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ISOLATION, REFINEMENT AND SCREENING OF HEAVY METALS TOLERANT BIOSURFACTANT-PRODUCERS MICROORGANISMS FROM EGYPTIAN SOIL

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Abstract

Due to the presence of many microbes has a high ability to produce biosurface tension reducer from inexpensive renewable substrates. Recently it was reported that biosurfactants have many properties and applications. This study aimed to isolate biosurfactant producer's microbes with heavy metals tolerance using different screening methods from different districts in Egypt. Hundred and seventy-one isolates were isolated from 10 different soil samples in different regions in Egypt. Soil analysis was performed to check for soil nutrients, pH level, the percentage of organic matter, and the volumetric distribution of soil granules. The total microbial count has assessed, and the morphological characterization of microbial isolates has identified. The lipase activity, the Parafilm-M method, Oil spreading technique, and the Emulsification index (E24), were performed. Organic matter percentage of soil samples were ranged from 0.07% to 0.35%. The most soil types were respectively, silt clay soil and sandy soil while the pH level was found to be neutral to alkaline. Out of 171 isolates, 80 (46.78%) isolates were showed high ability to degrade the used oil completely, while 91 (53.22%) isolates have a low ability to degrade the oil. Among those 80 isolates, only 21 isolates were categorized as strong isolates according to their results in oil degradation, Parafilm-M-and oil spreading tests. Our study reveals the high ability of biosurfactant production among Egyptian isolates isolated from highly nutrient-rich soils from different regions in Egypt. High resistance to different heavy metals was also shown which make our isolates a promising source for different applications.

Key words: Biosurfactant; Heavy-metals; E24; Oil degradation.

Introduction

Soil microorganism diversity plays a crucial role in the ecology of agriculture system, wherever soil microorganisms have many functions in improving soil structure and soil health (Wall *et al.,* 2012). Those soil microbial diversity provided soil structure by increased ecosystem functions which involved nutrient cycling, organic decomposition and soil aeration (Trivedi *et al*., 2013). Many bacterial soils have produced a variety of secondary metabolites which may be soluble or volatile compounds (Tyc *et al,* .2016). Production of antimicrobial agents is important for plant protection, in addition to enzymes and biosurfactants production which have antimicrobial, antipredation and cytotoxic properties.

Biosurfactants are amphiphiles molecules contain a hydrophilic moiety and hydrophobic moiety, also known as surface active agents which consisting of molecules having a polar water-soluble attached to a water-insoluble hydrocarbon chain (Saimmai *et al.*, 2012). Bioemulsifiers are another term of biosurfactants which recently become a motivating subject for many researchers in several domains, the amphiphilic biomolecules naturally generated by a large vary of living organisms like bacterium, filamentous fungi, yeast, and algae.

Biosurfactants classification according to their sources and their chemical compositions, where the origins of bio-surface active agents are typically classified into three classes; microbial, animal, and plant surfactants (Xu *et al.*, 2011). There are several groups of biosurfactants such as glycolipid which include rhamnolipids, trehalolipids, or glycolipopeptide and other surfactants; lipopeptide, polymeric surfactants, fatty acids, particulate biosurfactants and surface active antibiotics

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(Sivapathasekaran and Sen 2017).

Chemical surfactants are mainly produced from petroleum derivatives and products which require many steps of production and purification that make to gain disadvantages and considered as harmful to ecosystem and humans. With increased environmental awareness the global market has recently moved to produce microbial surfactants to replace petroleum surfactants (Santos *et al*., 2013).

Therefore most industries have headed to microbial biosurfactants as alternatives of synthesized surfactants. Due to its multiple properties which were not limited to similarity with manufacturer surfactants, their potential to soluble hazardous chemicals, their stability under hard conditions; extreme temperatures, pH level, and salinity, as well as their green household nature and ability to be degraded biologically by other microorganisms.

Biosurfactants attracted many attentions to application in different fields including agricultural, petroleum, bioremediation, biodegradation, pharmaceutical and cosmetics industrial sectors (Vijayakumar and Saravanan 2015). In addition to their role in fertility soil and plants promoters which its used for biological control of plant pathogens and bio-pesticides as well as increasing the nutrient solubility to plant (Adnan *et al.*, 2018) Also, fossil oil recovery at which microorganism enhance oil recovery was improved by using biosurfactants, metal remediation, hydrocarbon/ organic compound degradation and the soil washing technology which are alternative applications of biosurfactant (Karlapudi *et al.*, 2018).

Biosurfactant producing bacteria are very diverse and have been isolated from a wide variety of environments, including contaminated soil or water. It is reported that the production of biosurfactants by bacteria may play a role in the adaptation of these organisms to different environments; therefore, this could be a mechanism by which the bacteria can absorb heavy metals and become resistant to growth in polluted soils (Tanpinar *et al.*, 2014).

Nowadays, start some specifically field become more attractive to use biosurfactants in pharmaceutical as follow the major is an antimicrobial activity, anti-viral, anticancer and now recommended in cosmetics as antioxidants and anti-wrinkles or in facial cream (Lukic *et al.*, 2016). Recently, commercial and industrial statistics indicate raised productivity of bioemulisifiers and biosurfactants to achieve 476, 512.2 tons because of increasing demand. The main disadvantages of biosurfactant are the high cost throughout the production and final processing, but this problem has been overcome by exploiting low-cost materials such as agricultural residues and residues from the food, dairy, meat and animal industries (Bertrand *et al.*, 2018).

As previously, mentioned about many properties and applications of bio-surfactants as well as the wide range of microorganisms which have high potential to produce biosurfactants from cheap renewable substrates. The study aimed to the isolation of potent biosurfactant-producing microorganisms with their ability to resist heavy metal which in future will be used in different applications.

Materials and Methods

Samples collection

Ten different of soil samples were collected from different sites of various Egyptian governorates, three of soil which cultivated and non-cultivated soils were assembled from Sharqia (Zagazig). Three soils were imported from the construction site and garden was collected from Cairo. In addition, one soil sample was collected from each sewage contaminated, cleaning agricultural drainage and non-planted as well as workshop site from Giza. Fig. 1.

Soil analysis

The soil analysis was conducted according to (Rayment and Higginson 1992) to analyze soil nutrients, major elements *e.g.* nitrogen, phosphorous, and potassium, secondary nutrients *i.e.* SO^{-}_{4}, Ca^{++}, Mg^{++} and Na^{+} and minor nutrients such as Mn, Zn, and Fe as well as pH level, the organic matter percentage and the volumetric distribution of soil granules.

Total microbial count

The total microbial count in soil was estimated by using the serial dilution method (John *et al.* , 2018). Under sterile conditions making suspension of 10 gm of soil in 90 ml of distilled water and then shaking well the flask to mix soil. Then prepared the serial dilutions (10^{-1}-10^{-7}) One ml from each dilution was transferred into sterile Petri dishes. Then poured sterilized nutrient agar (for bacterial count) and sabaroud agar (for fungal count) into each Petri dish and the incubation conditions were suitable for bacterial growth (at 30 $^{R"}$ C for 48-72 hrs) and also fungal growth (at 28 $^{R"}$ C for 5-7 days). The total viable count has calculated by the following equation: (Colony count × dilution factor = CFU/ml).

Isolation biosurfactant-producing microorganisms

The isolation procedure has been performed using enrichment liquid method. An aliquot of 1 g of each soil was transferred in Erlenmeyer flasks containing 100 ml

sterilized mineral/minimal salt liquid media (MSM) composition is, in g/L as follows: 1000 ml of distilled water, 1.5 g of $NaNO_3$, 0.5g of $MgSO_4$, 0.01g of $FeSO_4$, 0.02 g of $CaCl_2$, 1g of Yeast extract, 1g of glucose, 5 ml of buffer solution was prepared as follows (g/100ml): 10 g of KH_2pO_4 and 10 g of K_2HpO_4 and the pH (7±1).The cultural MSM was supplemented with 1% of Motor oil as a sole carbon and energy source which was added to the individual in each flask (Paul Beulah, 2018).

The flasks were incubated in a shaker incubator at 35ºC and 121 rpm for 7days, and then 1 ml was transferred to sterile fresh MSM, incubated at the same conditions for another 7 days. After five times of transfer, an aliquot of 1 ml of culture was transferred in a sterile Petri dish and then poured MSM agar supplemented with Motor oil. The plates were incubated at 35±2ºC for a week in an incubator. After that, the colonies surrounded with emulsion were chosen and transmitted to nutrient agar plates, additionally to the pure isolates were streaked on nutrient agar slants for preservations at 4ºC (Geetha *et al.,* 2013).

Morphological characters

All isolates were undergone traditional identification which was accomplished by describing the morphological characteristics of colonies and Gram staining.

Enrichment technique

All pure isolates were tested for degradation of hydrocarbon as the primary indicator for biosurfactant production. The experiment was done as followed: a single pure colony was inoculated into 100 ml sterilized MSM broth with 1% motor oil, as only sole carbon source in 250 ml flasks and then incubated at 35ºC for 7 days under static conditions. After the incubation period the flasks were observed which one contains emulsion (Paul Beulah 2018). The microbial cell was removed by centrifugation at 10000rpm for 10 mins. Cell free supernatant was stored at -20°C.

Screening of the strong biosurfactant producers:

The biosurfactant activity was determined by different techniques; Parafilm-M- test, Oil spreading technique, and Emulsification index (E24) (Shekhar *et al.,* 2018).

Parafilm-M-test

25µl of each supernatant cell-free were placed on Parafilm M strip as hydrocarbon surface. After air drying, drops diameter were measured. Tween 20% and distilled water were used as positive and negative controls (Youssef *et al.*, 2004).

Oil spread technique

50 µl of oil was added to a Petri dish (10 cm^2) containing 40 ml of distilled water, and then about 20 µl of the supernatant was gently placed on the center of the oil layer. The diameter of the clearing zone was measured to evaluate ODA (oil displacement area) by using the following equation: ODA 3.14 r^2 (Rodrigues *et al.,* 2006).

Emulsification index (E24)

Table 1: Soil analysis parameters.

Sample NO.	Organic matter	Volumetric distribution of soil granules (%)				Soil type	pH 1:2.5	EC ms/cm	Ions per ml/L			Cations ml/L				KPN ppm			Minor Nutrients ppm			
	(%)	Coarse sand	Fine sand	Celt	Clay				Hco_3^-	Cl^-	So_4^-	Ca^{++}	Mg^{++}	Na^{++}	K^+	K	P	N	Cu	Mn	Zn	Fe
1	0.33	20.0	27.5	27.2	25.3	Silt Clay	7.65	0.9	0.5	7.2	1.3	2.4	1.6	4.5	0.5	213.6	11.0	41.6	0.7	3.3	3.0	10.0
2		7.8	22.3	35.6	34.3	Silt Clay	7.55	0.4	0.3	2.7	1.0	1.5	0.7	1.5	0.3	255.0	10.7	43.5	0.9	3.7	3.2	11.5
3	0.15	40.3	45.1	10.6	4.0	Sandy	7.71	4.5	0.7	38.8	5.5	10.0	6.0	28.3	0.7	175.0	1.0	20.3	0.3	1.7	0.5	4.7
4	0.23	7.3	16.8	38.3	37.6	Silt Clay	7.74	3.1	0.7	28.3	2.0	7.6	3.3	20.0	1.1	210.7	9.2	38.7	0.4	3.0	2.4	8.2
5	0.35	5.0	20.2	37.5	42.3	Silt Clay	7.61	0.5	0.3	3.7	1.0	1.2	1.0	2.4	0.4	215.3	9.0	37.8	0.6	2.8	2.6	8.6
6	0.21	20.3	24.2	28.2	27.3	Silt Clay	8.0	33.5	1.3	326.5	7.5	30.1	13.0	290.3	1.6	217.2	9.3	36.5	0.5	2.7	2.9	8.9
7	0.11	39.1	40.3	15.6	5.0	Sandy	7.81	5.5	1.2	47.8	6.0	11.5	6.0	37.3	0.2	110.3	3.0	9.3	0.3	1.1	0.6	4.5
8	0.07	38.6	45.2	13.2	3.0	Sandy	7.69	1.8	1.0	14.8	3.2	5.8	3.5	9.5	0.2	111.0	2.4	7.0	0.3	1.1	0.7	4.8
9	0.13	27.3	0.9	23.5	21.3	Silt Clay	7.73	5.3	0.9	47.8	4.3	8.2	4.5	39.5	0.8	179.6	2.3	27.3	0.4	1.7		4.1
10	0.33	20	27.7	27	25.3	Silt Clay	7.73	1.3	0.7	8.1	4.2	3.3	2.2	6.6	0.9	229	10.3	87.6	0.3	1.7	0.9	10.6

An equivalent volume (3 ml) each of the supernatant and tested oil was mixed using a vortex at high speed for 2 minutes. The mixture was left to settle down for 24 hrs (Lai *et al.*, 2009). The emulsification activity has calculated the percentage of the emulsion layer of the volume:

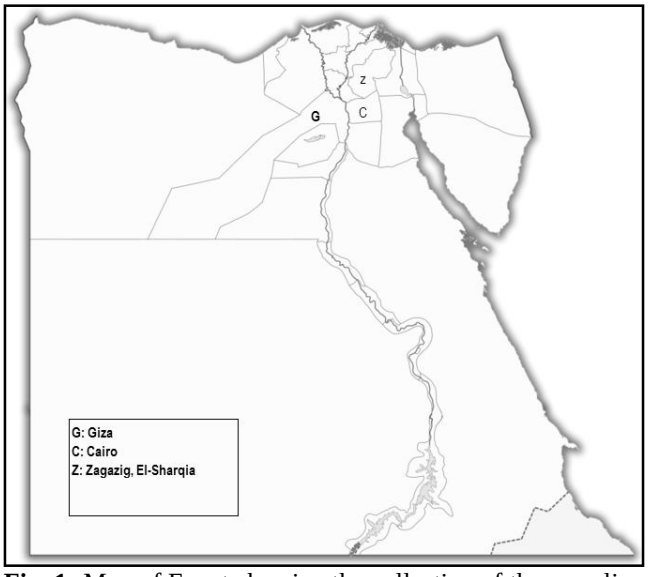

Fig. 1: Map of Egypt showing the collection of the sampling site.

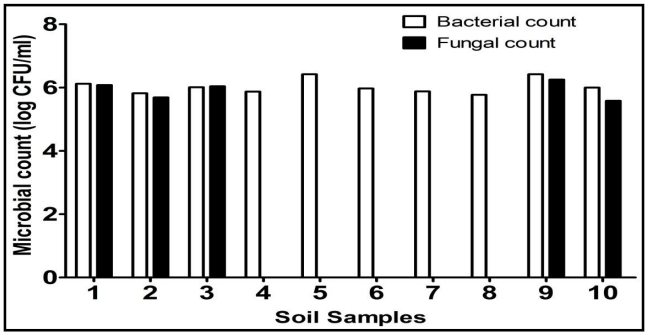

Fig. 2: Microbial count (bacterial and fungal) for different soil samples.

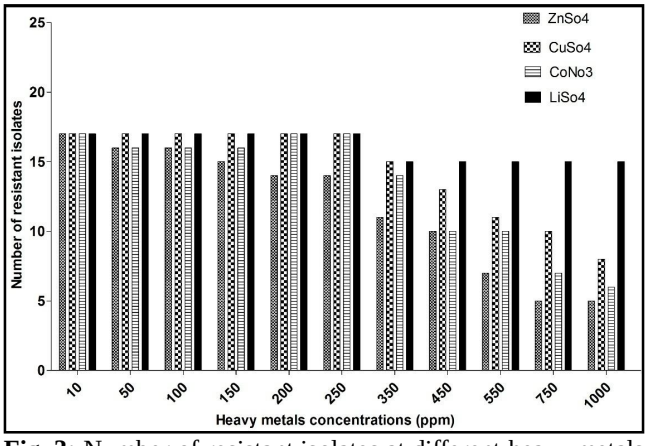

Fig. 3: Number of resistant isolates at different heavy metals concentrations.

$$E24\ (\%) = \frac{total\ height\ of\ emulsion\ layer}{total\ height\ of\ liquid} \times 100$$

Lipase activity

Different concentrations of Tween (Tween 20, 40, 60, and 80) were used to detecting the lipase activity according to (Kumar *et al.*, 2012). Tween medium composed of (g/l) : 1000 ml of distilled water, 1.50 g of yeast extract, 5 g of peptone, 1.50 g of beef extract, 5 g of $NaCl_2$, 10ml of (Tween 20, 40, 60 and 80), and 15 g of agar. The 21 isolates were streaked in the center and incubated for 24-72 hours at 35ºC. After incubation, the plates were flooded by a saturated solution of CuSo4. A clear zone around growth was considered a positive result.

Screening for Heavy Metal Tolerance

The twenty-one isolates were tested for its resistance or tolerance to four heavy metals ($ZnSO_4$, $CuSO_4$, $LiSO_4$, and $CoNO_3$) by agar plates (Rojas Pirela *et al.*, 2014). Different concentrations of heavy metal salt (10, 50, 100, 150, 200, 250, 350, 450, 550, 750 and 1000 ppm) were adding onto nutrient agar then autoclaved. The nutrient agar supplemented with heavy metals was poured into sterilized Petri dish. And then the selected isolates were streaked on heavy metal agar plates**.** Then plates were incubated at 35ºC for 72hrs. The growth was considered a positive result.

Antibiotic susceptibility test

The susceptibility of strains to different antibiotics was performed by disk diffusion method on Müller-Hinton agar according to (CLSI, 2017) recommendations using commercially available discs using: ciprofloxacin (5µg), cefotaxime (30µg), cefuroxime (30µg), meropenem (10µg), nalidixic acid (30µg), levofloxacin (5µg), gentamicin (10µg), imipenem (10µg), amikacin (30µg), tobramycin (10µg), cefepime (30µg), ampicillin (10µg) and Amoxicillin/Clavulanic (30µg).

Biochemical identification of microbial isolates

Selective 21 isolates were biochemically analyzed for the activities of catalase, oxidation, citrate utilization, urease test, and carbohydrate fermentation (Glucose, Galactose, Dextrose, Sucrose, and Maltose). The tests were used to identify the isolates according to (Jaysree *et al.*, 2011).

Results

Soil analysis

In our study, all soil samples were analyzed to check soil nutrients, soil type, organic matter, and pH level (Table 1). According to soil analysis, the percentage of the organic

matter of soil samples was ranged from 0.07% to 0.35%, the most distributed soil types were silt clay soil and sandy soil, while the pH level of most soil samples was found to be 7.55 to 8.

Total microbial count

Estimation of total microbial count in each soil was done according to the recommended serial dilution technique. The log number of the bacterial and fungal count was displayed in Fig. 2, at which the log number of the bacteria was ranged from 3.770 to 6.429. However, in some soil samples, the number of fungi was not detected.

Isolation of biosurfactant-producing microorganisms

In order to isolate the bacteria which degrades hydrocarbon and/or produce biosurfactant from different soils, mineral salt medium (MSM) was supplemented with motor oil which used as a sole carbon source, and as a result, a total of 171 pure isolates were obtained from all soil samples. The microbial count (bacterial and fungal) for the 10 soil samples was showed in Fig. 2.

Morphological characterizations of isolates

The traditional characterization was applied to the 171 isolates which include morphological characteristics such as elevation, margin, colony size, texture, color, appearance and optical. The colony size was defined as small, moderate, or large. Where the small size represented 61.40% of all isolates and 40% of the other isolates ranged in size from medium to large. Colony color was included orange, green, white, creamy and tan colors, whereas the colony texture was defined as either smooth or rough. In addition, 138 isolates were considered Gram-positive according to the Gram stain where 45.65% of them were defined as a spore former.

Screening of the strong biosurfactant producers

During oil degradation assay, out of 171 isolates, 80 (46.78%) isolates were showed high ability to degrade the used oil completely, while 91(53.22%) isolates have low ability to degrade the oil. Among those 80 isolates which were showed high ability in oil-degrading assay, only 21 isolates were categorized as strong isolates according to their results in oil degradation, Parafilm-M- and oil spreading tests (Table 2).

Table 2: Screening of biosurfactants from different soils by different methods.

Sample No.	Serial No.	Oil degradation	Parafilm M(cm)	Oil spreading	
				ODD(cm)	ODA(cm2)
S1	1*3	+	0.6	7.475	43.862
S1	2*2	+	0.7	6.1	29.209
S2	3*4	+	0.6	6.975	38.201
S2	4*4	+	0.55	6.75	35.766
S3	5*3	+	0.6	6.8	36.299
S3	6*6	+	0.55	5.575	24.389
S3	7*7	+	0.6	6.725	35.513
S3	8*5	+	0.5	6.35	31.653
S3	9*2	+	0.55	6.575	33.946
S4	10*2	+	0.6	4.425	15.377
S5	11*2	+	0.5	6.55	33.678
S5	12*2	+	0.65	5.35	22.468
S6	13*2	+	0.55	7.025	38.751
S9	14*1	+	0.6	6.475	32.921
S10	15*5	+	0.58	5.6	24.618
S10	16*1	+	0.6	6	28.26
S10	17*1	+	0.55	6.1	29.209
S10	18*1	+	0.65	6.25	30.664
S10	19*1	+	0.6	7	38.465
S10	20*1	+	0.55	7.075	39.304
S10	21*1	+	0.61	7.65	45.940
Distilled water	-	-	0.4	-	-
Tween20	-		0.6		

According to morphological characters and gram staining (*1= *Peudomonase* sp, *2= *Bacillus* sp, *3=*Enterobacteriaceae*, *4= Yeast,*5=Micrococcus, *6=*Lactobacillus*, *7=Actinomycetes.). * ODD (Oil Displacement Diameter), *ODA (Oil Displacement Area).

Different hydrocarbons (Motor oil, Kerosene, paraffin oil, Fried oil waste, and purified glucose) were used to detect the Emulsification index (Table 4).

Lipase activity was assessed by using four Tween concentrations (20, 40, 60 and 80) (Table 5). Out of 21 isolates, 16 isolates were showed positive lipase production ability while only 5 isolates were unable to hydrolyze Tween. Among the 16 isolates which were able to utilize Tween, 3 isolates had the ability to utilize all Tween types, 7 were able to utilize three Tween types, 5 isolates able to utilize two Tween types and only one isolate was able to utilize one type of Tween.

Resistance to different heavy metals was studied by different heavy metals concentrations (10 to 1000 ppm) Fig. 2, at which high resistance toward lithium was detected among the tested isolates.

Antimicrobial susceptibility

Antimicrobial susceptibility of the selected isolates toward the commercially used antibiotics showed in (Table 6). The antibiotic zones were measured and interpreted according to CLSI

Table 3: Emulsion layer of selected isolates in mm (millimeters).

Sample No.	Serial No.	Emulsion layer per mm														
		Motor Oil					Paraffin Oil					Frying Oil(waste)				
		M	D	P	F	G	M	D	P	F	G	M	D	P	F	G
S1	1	2.5	3	2.8	2.2	2.8	1.8	2	2.1	1.7	1.4	2.2	2.3	3.2	2.8	3.2
S2	2	3	3	3.3	2.9	3.1	2.1	2	1.7	2	0.9	1.2	2.8	3.7	3	2.2
S2	3	2.4	3	3.5	2.8	3.6	2	2	3.4	2	2	2.3	3.8	3	2.5	2.5
S3	4	3	2.7	3.5	3	2.8	2.2	2.3	2	2	1.6	2.3	2.3	2.5	2	2..3
S3	5	2.6	1.9	3	3	3.6	1.8	3	2	3	2	1.7	2.5	2.6	4	3.9
S3	6	2.6	3	3.5	2.9	3.5	1	3.5	3	2.2	1.9	2.2	3.5	3.4	4	2.2
S3	7	2.9	2.7	2.5	3.5	3.7	2	2.1	2.4	2.1	2	2.8	3.3	3.7	3.1	3.8
S3	8	2.9	3	3.6	2.4	3.5	3.6	1.6	3.5	2.7	1.7	2.4	2.2	2	2.8	3.9
S4	9	2.7	3.5	2.9	3.2	3.3	1.7	2	2	2.2	2	2.3	3.2	3.4	3.2	3.2
S5	10	2.6	2.4	3	2.7	2.4	1.8	2.1	1.8	2.1	1.5	2.2	2.3	2	2.7	2
S5	11	2.5	3.5	3.5	3.4	3.7	1.7	1.9	2.1	1.9	2	1.5	3.8	1.8	2.5	3.8
S6	12	2.5	1.2	1.2	0.8	1	1.7	1.9	2.3	2.2	2	3.8	2.2	3.9	4	3.8
S9	13	2.5	3	3.5	3.2	3.8	1.7	1.8	1.8	2.2	2.2	2	3.8	3.7	1.9	2.4
S10	14	2.1	2.9	3.2	3.9	3.6	1.7	1.9	1.9	1.9	2.1	2	3.2	1.9	3.8	3.8
S10	15	2.7	3.2	3	3.5	1.2	2	2	1.8	1.9	2.1	2.3	2.2	2	2	3.8
S10	16	3	2.5	3.6	3.4	3.7	1.8	2.2	2.1	2	1.8	1.9	1.9	2	1.7	2.1
S10	17	3.5	3.5	2.7	3.4	3.8	1.9	2	1.9	1.8	1.9	1.8	1.9	1.9	1.8	2
S10	18	3.4	3	3.4	2.7	2.6	2	2.2	2.1	2.2	2	1.8	1.9	1.7	1.7	3.8
S10	19	2.6	2.4	4	3	3.3	1.9	2	2	1.8	2	3.4	2.3	2.4	1.8	2.7
S10	20	2.5	3.4	3	3	3.7	1.8	1.9	1.7	2	2.2	1.9	2.8	2.2	2.1	2.3
S10	21	2.3	2.4	2	2.4	1.9	3	1.2	1.2	2.1	0.4	3	1.9	2	2.2	2.2

D* = Diesel, P* = Paraffin oil, M = Motor oil, F = Frying oil (waste), G = Glucose.

interpretations, and the results presented as resistant, intermediate and sensitive. High resistance rate was shown to nalidixic acid, cefuroxime, and ampicillin. The susceptibility of antibiotics showed 7 isolates were resistant against nalidixic acid, cefuroxime, and ampicillin while, high sensitivity rate was representing 17 isolates against imipenem, 15 isolates against meropenem, and finally 13 isolates against levofloxacin.

Discussion

Biosurfactants are surface-active, biodegradable non-toxic compounds produced by a wide range of microorganisms which show strong emulsification of hydrophobic compounds, therefore may play an important role in agriculture, petroleum, bioremediation, biodegradation, pharmaceutical and beauty care/or cosmetics industries (Banat *et al.,* 2010 ; Nayarisseri *et al*., 2018). In our study, the isolates producing biosurfactants were isolated from ten different soil samples from different regions in our country Egypt. Soil analysis included total organic matter, acidity level, major and minor nutrients among the ten soil samples were differently distributed Table 1, and according to this the microbial count diversity of bacteria and fungi were differently distributed Fig. 1 where there aren't any fungal count observed in soil samples 4, 5, 6, 7 and 8.

Fungi tend to be more sensitive to salt stress than bacteria (Gros *et al.,* 2003; Pankhurst *et al.,* 2001; Sardinha *et al.,* 2003; Wichern *et al.,* 2006), thus the bacteria/fungi ratio can be increased in saline soils. Differences in tolerance of salinity between microbe's lead to changes in community structure compared to non-saline soils (Gros *et al.,* 2003; Pankhurst *et al.,* 2001).

Microbial Soil communities are directly associated with soil biogeochemical processes, however, the community structure is considered to be a key determinant of the functions: Direct change in microorganism community structure may alter microbial functions and soil nitrogen element and carbon dynamics (Yan *et al.,* 2017). Faoro *et al.,* (2010) Indicates that the decrease in microbial biodiversity of soil samples is associated with a complex interaction of several factors, while the increase in biodiversity is mainly associated with the rise and, to a lesser extent, Ca^{2+} / Mg^{2+}. Saline soils generally include salts e.g. chloride and sulfates of Na, Ca, Mg and K. And the pH of saline soils is generally below 8.5. While, the normal desired range is 6.0–7.0 (Sharma *et al.,* 2016).

In soils, the pore water contains a variety of dissolved ions such as Na^{+}, Ca^{2+}, NH^{+}_{4}, Cl^{-}or SO^{2+}_{4}. As soil water

Table 4: Emulsification index of selected isolates after 24 hours.

Sample No.	Serial No.	Emulsion index (%) after 24 hours														
		Motor Oil					Paraffin Oil					Frying Oil(waste)				
		M	D	P	F	G	M	D	P	F	G	M	D	P	F	G
S1	1	62.5	75	70	55	70	45	50	52.5	42.5	35	55	57.5	80	70	80
S2	2	75	75	82.5	72.5	77.5	52.5	50	42.5	50	22.5	30	70	92.5	75	55
S2	3	60	75	87.5	70	90	50	50	85	50	50	57.5	95	75	62.5	62.5
S3	4	75	67.5	87.5	75	70	55	57.5	50	50	40	57.5	57.5	62.5	50	57.5
S3	5	65	47.5	75	75	90	45	75	50	75	50	42.5	62.5	65	100	97.5
S3	6	65	75	87.5	72.5	87.5	25	87.5	75	55	47.5	55	87.5	85	100	55
S3	7	72.5	67.5	62.5	87.5	92.5	50	52.5	60	52.5	50	70	82.5	92.5	77.5	95
S3	8	72.5	75	90	60	87.5	90	40	87.5	67.5	42.5	60	55	50	70	97.5
S4	9	67.5	87.5	72.5	80	82.5	42.5	50	50	55	50	57.5	80	85	80	80
S5	10	65	60	75	67.5	60	45	52.5	45	52.5	37.5	55	57.5	50	67.5	50
S5	11	62.5	87.5	87.5	85	92.5	42.5	47.5	52.5	47.5	50	37.5	95	45	62.5	95
S6	12	62.5	30	30	20	25	42.5	47.5	57.5	55	50	95	55	97.5	100	95
S9	13	62.5	75	87.5	80	95	45.5	45	45	55	55	50	95	92.5	47.5	60
S10	14	52.5	72.5	80	97.5	90	42.5	47.5	47.5	47.5	52.5	50	80	47.5	95	95
S10	15	67.5	80	75	87.5	30	50	50	45	47.5	52.5	57.5	55	50	50	95
S10	16	75	62.5	90	85.5	92.5	45	55	52.5	50	45	47.5	47.5	50	42.5	52.5
S10	17	87.5	87.5	67.5	85	95	47.5	50	47.5	45	47.5	45	47.5	47.5	45	50
S10	18	85	75	85	67.5	65	50	50	52.5	55	50	45	47.5	42.5	42.5	95
S10	19	65	60	100	75	82.5	47.5	50	50	45	50	85	57.5	60	45	67.5
S10	20	62.5	85	75	75	92.5	45	47.5	42.5	50	55	47.5	70	55	52.5	57.5
S10	21	57.5	60	50	60	47.5	75	30	30	50	10	75	47.5	50	55	55

D*=Diesel, P*= Paraffin oil, M= Motor oil, F= Frying oil (waste), G= Glucose

content decreases, dissolved ions become more intensified (Rath *et al.,* 2015).

There are a number of soil characteristics that are often directly or indirectly related to soil pH, and these factors may drive the observed changes in community composition as the hydrogen ion concentration varies by many orders of size across the soils in this study (Lauber *et al.,* 2009). It has additionally been according those changes in soil environment like soil moisture, pH and temperature referred to indirectly by plant characteristics can have an effect on the soil microorganism diversity and composition (Nagendran *et al.,* 2014).

Soil pH may act as an environmental filter for instance by stressing bacterial cells which results in the selection of specific bacterial groups (Atlas and Bartha 2005; Fierer and Jackson 2005) stated that many bacteria and fungi have pH optima near neutral. Another factor is the water availability which affects the osmotic status of bacterial cells and can indirectly regulate substrate availability, diffusion of gases, soil pH, and temperature. Also, periods of moisture limitation typical of boreal environments may affect bacterial communities through starvation, induced osmotic stress, and resource competition, selecting for individual bacterial groups that are tolerant to moisture-limited conditions (Treves *et al.,* 2003; Dimitriu and Grayston 2010).

Oil degradation assay was applied to all the 171 pure isolates and as a result, 21 isolates were classified as strong biosurfactant producers. Nievas *et al.,* 2008 reported that by introducing biosurfactant producing bacteria to a contaminated culture system, enhanced biodegradation can be achieved through mobilization, solubilization, or emulsification of hydrocarbons. While according to Franzetti *et al.,* (2008) those microbes which can take hydrocarbon by direct uptake mode do show high surface hydrophobicity. But recently in 2016, Sumathi and Yoganan than found that biosurfactant produced by *Pseudomonas aeruginosa* presented 50% hydrophobicity.

To select the most microorganisms which were able to produce biosurfactants among those 21 isolates, different recommended laboratory tests were performed including Parafilm-M-test, Oil spread technique, Emulsification index (E24) and Lipase activity test (Hassanshahian 2014; Shekhar *et al.,* 2018). Recently, different reports for the best and accurate biosurfactant screening methods were founded. In 2004, Youssef *et al.,* reported that the oil spreading test is a better predictor

Table 5: Lipase activity of selected isolates at different concentrations of Tween.

Sample No.	Serial No.	Tween concentration			
		Tween 20	Tween 40	Tween 60	Tween 80
S1	1	-	-	-	-
S2	2	-	-	-	-
S2	3	-	+	+	+
S3	4	-	+	+	+
S3	5	-	-	-	-
S3	6	-	+	+	-
S3	7	-	+	+	+
S3	8	-	+	+	+
S4	9	-	-	-	-
S5	10	-	-	+	-
S5	11	-	+	+	+
S6	12	-	-	-	-
S9	13	-	+	+	-
S10	14	-	+	+	-
S10	15	-	+	+	-
S10	16	+	+	+	+
S10	17	+	+	+	+
S10	18	+	+	+	+
S10	19	-	+	+	+
S10	20	+	-	+	+
S10	21	+	-	+	+

of biosurfactant production than the drop collapses method because it is very sensitive, requires a small sample volume. While in a different opinion of Techaoei *et al.,* (2007) stated that the bacterial strain was selected for emulsification index and Parafilm M tests suggesting that these methods are better-predicted biosurfactant production than the drop collapse method because they are very sensitive for detection and have several advantages in requiring a small volume of samples, and also they are rapid and easy to be carried out, and do not require specialized equipment.

According to Sari *et al.,* 2014 and Korayem *et al.,* 2015, Parafilm-M test and surface tension determination are both physical methods widely applied for identification of biosurfactant-producing microorganisms. On the other hand, Walter *et al.,* (2010) reported that the emulsification index is a reliable method used for detection of bioemulsifier producers. Femi-Ola *et al.,* (2015) reported that the emulsification activity by using Kerosene was showed the % emulsification of was 51.61 % and 53.13% for *Bacillus* spp and *Pseudomonas* spp.

Among our isolates, the largest ODD/ODA results of oil spreading based on ODD (Oil Displacement Diameter) and ODA (oil displacement Area) were 7.65 (cm)/45.940 (cm^2), which considered higher than that of 6.5 cm/33.18 cm^2 reported by Yalçin *et al.,* (2018).

Table 6: Antibiotic resistance pattern of selected isolates.

Sample No.	Serial No.	Antibiotics												
		NA	CIP	LEV	IPM	MEM	AK	CN	TOB	CXM	CTX	FEP	P	AMC
S1	1	I	S	S	S	S	S	S	R	R	S	I	R	S
S1	2	1.4	2.2	2.4	S	3.7	2.1	2.2	2.3	S	2	1.7	2	2
S3	5	I	S	S	S	S	R	S	R	R	R	R	R	R
S3	6	R	3.2	3.2	R	S	S	4	3	2	4.4	4	3.2	3.2
S3	7	R	3.6	S	S	S	3.4	R	R	R	R	R	R	R
S3	8	R	3.1	S	S	S	S	3.4	3	1.3	S	3.4	2.8	3
S3	9	R	1.2	1.3	4	2.5	2.1	2.6	1.5	1.6	4.2	4	2.1	2
S4	10	1.7	3	3	S	3.4	2.4	2.3	2.3	R	2	1.9	1.8	1.6
S5	11	R	2.7	S	S	S	2.5	2.5	2.3	0.9	3	2.1	2	2.5
S5	12	R	3	2.5	S	S	1.5	2.4	1	2.5	4.6	4	2.7	2.5
S6	13	R	2	2	3	2.4	2.6	2.5	1.2	1.7	2.5	2	2.8	2.5
S9	14	R	S	S	S	S	I	R	S	R	1.6	S	R	R
S10	15	R	S	S	S	S	S	S	S	R	S	S	3	3
S10	16	R	S	S	S	S	R	R	R	R	1.4	S	R	R
S10	17	R	S	S	S	S	I	S	S	R	1.9	S	R	R
S10	18	R	S	S	S	S	I	S	S	R		S	R	R
S10	19	1.3	S	S	S	S	S	S	S	R	2.7	S	R	0.7
S10	20	1.2	S	S	S	S	S	S	S	R	2.6	S	R	0.7
S10	21	1.6	S	S	S	S	S	S	S	R	2.4	S	R	1.2

Ciprofloxacin (CIP 5), Cefotaxime (CTX 30), Cefuroxime (CXM 30), Meropenem (MEM 10), Nalidixic acid (NA30), Levofloxacin (LEV 5), Gentamicin (CN 10), Imipenem (IPM 10), Amikacin (AK 30), Tobramycin (TOB 10), Cefepime (FEP30), Ampicillin (P10), Amoxicillin/Clavulanic (AMC 30). R: resistant, I: intermediate, S: sensitive.

Different Tween utilization ability was obtained by using Tween 20, 40, 60 and 80 (Table 3). Kumar *et al.,* (2012) explain this result by that Tween 80 contains esters of oleic acid which can be degraded by lipase while Tween 20 contains esters of lower chain fatty acids.

In our study, high tolerance was showed toward the four used heavy metals at different concentrations Fig. 2. Some levels of resistance and the tolerance that was found among the isolates were probably due to the metal contamination in the soil. Heavy metals and other toxicants have been suggested to play an important role in promoting antibiotic resistance (Benmalek and Fardeau 2016). Bacteria have adapted to the existence of heavy metal ions in their environmental (Laneva 2009). Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological, and/ or genetic adaptation including morphological, changes of cells, as well as environmental modifications of metal speciation. Generally, long term exposure of heavy metals to microorganisms enforces a selection pressure which facilitates the proliferation of microbes, tolerant/resistant to metal stress. There are five main mechanisms of heavy metal tolerance/resistance extracellular barrier, active transport of metal ions (efflux), extracellular sequestration, intracellular sequestration, and reduction of metal ions. This adaptive mechanism of metal resistance has been explored by assaying habitats exposed to anthropogenic or natural metal contamination over an extended period of time (Maitra 2016; Laneva 2009).

The high levels of resistance and wide broad tolerance which was found between isolates are probably attributed to the high metal contents of soils (Abou-Shanab *et al.,* 2007). Bacterial extracellular polymers, such as polysaccharides, proteins, and soil substances protect the bacterial cell from any unfavorable conditions which further more increase its resistance to awide range of antibacterial agents (Mohamed *et al.,* 2019; Mohamed *et al.,* 2018). These substances thus detoxify metals by complex formation of an effective barrier surrounding the cell (Rajkumar *et al.,* 2010).

To survive beneath metal-stressed conditions, microorganisms have evolved many kinds of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation, and complication of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Issazadeh *et al.,* 2013).

It was suggested that the tolerance Cu may be due to the ability of the isolates to accumulate copper ions in its cell wall thus preventing its entry into the cell. However, at higher concentrations, there is oxidation of lipid membranes, damage to nucleic acids and generation of free radicals from hydrogen peroxide (Onuoha *et al.,* 2016). But about nickel and cobalt, it was reported that resistance is linked to the outer membrane efflux system and cytoplasmic membrane efflux systems(Mikolay and Nies 2009; Spain and Alm 2003). Also, lithium ions (Li+), known as a toxic alkali metal cation, is at least partly detoxified by Li+ efflux via a proton antiporter in *E. coli* (Inaba *et al.,* 1994).

In this study, the susceptibility of antibiotics showed isolates were resistant against nalidixic acid, cefuroxime, and ampicillin while, some of isolates were showed sensitivity against some of antibiotics such as levofloxacin, impenem and meropenem (Andy and Okpo 2018) demonstrated that 9 isolates of *Pseudomonas aeruginosa*, 13 isolates of *E. coli*, and 12 isolates *Klebsiella* were resistant to cefuroxime.

Conclusion

Our study reveals the high ability of biosurfactant production among Egyptian isolates isolated from highly nutrient-rich soils from different regions in Egypt. High resistance to different heavy metals was also shown which make our isolates a promising source for different applications.

Abbreviations

E24: Emulsification Index; MSM: Mineral/Minimal salt media; ODA: Oil displacement area; ODD: Oil displacement diameter; CIP: Ciprofloxacin; CTX: Cefotaxime; CXM: cefuroxime; MEM: Meropenem; NA: Nalidixic acid; LEV: Levofloxacin; CN: Gentamicin; IPM: imipenem; AK: Amikacin; TOB: Tobramycin; FEP: Cefepime; P: Ampicillin; AMC: Amoxicillin/Clavulanic; ZnSo4: Zinc sulfate; CuSo4: Cupric sulfate; CoNo3: Cobalt Nitrate; LiSo4: Lithium sulfate.

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