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Screening and Identification of Efficient Biosurfactant Producing Bacteria for some Medical Applications

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Abstract

Biosurfactant is a shortcut term for surface-active agent produced by microorganisms and has ability to reduce surface tension between immiscible matters. This study was aimed to isolate, screen and identify biosurfactant producing bacteria from oil-polluted sites from south of Jeddah, Saudi Arabia. Out of the total 42 bacterial isolates, 4 biosurfactant producing bacterial strains were detected through different screening tests. The isolates were identified by 16S rRNA gene sequencing. These active isolates named EMB6, EMB 18, EMB 19, EMB 24. They were displayed the surface tension ≥ 40 mN/m, scored 30% in BATHA assay, they were showed positive activity with drop collapse test, also with oil displacement test (> 2 cm), and produce dark blush halo on CTAB assay. These isolates were identified using 16 S rRNA. The isolate EMB 6 was closely related to *Klebsiella quasivariicola* (98.24%); isolates EMB 18, EMB 19 and EMB 24 were closely belong to *Pseudomonas aeruginosa* (99.41%); (96.19%) and (97.51%) respectively. Results in this study proved that these isolates had an outstanding potential for production of biosurfactant which can be used in food and medical applications.

Keywords: Biosurfactant; CTAB; *Klebsiella; Pseudomonas*; 16 S rRNA; Phylogenetic Tree

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Introduction

Surfactant is a shortcut term for surface-active agent and it is known as a chemical molecule can modify the interface between various phases of matter (liquid-gas, liquid- liquid, and liquid-solid). Moreover, it has ability to reduce surface tension between immiscible matters. There by, surfactant is widely utilized in the manufacture of many commercial products such as laundry detergents, wetting agents, emulsifiers, foaming agents and dispersants. Consumption of surfactants is probably greater than 13 million tons per year worldwide [1]. Nearly 2.7 million tons of surfactants produced are petroleum derived [2].

Surfactant from petrochemical origin has non-biodegradable and toxicity nature. As a result, accumulation of such products in the environment enacts conflicting effects on natural environment resources. Synthesized surfactants containing wastewater are discharged into the environment, resulting in harming aquatic life, polluting the water and decreasing primary productivity of water bodies [3]. In another hand, surfactant from biological origin has ecological compatibility nature. Surfactant from biological source such as microorganisms is called biosurfactant. Biosurfactant may produce by microorganisms as a secondary metabolite in their environment. It has unique properties such as resilience to pH, temperature and high salt concentration, biodegradability, low poisonous quality, emulsifying and demulsifying capacity and antimicrobial action [4].So, they are suitable agents for different bioremediation technologies [5] and for different commercial applications. As mentioned in a review by Santos *et al.* [6] the production of biosurfactant from microbes can be from renewable substrates such as: vegetable oils, whey, molasses, starchy substrates and animal fat. The production of biosurfactant dependson various factors such as carbon source, nitrogen source, carbon to nitrogen (C:N) ratio, pH, temperature, agitation, and oxygen availability [7].

Biosurfactants are produced by a diverse group of microorganisms mainly bacteria, fungi, and yeasts. These microorganisms were isolated from soils or water samples which are contaminated with hydrophobic organic compounds such as oil. Mainly, bacteria play important role in biosurfactant production. Different bacterial species were isolated and involved in biosurfactant production: *Pseudomonas aeruginosa* and *Serratia rubidea* [8]; *Bacillus amyloliquefaciens* [9]*; Comamonas aquaticawas* [10]; *Agrobacterium rubi* [11]. Studies involving biosurfactants began in the 1960s and the use of these compounds has expanded in recent decades [12].

The main objective of the present study is to isolate, purify, screen, and identify efficient bio surfactant-producing bacteria from oil contaminated samples isolated from southern seashore in Jeddah city.

Materials and Methods

Isolation of Biosurfactant Producing Bacteria

Twenty different oil contaminated samples (water & soil) were collected from southern region of Jeddah city seashores. All samples were placid in sterile bottles and transported on ice container to the laboratory and stored at 4℃ until they were analyzed. For soil samples, ten grams of each samples were transferred to 250 ml Erlenmeyer Flask containing 100 ml physiological saline (1% NaCl), then they were agitated 2 hrs. at 25℃ and let stand for one hours. Five milliliters of the soil suspension transferred into 45 ml of Mineral Salt medium containing 1% model hydrocarbon compounds (diesel oil) as the sole of carbon and energy source in 250 ml Erlenmeyer flask [13]. The component of modified mineral salt medium was as follows (g/l): 20 of NaCl, 2.0 of KH_2PO_4, 1.0 of NH_4NO_3, 3.0 of Na_2HPO_4, 0.7 of $MgSO_4.7H_2O$. One ml/l of trace element solution was added to the mineral salt medium. The trace element solution was prepared as follows (mg/L): $ZnSO_4.7H_2O$, 10; $CuSO_4.5H_2O$, 0.50; $MnSO_4.H_2O$, 0.50; $CaCl_2$, 20; $FeCl_3$, 30 and the solution was adjusted to pH 7.0 [14]. For liquid samples, 5 ml/45 ml were transferred directly to the same mineral salt medium that mentioned above. After that all samples were incubated in a rotary shaker at 37℃ and 120 rpm for 7 days, and then transferred to fresh medium, incubated at the same conditions for another 7 days. After four subcultures, samples were serially diluted using sterile saline solution (0.85% NaCl) and spread on Nutrient Agar plates [15]. After incubated for 24 hrs. at 37℃, Different bacterial isolates were selected, purified several times and preserved in Nutrient Agar slant at 4℃.

Screening of Isolation Bacteria for Biosurfactant Production

To screen the isolated bacteria for biosurfactant production, a loop full of each purified isolate was inoculated into 25 ml Nutrient Broth as a seed media in a 100 ml Erlenmeyer flask, incubated for 24 h at 37℃ and 120 rpm. After incubation period, one ml from bacterial liquid culture was transferred to 50 ml Mineral Salt Medium accompanied with 1% diesel oilas a sole of energy and carbon source and incubated at 37℃ and 120 rpm for 168 hrs. After fermentation period, cells were removed from each bacterial culture by centrifugation (4800 g at 4℃ for 30 min). The sample supernatant was used for Cetyl trimethyl-ammonium bromide (CTAB),drop collapse, oil displacement and emulsification assays. Bacterial pellets were used for Bacterial Adhesion to the Hydrocarbon (BATH) assay.

Hemolytic Activity

The formation of clear zone on blood agar plate is a qualitative method used as an indicator of biosurfactant production [16-18]. Blood agar plates were inoculated with bacterial culture grown in mineral salt medium and examined for hemolysis activity after incubated for 48-72 hrs. at 37℃.

CTAB Assay

Cetyltrimethyl-ammonium bromide (CTAB)-methylene blue agar plates was established by Siegmund and Wagner [19] for detection of anionic surfactants. A well was punctured into the CTAB agar plate using a sterile cork borer and filled with 50μL of the culture supernatant. Then, the CTAB agar plates were incubated for 48-96 hrs.at 30℃. After incubation period, the appearance of dark bluish or greenish halos around the wells was observed to suggest the production of anionic biosurfactant [20].

Drop Collapse

This assay developed by Jain D, et al. (1991) [21] and it relies on the destabilization of liquid droplets by surfactants. Five µl of mineral oil was added to glass slide and it was equilibrated for one hour at room temperature, then 10 µl of the culture supernatant was added to the surface of oil. The shape of the drop on the surface of oil was inspected after 1 min. Drops with flat shape imply the present of biosurfactant in the sample.

Oil Displacement Test

The oil displacement test was performed by filling a petri dish with 20 mL of distilled water, then 20 µL of crude oil was added, then the same amount of bacterial supernatant was added on the top of crude oil layer. After 30-60 sec, the displacement of oil was observed and measured [10,22]. The presence of surfactant in the sample was detected by the formation of a clear zone due to oil displacement.

BATH Assay

Microbial surface hydrophobicity was assessed by the Bacterial Adhesion to the Hydrocarbon Method (BATH) described by Rosenberg M, *et al.* (1980) [23]. BATH assay based on decrease in the absorbance of the lower aqueous phase was used an index for measuring the bacterial adherence to hydrocarbon. The degree of hydrophobicity was calculated as:

- **% Bacterial adherence = (1-$OD_{aqueous\ phase}$/ $OD_{original}$)× 100**

The cell pellets that were collected from the bacterial growth in mineral salt medium after 168 h incubation period, were washed twice and suspended in a phosphate urea buffer solution. Then, they were diluted using the same buffer solution to an optical density ($OD_{orginal}$) of approximately 1 at 600 nm. Diesel (0.5 ml) was added to 5 ml of microbial suspension and vortexes for 2 min. The optical density of aqueous phase was measured ($OD_{aqueous\ phase}$) after 10 min. The ability of adhering to hydrocarbon is acharacteristic feature of biosurfactant producing microorganisms.

Surface Tension of the Liquid

The Du-Nouy-Ring assay is one of the variety methods was used for this purpose and widely applied for screening of biosurfactant producing microbes. The supernatant was used for measuring surface tension under room temperature using KRUSS FORCE TENSIOMETER-K6. Microbial candidates for biosurfactant production are expected to decrease surface tension to around 35 mN/m [24,25].

Characterized and Identification of the Selected Isolates of Bacteria

Purified isolated bacterial cells morphological shape were observed with Gram staining under a microscope (oil immersion, 100×). Genomic DNA of selected isolate was extracted according to the method described by Asubel FM, et al. (1987) [26]. Universal bacterial primers corresponding to *Escherichia coli* positions 27F and 1492R were used for PCR amplification of 16S rRNA gene.

Results

Bacterial Isolates

Forty-two bacterial isolates were isolated and purified from different collected oil contaminated samples. Gram stain and cell microscopical examine shows 47% of isolates were gram -ve, 53% were

gram +ve, 14.4% have cocci cells shape and 16.6% have short rods cells shape, 69% have rods cells shape, and 16.6% were spore forming bacteria. Growth on MacConkey agar medium suggested that 45% of isolates had lactose-fermenting capability while 55% were non-lactose-fermenting colonies, and 53% had no growth on MacConkey agar medium (Figure 1).

Screening Bacterial Isolates for Biosurfactant Production

All the 42 isolated bacterial strains were subjected to screening for their biosurfactant production (Figure 2). Almost eighty three percent of isolated bacterial strain were able to grow on mineral salt medium with diesel oil as a soul of carbon source. Approximately 5% of screened strains had hemolytic activity on blood agar plate. Among the screened bacterial strains, 83.4% showed complete spreading on oily surface comparing with water as control. Likewise, for oil displacement test 83.4% of the isolates were showed positive activity on displacement the crude oil. On the other hand, 19% of screened strains were produced dark bluish halo around the well in CTAB Agar Assay and 81% tested negative by this assay. As well for BATH assay, 19% of screened bacterial isolates had more than 25 percent in this assay. Based on the screening methods, the efficient bacterial strains were EMB6, EMB 18, EMB 19, EMB 24. These strains were displayed the surface tension ≥ 40 mN/m (Figure 3) and approximately scored 30% with BATHA assay (Figure 4).

Beside they were showed positive activity (formation of flat drop) with drop collapse test (Figure 5). Also, the selected strains formed clear zone in oil displacement test with diameter more than 2 cm (Figure 6). Additionally, the tested bacterial strains were able to produce dark blush halo on CTAB assay (Figure 7).

Consequently, the bacterial strains EMB6, EMB18, EMB19, and EMB 24 were selected for molecular identification. The cell morphology for these selected bacterial strains were no spore forming gram negative rod cells (Figure 8).

Molecular Identification

Molecular identification of the selected isolates was performed based on the 16S rRNA gene sequences, using the GenBank BLAST tool. It was found that EMB 6 was closely related to *Klebsiella quasivariicola* (98.24%); EMB18: *Pseudomonas aeruginosa* (99.41%); EMB19: *Pseudomonas aeruginosa* (96.19%) and EMB24 *Pseudomonas aeruginosa* (97.51%) (Figures 9, 10, 11, and Figure12).

Figure 1: Pie charts showed the character of all isolated bacteria, the ratio of: a) Gram stain, b) bacterial cell shape, c) spore forming bacteria, d) lactose fermentation by screened bacterial strains and e)bacterial growth on MacConkey agar medium.

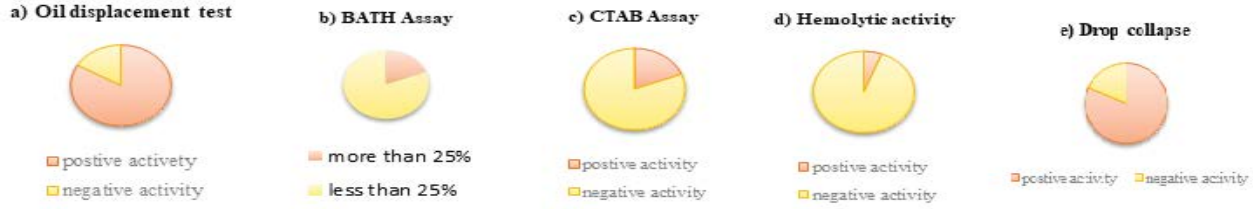

Figure 2: Pie chart showed the result of screened test among isolated bacterial strains, **a)** ratio of oil displacement test, **b)** BATH assay, **c)** CTAB assay, **d)** Hemolytic activity, and **e)** drop collapse assay.

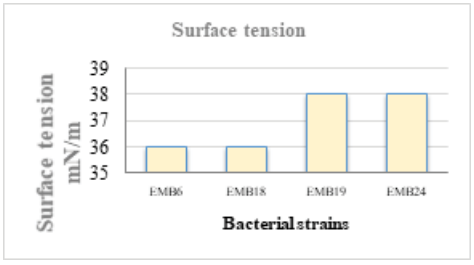

Figure 3: Surface tension for the four selected bacterial isolates EMB6, EMB18, EMB19 & EMB 24.

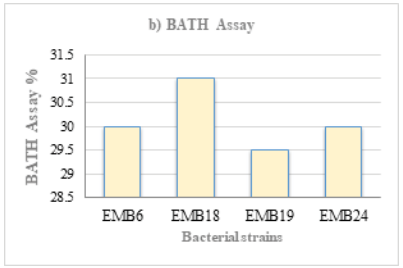

Figure 4: BATH Assay of the four selected bacterial isolates.

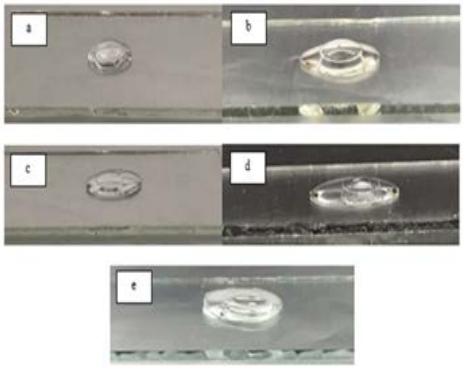

Figure 5: Drop collapse assay a) negative control distillated water, b) bacterial strain EMB6, c) bacterial strain EMB18, d) bacterial strain EMB19, and e) bacterial strain EMB24.

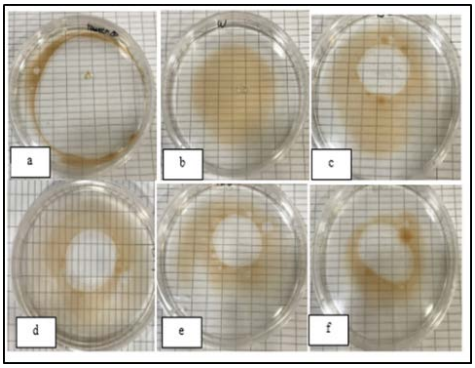

Figure 6: Indicates result of oil displacement test for selected bacterial strains a) positive control (tween 80), b) negative control distillated water, c) bacterial strain EMB6, d) bacterial strain EMB18, e) bacterial strain EMB19, and f) bacterial strain EMB24.

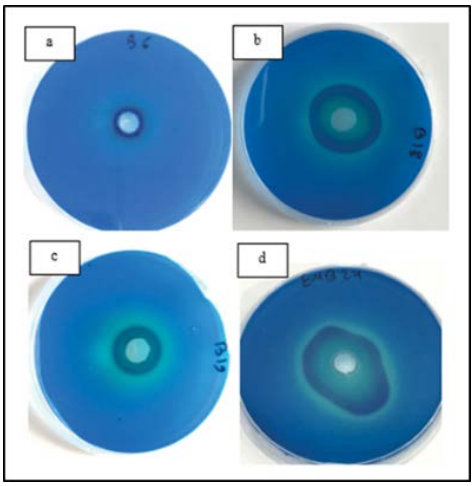

Figure 7: CTAB assay for selected strains a) bacterial strain EMB6, b) bacterial strain EMB18, c) bacterial strain EMB19, and d) bacterial strain EMB24.

Discussion

The present study was aimed to isolate biosurfactant producing bacteria from oil contaminated samples from Jeddah city, Saudi Arabia. Occurrence of biosurfactant-producing bacteria in oil- contaminated environments was reported by many researchers [10,27-29]. Isolation of bacteria which have capability to produce biosurfactant was done by enrichment culture method, which minimal media was supplemented with hydrocarbon (diesel oil) as sole carbon source. Most bacterial isolated strains (84.4%) have been shown to be able to use hydrocarbons (diesel oil) as their sole carbon source and they could produce biosurfactant as a secondary metabolite in the culture media.

In terms of screening the activity of biosurfactant produced by

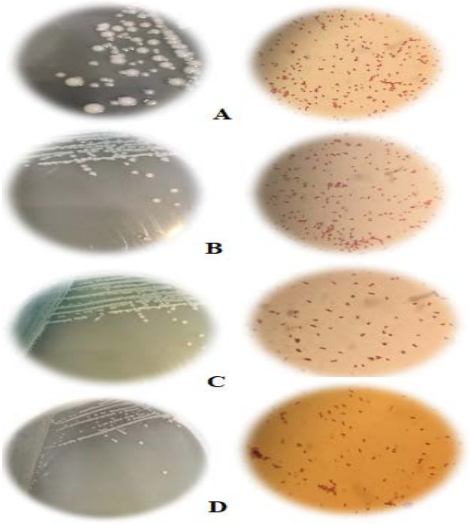

Figure 8: Illustrates bacterial colony and cell morphology for selected bacterial strains: a) EMB6, b) EMB18, c) EMB19, andd) EMB 24.

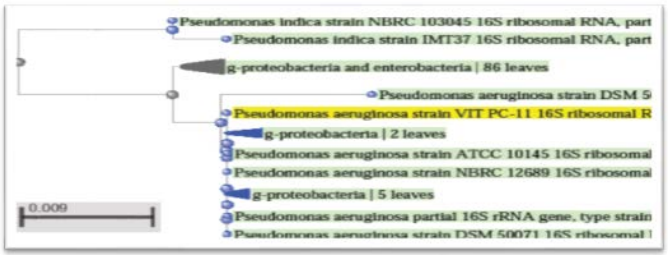

Figure 9: The phylogenetic tree of Klebsiella quasivariicola EMB6 compared to other genera.

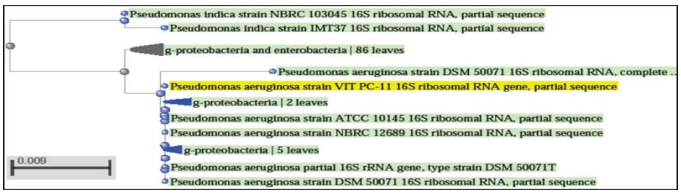

Figure 10: The phylogenetic tree of Pseudomonas aeruginosaEMB18 compared to other genera.

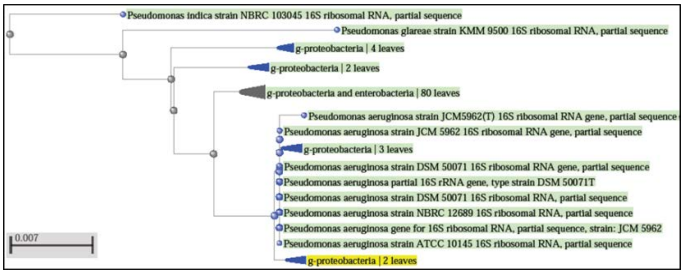

Figure 11: The phylogenetic tree of Pseudomonas aeruginosa EMB19 compared to other genera.

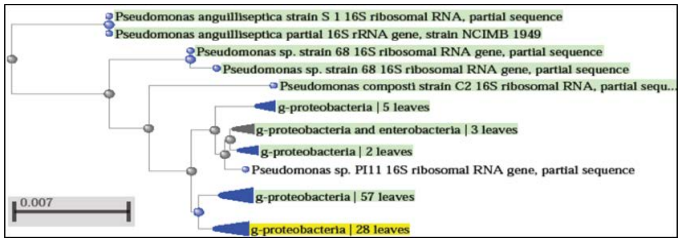

Figure 12: The phylogenetic tree of Pseudomonas aeruginosa compared to other genera EMB 24.

isolated bacteria, there are many different techniques that can be used, both qualitative and quantitative types in this study, different qualitative investigates have been applied such as: hemolytic test, drop-collapse test, and CTAB agar assay. Furthermore, quantitative screening method have been used including oil displacement test, BATH assay and surface tension measurement.

Hemolytic activity of biosurfactants was first discovered when Bernheimer AW, et al. (1970) [30] reported that the biosurfactant produced by *B. subtilis*, surfactant, lysed red blood cells. Although, according to Jain D, et al. (1991) [21] hemolytic method has some limitations. First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Among 42 tested strains only 5% showed positive hemolytic activity.This finding was supported by Plaza G, et al. (2006) [31] who confirmed the poor specificity of this method. In some studies strains with positive hemolytic activity were found negative for biosurfactant production [32].

Moreover, drop collapse method is a sensitive and rapid to perform method which requires small quantity of screened samples. From 42 strains screened, 83.4% strains were positive for drop collapse activity. This assay has been applied several times for screening purposes [20,33,34].

Additionally, CTAB agar assay is a specific screening method for anionic biosurfactants. It is used for the detection of glycolipid-type biosurfactant production by the bacterial colonies in the culture plate directly [35]. The CTAB agar assay has been applied in several studies [36,37]. Dark bluish ring formation on CTAB agar by EMB6, EMB 18, EMB19, EMB24 supernatant, indicated the ability of biosurfactant production by selected strains.The oil displacement test also is a rapid and easy method to check the presence of biosurfactant in the cell free culture broth. In addition, this method can detect even low activity and quantity of biosurfactant present. Morikawa M, et al. (2000) [22] reported that the area of oil displacement in oil spreading assay is directly proportional to the concentration of the biosurfactant in the solution. Among 42 investigated strains, 83.4% found positive for oil displacement assay.The Strains with positive drop collapse were found positive for oil displacement assay this finding in agreement with Thavasi R, et al. (2011) [16]. The selected bacterial strains were showed spreading the crude oil by more than 2 cm EMB6 (3.0 ± 0.2), EMB 18 (3.5 ± 0.5), EMB 19 (2.7 ± 0.3), and EMB 24 (3.0 ± 0.3).

For BATH assay, the selected strains showed hydrophobicity as follow: EMB 6 (30%±1.3); EMB18 (31%±1.5); EMB 19 (29.5%±0.8) and EMB24 (30%±1.2). The cell surface hydrophobicity was related to the biosurfactant secreted on the cell surface, helping adhesion of microorganisms to the hydrocarbons, and resulting in the effective degradation [23,38]. According to Meliani A, et al. (2014) [39], the best biodegradation of hydrocarbons was observed when cells had hydrophilic-hydrophobic properties ((hydrophobicity was approximately 30%) this support the current finding.

The result of screening test drop collapse, CTAB agar assay, oil displacement and BATH assay, implies that the selected isolates (EMB6, EMB18, EMB19, EMB24) had ability to produce surface active compounds. To confirm the ability of selected bacteria to produce biosurfactants, measurement of surface tension activity was performed. All selected bacterial isolates were able to lower the surface tension, presumably via biosurfactant production. It wasobserved the reduction of surface tension values to 36 mN/m, 36.3 mN/m and 38 mN/m, 39.7 mN/m for EMB6, EMB18, EMB19, EMB 24 respectively. Cooper D, et al. (1986) [40] considered a culture as promising if it reduces the surface tension of a liquid medium to 40 mN/m or less. The finding of present research achieved reduction in surface tension to less than 40 mN/m. Ahmad Z, et al. (2016) [28] found the *Klebseilla* sp. showed higher surface tension reduction activity (35.15 mN/m). This agree with the present finding for *Klebsiella quasivariicola* (EMB6).

The selected bacterial isolates were identified as (EMB6) *Klebseilla quasivariicola*. None of the previous studies reported the potential of to *Klebseilla quasivariicola* produce biosurfactant. Whereas, the bacterial strains EMB18, EMB19, EMB24 were identified as *Pseudomonas aeruginosa*. Contrariwise, biosurfactant producing *Pseudomonas aeruginosa* strains have dominant existence in hydrocarbon polluted environment and was reported by many researchers [22,41-43].

Conclusion

The bacterial isolates EMB6, EMB18, EMB19, and EMB24 showed high potential to produce biosurfactant. EMB 6 which was found to be closely related to *Klebseilla quasivariicola*, EMB18, EMB19, EMB24 to *Pseudomonas aeruginosa* the most talented biosurfactant producer based on the applied screening methods. These results disguised that oil contaminated sites contain bacteria capable of producing biosurfactant that could effectively lower the surface tension.

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