant **(Carrillo, *et al.*, 1996; Youssef *et al.*, 2004).** The observation was recorded as α = greenish halo (incomplete hemolysis, β = clear zone (complete hemolysis and γ = no hemolysis.

3- Drop Collapse Assay

The assay was carried out as described by **Jain *et al*. (1991).** A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after one minute. If the drop of supernatant collapsed and spread on the oil coated surface, it signifies the presence of biosurfactant (positive). But if the drop remains after one minute, it was documented as negative. This test was simultaneously carried out on distilled water as control.

4- Emulsification index (E_{24})

Emulsification activity was measured by vortexing 2 ml of culture supernatant with 3 ml of mineral oil for 2 min, obtained maximum emulsification after 24 h., emulsification index was calculated according to the following equation (**Cooper and Goldenberg 1987; Viramontes-Ramos *et al*., 2010).**

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

5- Blue agar plate method (BAPM)

Pure culture of each isolate was streaked in mineral salt agar medium containing carbon source (2% glucose), 0.2 g cetyl trimethyl ammonium bromide (CTAB) and 0.005 g methylene blue, after incubation period, a dark blue halo around the culture was formed indicating the anionic biosurfactant form an insoluble complex with this cationic bromide salt, and the complex is revealed using methylene blue present in the agar. The halo intensity is proportional to quantity of biosurfactant produced **(Siegmund and Wagner, 1991; Satpute *et al*., 2008).**

6- Surface tension

The measurement of surface tension was conducted using a du Nouy ring tensiometer for the most potent biosurfactant bacterial isolates according to **Ozdemir *et al*., (2004).**

Identification of bacterial isolates.

Identification of most potent bacterial isolates selected based mainly on morphological and biochemical test according to **Bergy's manual of systematic bacteria (2005a&b),** and VITEK2 System Version: 05.02 methods were used.

RESULTS:

Screening methods for biosurfactant producing bacteria:

During this study, sixty four (64) purified bacterial isolates obtained from polluted soil samples, classified as the following, thirty four (34) purified bacterial isolate were gram positive rod shape, ten (10) isolates gram positive cocci, eighteen (18) isolates gram negative rod shape and two (2) isolates gram negative cocci representative as 53.12%, 15.62%, 28.13% and 3.13% respectively as shown in table, 1 and represented graphically in Fig. 1.

Five different screening methods were used for determined the activity of these isolated in biosurfactant production. The result showed that, thirty six comprising gram positive and gram negative (Table 1) were positive for biosurfactant production in the hemolytic assay. Among 36 hemolytic isolates, twenty six (26) isolates showed α hemolytic by 40.62% from which, eleven (11) α-hemolytic bacterial isolates didn't show biosurfactant activity in the remaining screening methods by 17.19% and ten (10) isolates were β hemolytic by 15.62%. According to data recorded in table 1, twenty eight (28) bacterial isolates by 43.76% did not show any hemolytic activity (γ hemolytic) and did not show biosurfactant production in other three screening methods (oil spread method, and drop collapse method). Low emulsification activity was also observed for these twenty eight isolates ranging between (3.08 to 1.63%). Bacterial isolate 5.2 and 12.2 did not show biosurfactant production in screening methods hemolytic assay, blue agar plate and drop collapse but low show biosurfactant activity in oil spread methods and emulsification activity (table 1, Fig. 2 A, B, C, D and F).

Only six bacterial isolates showed positive biosurfactant activity with blue agar plate technique by 9.38% of total isolates .Another biosurfactant screening method was drop collapse in which only twenty one bacterial isolates showed positive results by 32.81%.

Table 1: Preliminary characterization, qualitative and quantitative screening assay for biosurfactant producing bacterial isolates.

Bacterial isolate no	Gram stain & shape	Oil spread (mm)	Hemolytic assay	Blue agar plate	Drop collapse	E24 (%)
1.1	G+ Rod	68	α	-	+	26.12
1.2	G- Rod	46	α	-	-	10.34
1.3	G+ Rod	0	γ	-	-	2.13
1.4	G+ Rod	43	β	-	+	9.32
1.5	G- Rod	0	γ	-	-	2.87
1.6	G- Rod	42	α	+	+	8.13
2.1	G- Rod	67	β	-	+	25.76
2.2	G+ Rod	0	γ	-	-	1.97
2.3	G+ Rod	82	α	-	+	37.23
2.4	G+ Cocci	0	α	-	-	2.43
3.1	G+ Rod	0	γ	-	-	2.75
3.2	G+ Rod	0	γ	-	-	1.76

Continue: Table 1.

3.3	**G- Rod**	**16**	**α**	**-**	**-**	**4.65**
3.4	**G+ Rod**	**0**	**α**	**-**	**-**	**2.35**
3.5	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.19**
3.6	**G+ Cocci**	**0**	**γ**	**-**	**-**	**3.05**
4.1	**G- Rod**	**83**	**β**	**+**	**+**	**39.13**
4.2	**G- Rod**	**94**	**β**	**+**	**+**	**41.32**
4.3	**G+ Rod**	**42**	**α**	**-**	**+**	**13.36**
4.4	**G+ Rod**	**31**	**α**	**-**	**+**	**9.13**
4.5	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.58**
4.6	**G+ Rod**	**37**	**α**	**-**	**+**	**9.65**
4.7	**G- Rod**	**0**	**γ**	**-**	**-**	**3.15**
5.1	**G+ Cocci**	**92**	**β**	**-**	**+**	**36.73**
5.2	**G+ Rod**	**27**	**γ**	**-**	**-**	**8.65**
5.3	**G+ Rod**	**0**	**α**	**-**	**-**	**2.43**
5.4	**G+ Cocci**	**0**	**γ**	**-**	**-**	**2.76**
6.1	**G+ Rod**	**61**	**α**	**-**	**+**	**29.25**
6.2	**G- Rod**	**52**	**β**	**+**	**+**	**22.32**
6.3	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.74**
6.4	**G+ Rod**	**0**	**α**	**-**	**-**	**1.87**
6.5	**G+ Cocci**	**0**	**γ**	**-**	**-**	**2.73**
6.6	**G+ Rod**	**0**	**γ**	**-**	**-**	**1.63**
7.1	**G- Rod**	**47**	**β**	**-**	**+**	**17.63**
7.2	**G+ Rod**	**0**	**α**	**-**	**-**	**2.61**
7.3	**G+ Rod**	**36**	**α**	**-**	**-**	**12.37**
7.4	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.32**
7.5	**G+ Rod**	**0**	**α**	**-**	**-**	**2.56**
7.6	**G- Rod**	**0**	**γ**	**-**	**-**	**2.9**
8.1	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.75**
8.2	**G+ Cocci**	**55**	**β**	**-**	**+**	**25.39**
8.3	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.51**
8.4	**G- Cocci**	**0**	**γ**	**-**	**-**	**2.38**
8.5	**G+ Rod**	**0**	**γ**	**-**	**-**	**3.08**
9.1	**G- Rod**	**21**	**α**	**-**	**-**	**8.75**
9.2	**G- Rod**	**50**	**β**	**+**	**+**	**21.76**
9.3	**G+ Cocci**	**0**	**γ**	**-**	**-**	**2.41**
9.4	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.67**
9.5	**G- Rod**	**0**	**γ**	**-**	**-**	**2.43**
10.1	**G+ Cocci**	**65**	**α**	**-**	**+**	**28.23**
10.2	**G- Rod**	**60**	**β**	**+**	**+**	**26.43**
10.3	**G- Rod**	**24**	**α**	**-**	**+**	**11.31**
10.4	**G+ Rod**	**0**	**α**	**-**	**-**	**1.87**
10.5	**G- Cocci**	**0**	**γ**	**-**	**-**	**2.31**
10.6	**G+ Rod**	**0**	**α**	**-**	**-**	**2.4**
11.1	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.24**

Continue: Table 1.

11.2	**G+ Cocci**	**0**	**α**	**-**	**-**	**2.54**
11.3	**G- Rod**	**21**	**α**	**-**	**+**	**7.68**
11.4	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.62**
12.1	**G+ Cocci**	**0**	**α**	**-**	**-**	**2.8**
12.2	**G- Rod**	**4**	**γ**	**-**	**-**	**5.36**
12.3	**G+ Rod**	**0**	**α**	**-**	**-**	**2.47**
12.4	**G+ Rod**	**14**	**α**	**-**	**+**	**6.65**
12.5	**G+ Rod**	**0**	**γ**	**-**	**-**	**2.58**

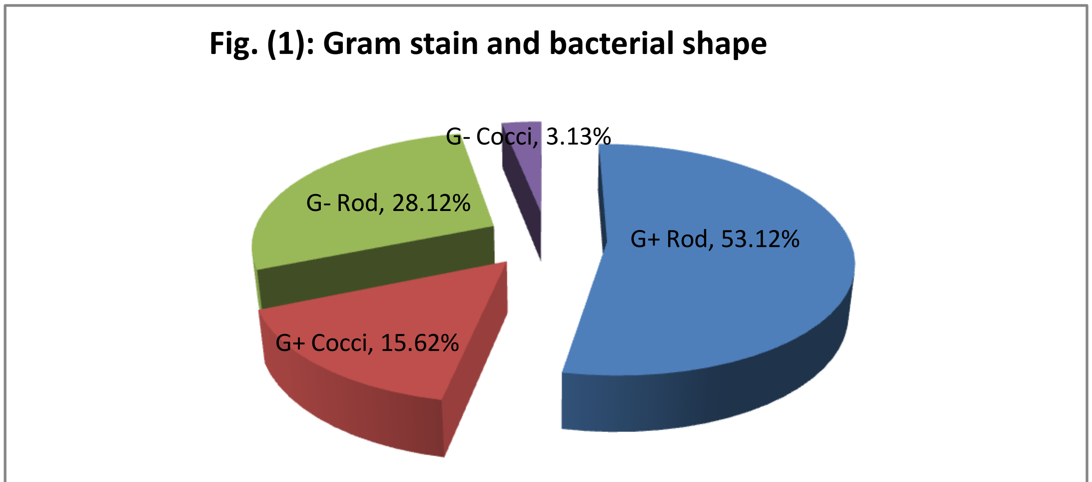
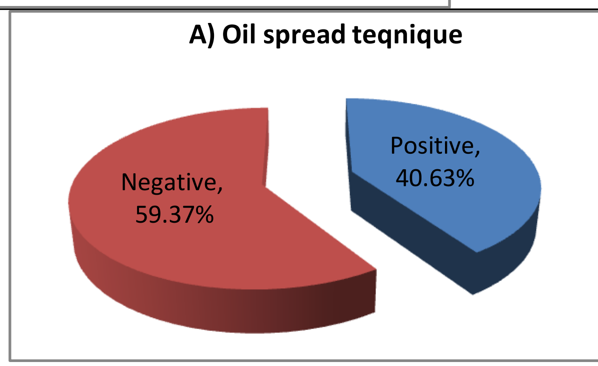
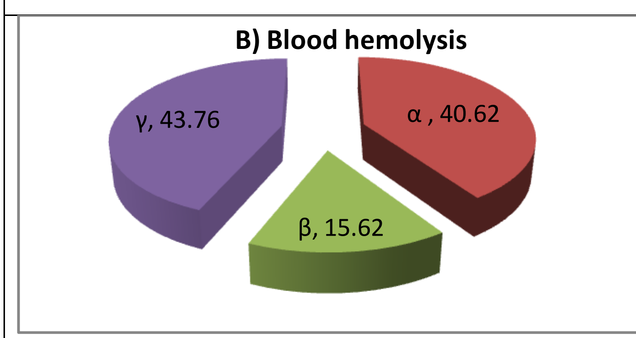
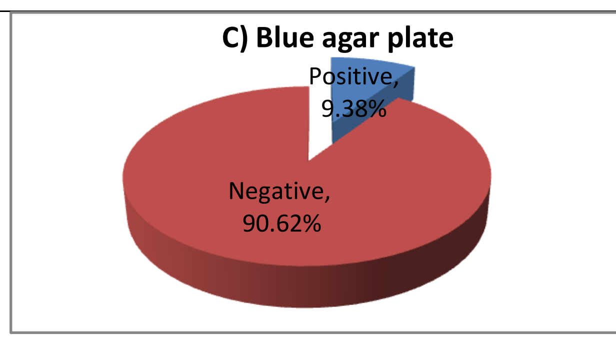


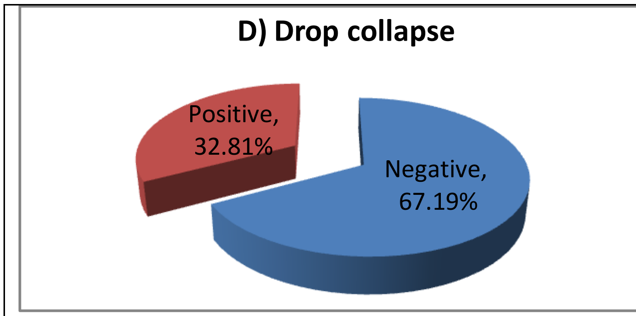
Fig 2: assessment of different screening methods by totally bacterial isolates, A) Oil spread , B) hemolytic activity, C) Blue agar plate, D) Drop collapse, E) E_{24} .

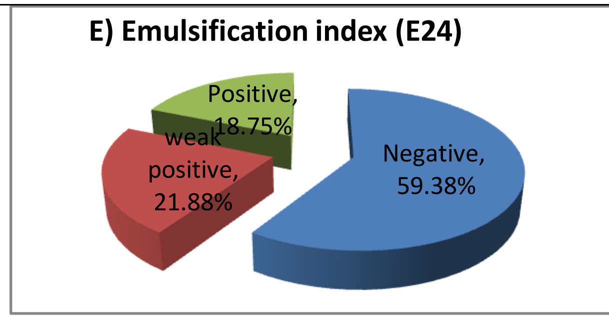


N.B: Negative less than 5 mm









N.B: Negative less than 5 %, weak positive (5-20%) & Positive More than 20 %

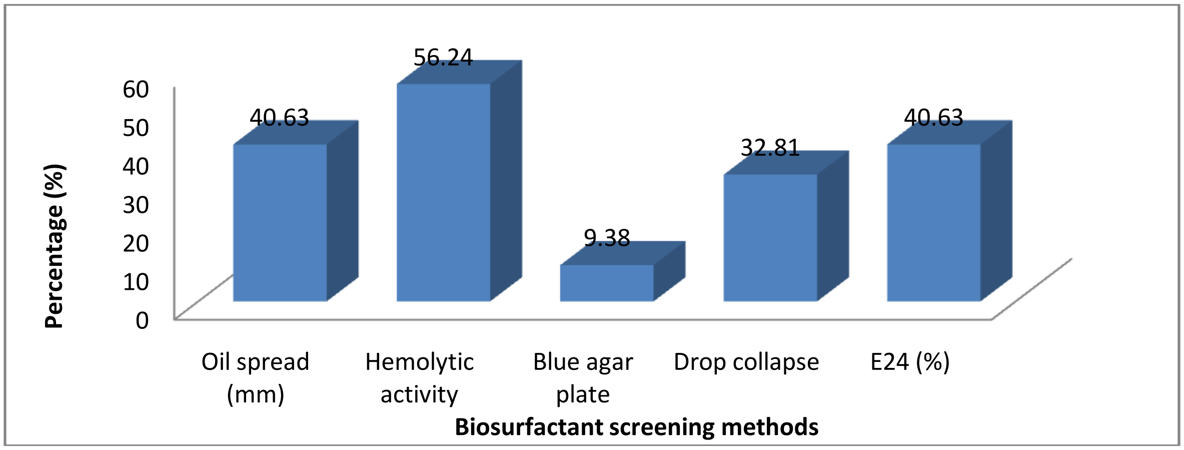
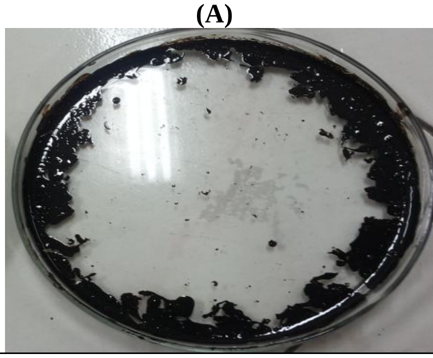
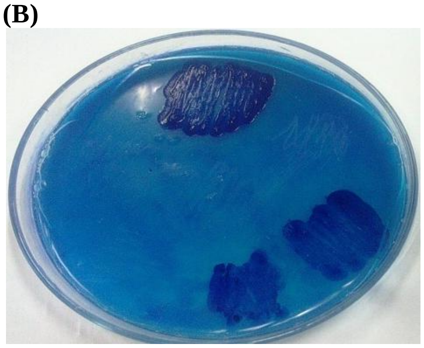


Fig. 3: Comparative of different biosurfactant screening methods among bacterial isolates.

(A)



(B)



(C)

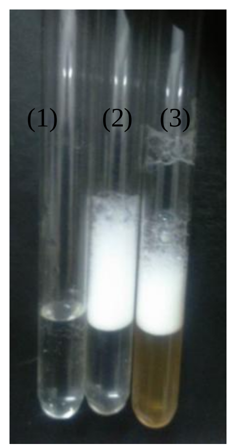


Fig.4: Different biosurfactant screening methods, A) Oil spreed technique, B) Blue agar plate, C) Emulsification index(E_{24}) in which, 1) dist. water as negative control, 2) SDS + mineral oil as positive control and 3) bacterial supernatent + mineral oil.

According to data reported in table 1, select ten most potent biosurfactant bacterial isolates which listed in table 2. Seven (7) most potent isolates show high biosurfactant activity in four screening methods (oil spread, hemolytic activity, drop collapse and emulsification activity), while other three most potent isolates show high biosurfactant activity in these screening methods besides blue agar plate. For these ten most potent bacterial isolates, surface tension was measured by tensiometer and showed high biosurfactant activity ranging between 36.4mN/m to 49.2mN/m when compared with positive and negative control which recorded 27mN/m and 72mN/m respectively.

Table 2: Most potent biosurfactant bacterial isolates.

	Isolate No.	Gram stain & cell shape	Oil spread (mm)	Hemolytic activity	Blue agar plate	Drop collapse	E_{24} (%)	Surface tension (mN/m)
1	1.1	G+ Rod	68	α	-	+	26.12	42.4
2	2.1	G- Rod	67	β	-	+	25.76	44.3
3	2.3	G+ Rod	82	α	-	+	37.23	38.6
4	4.1	G- Rod	83	β	+	+	39.13	36.4
5	4.2	G- Rod	94	β	+	+	41.32	37.3
6	5.1	G+ Cocci	92	β	-	+	36.73	38.6
7	6.1	G+ Rod	61	α	-	+	29.25	46.7
8	8.2	G+ Cocci	55	β	-	+	25.39	48.1
9	10.1	G+ Cocci	65	α	-	+	28.23	47.2
10	10.2	G- Rod	60	β	+	+	26.43	49.2
	Positive control (SDS 1 g L^{-1})		85	β	+	+	64.56	27
	Negative control (DW)		0	γ	-	-	1.49	72

Identification of the most potent biosurfactant isolates

Identification of ten most potent biosurfactant isolates was done by morphological, physiological and biochemical methods as showed in Table 3 and 4, besides VITEK 2 as showed in table 5. VITEK 2 method identified the most potent isolates as *Bacillus pumilus* 1.1 with probability 90%, *Acinetobacter baumanii* 2.1 with probability 93%, *Staphylococcus lentus* 5.1 and 8.2 with identification probability 88%. Isolate code 2.3 and 6.1 identified as *Bacillus cereus* with probability 98% and 99% respectively. Isolates code 4.1, 4.2 and 10.2 identified as *Pseudomonas aeruginosa* with probability 95%, 99% and 99% respectively, while isolate 10.1 identified as *Micrococcus luteus* with probability 99% as showed in Table 5.

Table 3: Morphological and biochemical characterization for the best biosurfactant producing bacterial isolates (Gram positive isolates) as compared to Bergy's (2005b).

Tests	*Bacillus pumilus*		*Bacillus cereus*			*Staphylococcus lentus*			*Micrococcus luteus*	
	Ref.	Isolate (1.1)	Ref.	Isolate (2.3)	Isolate (6.1)	Ref.	Isolate (5.1)	Isolate (8.2)	Ref.	Isolate (10.1)
Gram stain	G+	G+	G+	G+	G+	G+	G+	G+	G+	G+
Cell shape	Rod	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	cocci
Spore formation	+	+	+	+	+	+	-	-	-	-
Pigment	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
H2S production	+	-	+	+	+	-	-	-	-	-
Urease production	-	-	d	+	+	+	-	-		-
Voges Proskauer	+	+	+	+	+	-	-	-	+	-
Methyle red	-	-	-	-	-	+	-	-	-	+
Indol production	-	-	-	-	-		-	-		-
Citrate utilization	+	+	+	+	+		+	+		+
Nitrate reduction	-	-	d	+	+	-	+	+	+	-
Starch hydrolysis	-	+	+	+	+		+	+		-
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+
Lipid hydrolysis	+	+	+	+	+	+	+	+	+	+
Growth on MacConky	-	-	-	-	-	-	-	-	-	-

Table 4: Morphologichal and biochemical characterization for the best biosurfactant producing bacterial isolates (Gram negative isolates) as compared to Bergy's (2005a).

Tests	*Acinetobacter baumanii*		*Pseudomonas aeruginosa*			
	Ref.	**Isolate (2.1)**	**Ref.**	**Isolate (4.1)**	**Isolate (4.2)**	**Isolate (10.2)**
Gram stain	G-	G-	G-	G-	G-	G-
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Spore formation	-	-	-	-	-	-
Pigment	-	-	Green/brown	green	green	-
Motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
H2S production	-	-	-	-	-	-
Urease production	+	+	d	-	-	-
Voges Proskauer	-	-	+	-	-	-
Methyle red	+	+	-	-	-	+
Indol production	-	-	+	-	-	-
Citrate utilization	-	-	+	+	+	+
Nitrate reduction	-	-	+	-	-	-
Starch hydrolysis	-	-	-	-	-	-
Casein hydrolysis	-	-	+	+	+	+
Lipid hydrolysis	+	+	+	+	+	+
Growth on MacConky	+	+	+	+	+	+

Table 5: Final results for identification of bacterial isolates by VITEK2.

No.	Bacterial isolate code	Isolate name	Identification Probability by VITEK2
1	**1.1**	***Bacillus pumilus***	90 %
2	**2.1**	***Acinetobacter baumanii***	93 %
3	**2.3**	***Bacillus cereus***	98 %
4	**4.1**	***Pseudomonas aeruginosa***	95 %
5	**4.2**	***Pseudomonas aeruginosa***	99 %
6	**5.1**	***Staphylococcus lentus***	88 %
7	**6.1**	***Bacillus cereus***	99 %
8	**8.2**	***Staphylococcus lentus***	88 %
9	**10.1**	***Micrococcus luteus***	99 %
10	**10.2**	***Pseudomonas aeruginosa***	99 %

DISCUSSION:

Biosurfactant play a key role in emulsifying hydrocarbons, considered very suitable alternative to chemical surfactants due to their properties like eco-friendly, less/no toxicity, biodegradability, high specificity ,and synthesis from cheaper renewable substrates **(Mohan *et al*., 2006).** Therefore, search of biosurfactant producing microorganisms is an important area of research. It is important to note that most of the researchers have used maximum two or three screening methods for selection of biosurfactant producers (**Bodour and Miller, 2000**), we suggest a single method is not suitable to identify all type of biosurfactant, therefore application of more than one method required for effective screening.

In this study, six different screening methods were assessed for used to selecting biosurfactant producing bacteria, it was seen that it is not very useful to use hemolytic activity only to select biosurfactant producing bacteria **(Urum and Pekdemir, 2004).** The present data of α- hemolysis recorded 17.19% negative for biosurfactant activity which in well agreement with **Noha *et al.* (2004)** which reported 16% of false positive results in hemolytic activity for biosurfactant screening may be due to lyses blood agar by bacterial virulence factors. While it was regarded by some authors as indicative for biosurfactant production and used as a preliminary method for bacterial screening **(Bento *et al*., 2005; Nasr *et al*., 2009).**

The oil- spreading method is rapid and easy to carry out, requires no specialized equipment, and only requires a small volume of sample **(Plaza *et al*., 2006).** In this study, 40.63% of isolates were found to be positive by oil-spreading method, while **Shoeb *et al*., (2015)** reported that 44% exhibited positive results for oil-spreading test.

The CTAB agar plate method is a semi quantitative assay for the detection of extracellular glycolipids or other anionic surfactants only (**Pinzon and Ju, 2009; Erum *et al*., 2012).** This assay was developed by **Siegmund and Wagner (1991)**. in the present study revealed nearly 9.38% of isolates as positive, **Shoeb, *et al*. (2015)** recorded that 52.8% of isolates give positive results in CTAB.

The drop-collapse method is a sensitive and easy to perform method and has several advantages in requiring a small volume of samples, being rapid and easy to carry out, and not requiring specialized equipment. **Bodour *et al*. (2003)** reported that the drop collapse method may be used to detect biosurfactant producing microorganisms in natural environments. The present result recorded that 32.81 % from total bacterial isolates give positive results in drop collapse which less effective than oil spread and blood hemolysis methods.
The Biosurfactant production was detected by using emulsification index. Surface activity and emulsification activity have direct correlation **(Surachai *et al*., 2007).** Data of this method during this study was similar with oil spread methods being 40.63%, thus these two methods favor to be cheap, easily and reliable screening methods for biosurfactant producing bacteria

The high incident rate of *Bacillus* sp. and *Pseudomonas* sp. in oil contaminated waste when compared to other organisms isolated may be a direct correlation to their ability to produce emulsifiers to degrade oil rather than just a coincidence **(Femi-Ola *et al*., 2015).**
Bacillus species has proved to occur mainly in soil because of their spore forming ability; the spores have the ability to survive hard conditions in soil environment, also *Pseudomonas* sp. have the ability to produce biosurfactant in contaminated soil, similar observation were true with *Acinetobacter* sp. this results was matching with **Youssef *et al*., (2004).**

CONCLUSION:

Comparatively oil spread, drop collapse, emulsification index (E_{24}) and tensiometer methods considered highly special technique for detection of biosurfactant producing bacteria, while blue agar technique have high specificity to rhamnolipids biosurfactant only, in contrast hemolytic activity not favorable to use as the main criteria, but only as primary screening tool. Most of biosurfactant producing bacteria were belonged to genus *Bacillus* and *Pseudomonas*.

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