Original Article

# Screening And Characterization Of Biosurfactant-Producing Bacteria Isolated From Oil-Contaminated Soil

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Date Of Publication 22 October, 2022

**Doi:** 10.47750/pnr.2022.13.S08.164

## Abstract

Some bacterial isolates make biosurfactants, which are chemical substances that help oil biodegrade. 13 gram positive and gram negative bacteria isolates were isolated from various sources of oil polluted soil and identified using various ways before being submitted to select the active isolate for biosurfactant synthesis. Several approaches were used to screen isolates for biosurfactant-producing bacteria in this investigation. generating biosurfactants On hemolysis activity, cetyl trim ethyl ammonium bromide (CTAB) agar, oil spreading test, drop collapse assay, emulsification index, BATH (Bacterial adhesion to hydrocarbons) assay, and foaming activity. The biosurfactant productivity was shown to be highest in (*Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, Micrococcus luteus, Delftia acidovorans, Pseudomonas putida, Pseudomonas aeruginosa, and Pseudomonas stutzeri*).

**Keywords:** Bacteria, Biosurfactant, Screening of Biosurfactant

## INTRODUCTION

Biosurfactants are a broad category of biological compounds produced by bacteria, fungus, and yeast (Nitschke et al., 2018). They are a modern classic of surface active molecules with a wide range of physical, chemical, and biological properties that are used in a wide range of commercial applications (Singh *et al.*, 2018).Agriculture, food, cosmetics, and medicines are just a few of the industries that use surfactants. The bulk of these surfactants are created chemically, and they may be detrimental to the environment (Sauvageau *et al.*,2012). Despite the fact that most biosurfactants have emulsifying characteristics, bio emulsifiers do not always lower surface tension. Biosurfactants have several features over chemically created surfactants, including improved biodegradability, lower toxicity, environmentally friendly nature, improved foaming action, and durability at extreme pH, salinity, and temperature (Madhu and Prapulla,2014).

Microbial surfactants, also known as secondary metabolites, aid in nutrition transport, microbe-host interactions, and act as biocide agents, which protect biosurfactant-producing microbes (Joshi *et al.*, 2013). They also have a function in the pathogenicity of bacteria and the production of biofilms (Chen *et al.*,2007). Bacteria are the most frequent microorganism that produce biosurfactants, but yeasts and filamentous fungi also do so. Microorganisms that thrive on water-insoluble hydrocarbons, as well as water-soluble molecules such as glucose, sucrose, glycerol, or ethanol, can create these compounds, which can be ejected or remain attached to the cell wall. Bacteria and yeasts produce the majority of biosurfactants (Ebrahimi and Tashi, 2012). Glycolipids and phospholipids are two examples of surface-active molecules produced by bacteria (Rosenberg and Ron, 1999). Pharmaceuticals & medicine, agriculture, food, and cosmetics are all industries that use biosurfactants (Kamal-Alahmad,2015). They're also effective in bioremediation of contaminated sites since they have physiological functions that improve the bioavailability of hydrophobic substances and can bind to heavy metals, promoting improved chemical pollutant degradation (Van Hamme *et al.*,2006). *(Bacillus, Burkholderia, Flavobacterium, and Pseudomonas*) are among the bacterial genera that have been found to produce biosurfactants; microorganisms are generally thought to be generous biosurfactant makers (Thavasi,2011).

Biosurfactant-producing bacteria are grown in oil-contaminated conditions, where they make Biosurfactants using hydrocarbons as a carbon source. This usually turns them into harmless compounds or causes them to mineralize (Femi-Ola *et al.*,2015). A combination of screening tests, such as (hemolytic activity, oil spreading test, drop collapse test, foaming activity, and emulsification index), is required to find prospective biosurfactant manufacturers (Akintokun *et*

*al.*,2017). A combination of numerous screening techniques, which were successfully investigated in the current work, is necessary for effective detection of prospective biosurfactant producers.

## MATERIALS AND METHODS

### Sample Collection And Chemicals

A soil sample taken from an oil-contaminated site. In January 2021, samples were collected aseptically from several sites using a soil auger to a depth of 2-5 cm and stored in sterile aluminum foils before being transferred to the laboratory and stored at 4°C for 24 hours.

### Enrichment, Isolation, And Identification Of Pahs-Degrading Bacteria Samples Are Cultivated In BSM (Basic Salt Medium)

10 g of oil-contaminated soil was placed in 100 mL of mineral basic salt medium (MBS), pH 7.0, containing (g/L) (1.0 (NH4)2SO4, 0.8 K2HPO4, 0.2 KH2PO4, 0.2 MgSO4 7H2O, 0.1 CaCl2 2H2O, 0.005 FeSO4 7H2O) (Juhasz and Naidu, 2000). The flasks were shaken for seven days at 150 rpm at 30°C on an orbital shaker. To isolate bacterial strains, MBS agar plates coated with the same PAHs as the only carbon source were utilized. Individual colonies with different morphologies were selected. The ability of each isolate to degrade PAHs were examined. Using sterile distilled water as diluents, all of the homogenized mixture was serially diluted. For the serial dilution, six test tubes holding 9ml of distilled water were used. The following screening procedures were used to investigate for Biosurfactant production in the selected isolates (Saravanan and Vijayakumar,2012).

### Screening Of Isolates For Biosurfactant Production

Bacteria were grown in a flask (500 ml capacity) with 100 ml MSM containing (gl/1) 1.0 K2HPO4, 0.2 MgSO4.7H2O, 0.05 FeSO4.7H2O, 0.1 CaCl2.2H2O, 0.001 Na2MoO4.2H2O, 30 NaCl, and crude oil (1.0 percent, w/v) .A loopful of bacteria was inoculated into culture flasks containing sterilized mineral salt medium and incubated in a shaker incubator for seven days at 200 rpm at 30°C. After incubation period, the culture broth from each flask was centrifuged at 6000 rpm ,4°C for 15 minutes, after that the supernatant was filtered through Millipore ( size0.45m). This supernatant was used for different methods to estimate biosurfactant production.

## SCREENING METHODS FOR BIOSURFACTANT PRODUCTION

### 1- Hemolysis Test

By plate assay using blood agar medium, 13 isolates of bacterial species (6 gram positive and 7 gram negative) were evaluated to give higher yielding isolates for biosurfactant synthesis (Carrillo *et al.,* 1996). To test for hemolytic activity, each isolate was streaked on blood agar medium and incubated at 37 °C for 24–48 hours. Visual inspection of the plates revealed zones of clearing surrounding the colonies, indicating biosurfactant production.

### 2- CTAB Agar Plate Method

All 13 isolates (six gram positive and seven gram negative) grew on CTAB(cetyl trimethylammonium Bromide) (/methylene blue agar medium with (g/l) (Agar-Agar 15g, CTAB 0.2g, Glucose 5g, Methylene blue 0.005g, Peptone10g, and Yeast extract 0.5g, pH was adjusted to 7.3). Using a cork borer, a hundred microliter of previously activated bacterial culture was inserted into both well prepared in MSM with CTAB/methylene blue and incubated at 37°C for two days. Biosurfactant secretion was suggested by the formation of a dark blue halo zone around the colony. The radius of the inhibitory region was measured in mm.

### 3- Oil Spreading Test

This experiment was demonstrated according to the procedure given by Morikawa *et al.*, 2000. 20 mL D.W was put into a plastic Petri dish, and 20 µl of crude oil covered the top of the water. After that, 10 µl of cell-free culture broth(supernatant) was sprayed onto the oil surface. The oil will be replaced by an oil-free clearing zone, whose diameter shows surfactant activity, also known as oil displacement activity. Water was used as a negative control.

### 4- Drop Collapse Test

This test was used for screen biosurfactant synthesis was described by Batista (2006). The interfacial tension between the drop containing the surfactant and the parafilm surface is lowered in this experiment, resulting in the drop spreading. were pipetted as a droplet on the parafilm. As a negative control, distilled water was used. It was noticed that the droplet flattens and spreads across the parafilm surface. The parafilm was covered with 2 microliters of crude oil, and 13 of the 48-hour cultures were transferred to the oil-coated parafilm sections, where they were allowed to equilibrate for 24 hours. The result was considered positive for biosurfactant production when the drop was flat, but negative when the drop was rounded, indicating a lack of biosurfactant synthesis (Youssef,2004).

### 5- Emulsification Index Test

The ability of isolates to emulsify crude oil was measured using an emulsification index (E24). 1.5 mL hydrocarbon was combined with 1.5 mL cell-free broth for 2 minutes in a test tube before being vortexed at high speed for 2 minutes and

set aside for 24 hours. The following equation (Asfora Sarubbo *et al.*, 2006) was used to calculate the percentage of the emulsification index:

**(E24 = Height of emulsion formed × 100/total height of solution).**

### 6- Bacterial Adhesion To Hydrocarbons (BATH)

The BATH assay can be used to determine the cells' hydrophobicity. The suspension's absorbance was measured at 600 nm (A0), then 100 µl of crude oil was mixed with 2 ml of cell culture in test tubes and mixing by vortex for 3 minutes. The crude oil and aqueous phase were allowed to separate for 1 hour after mixing. The aqueous phase was removed with care. The O.D of the aqueous phase was then estimated in a spectrophotometer at 600nm (A1) . The following formula was used to compute hydrophobicity, which is given as a % of cell adhesion to crude oil:

**H% = (1-A/A0) *100**

### 7- Foaming Activity

Bacterial isolates were cultivated separately in flasks volume 250 mL with 100 mL nutrient broth medium in each flask. The flasks were incubated for 72 hours at 37°C and 200 rpm in a shaker incubator. Foam activity is measured in the graduated cylinder as the period of foam stability, foam height, and foam form.

## RESULTS AND DISCUSSION

### Screening Methods For Biosurfactant Production

### 1- Hemolysis test

Based on their ability to utilize crude oil as their only carbon source, a total of 13 G-ve and G+ve bacterial species were discovered from oil-contaminated soils. On solid MBS medium, the colony form, colony color, and development levels of these isolates differ. Seven G-ve (*Acinetobacter baumannii, Pseudomonas putida, Delftia acidovorans, Enterobacter cloacae, Pseudomonas aeruginosa, Pseudomonas stutzeri, and Pandoraea spp.)* and six Gram positive bacteria (*Micrococcus luteus, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus lentus, Staphylococcus lugdunebsis and Kocuria kristinae*). Because of their ability to lower the surface tension of petroleum hydrocarbons, biosurfactants play an essential role in oil recovery (Roy,2017). Finding biosurfactant-producing bacteria to increase and facilitate oil bioremediation was considered an important goal of the current study for this purpose. Blood hemolysis, blue agar plate, oil spreading technique, drop collapse assay, Bacterial adhesion to hydrocarbons (BATH), emulsification behavior, and foaming activity were used to screen all of the pre-identified isolates for biosurfactant production.

A primary way for screening a biosurfactant manufacturer is to use a hemolytic activity assay. Blood agar plates were used to screen all isolates. In Figure 1, eight isolates *(Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, Micrococcus luteus ,Delftia acidovorans, Pseudomonas putida, Pseudomonas aeruginosa, and, Pseudomonas stutzeri*,) showed positive results for hemolytic activity by forming a clear zone Biosurfactant production is frequently tested using the blood agar method. In the hunt for biosurfactant-producing bacteria, hemolytic activity appears to be an effective screening criterion (Carter, 1984). The isolates in our experiments demonstrated good hemolytic activity, which is used as a major screening approach for biosurfactant-producing bacteria. Verification is also necessary for other monitoring measures that showed surface activity, like; oil spreading test and the ability to emulsify hydrocarbons (Youssef *et al.*, 2004).

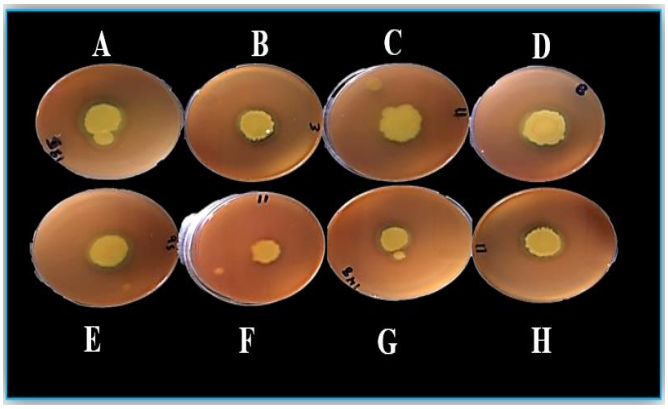


**Figure 1:** Biosurfactant producing isolates on blood agar medium (A: Kocuria kristinae ,B: Staphylococcus warneri, C: Staphylococcus haemolyticus, D:Micrococcus luteus, E:Delftia acidovorans, F:Psedomonas putida, G: Pseudomonas aeruginosa ,and H: Pseudomonas stutzeri).

### 2- CTAB Agar Plate Method

The blue agar plate method is a semi-quantitative agar plate method that uses the cationic surfactant CTAB and the basic color methylene blue to create an insoluble ion pair of anionic surfactants. All isolates were screened for biosurfactant production. Only Six isolates (Figure 2), both G+ve and G-ve, generated dark blue halos on CTAB agar, indicating biosurfactant synthesis. The CTAB agar plate method is a semi-quantitative test for extracellular glycolipids and other

anionic surfactants (Feigner *et al.*,1995). In a prior study, roughly 9.38 percent of isolates were found to be positive for CTAB agar plate test, and 52.8 percent of isolates were found to be positive for CTAB agar plate test (Noudeh *et al.*,2007).

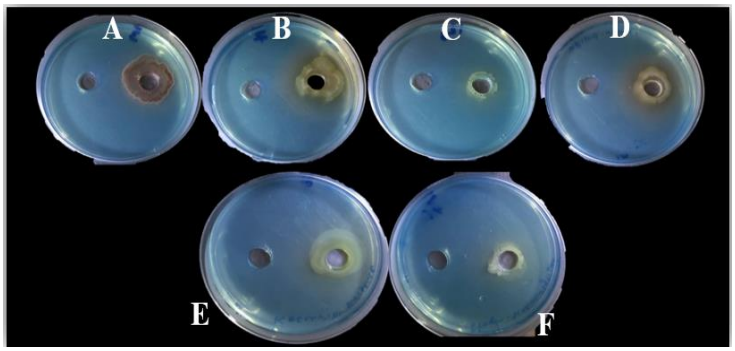


**Figure 2.** Biosurfactant producing by gram negative and gram positive bacteria (A: *Delftia acidovorans, B:Psedomonas putida, C:Pseudomonas aeruginosa ,D: Pseudomonas stutzeri, E:Kocuria kristinae ,and F: Staphylococcus warneri,*). bacterial isolate on CTAB agar medium.

### 3- Oil Spreading Test:

The oil spreading assay confirmed the drop collapse assay's findings, with organisms that tested positive for the drop collapse assay also testing positive for the oil spreading test. According to Morikawa *et al.*,2000, the oil displacement area is proportional to the amount of surface-active chemical in the solution. This study, however, only performed a qualitative assessment to check for the presence of surfactant. According to the findings (Table 1,Figure 3), gram positive (*Staphylococcus lentus, Staphylococcus lugdunebsis*) and gram negative (*Acinetobacter baumannii, Enterobacter cloacae, and Pandoraea sp*.) bacteria gave negative results in the oil spreading assay, whereas *Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, Micrococcus luteus, Delftia acidovorans, Pseudomonas putida , Pseudomonas aeruginosa, and Pseudomonas Stutzeri)* gave positive result with a diameter of 3.5 ,3.1,2.6,2.2,3.2,2.7,2, and 1.9mm respectively.

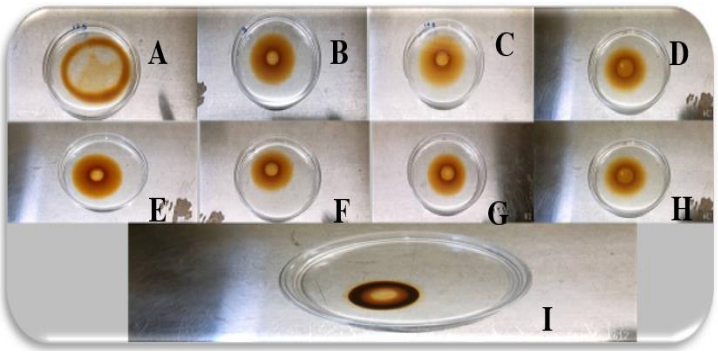


**Figure 3:** Oil displacement assay for *gram positive and gram negative bacteria* (A: *Kocuria kristinae ,B: Staphylococcus warneri, C: Staphylococcus haemolyticus, D: Micrococcus luteus , E:Delftia acidovorans, F:Psedomonas putida, G:Pseudomonas aeruginosa , and H: Pseudomonas stutzeri*) Producing biosurfactant, (I) negative control using only oil.

**Table 1:** Oil spreading, Drop collapse, Emulsification ,and BATH assay

| Bacterial isolates (gram positive) | Oil spreading test | Drop collapse test | Emulsification index(%) | BATH assay |
|---|---|---|---|---|
| **Kocuria kristinae** | +++ | ++ | **104%** | +++ |
| **Staphylococcus warneri** | +++ | ++ | **96.3%** | ++ |
| **Staphylococcus haemolyticus** | +++ | + | **88.5%** | ++ |
| **Micrococcus letus** | ++ | + | **81.8%** | + |
| **Bacterial isolates(gram negative)** | | | | |
| **Delftia acidovorans** | +++ | ++ | **96%** | +++ |
| **Pseudomonas putida** | +++ | ++ | **92%** | ++ |
| **Pseudomonas aeruginosa** | ++ | + | **87.5%** | ++ |
| **Pseudomonas stutzeri** | ++ | + | **76%** | + |

**Note:** In oil spreading (+) indicate diameter of 0.5-1.5 mm, (++) indicate diameter of 1.6 to 2.5 mm, (+++) indicate diameter of 2.6 to 3.5 mm; Drop collapse test: (+++)- drop collapse within 1 minute, (++) drop collapse after 1minute and (+) - drop collapse after 3 minutes of biosurfactant addition; BATH assay: (+++) cell adhesion > 90%, (++) - 60 to 89% cell adhesion, (+) 40 to 59% cell adhesion*).(* Anuraj *et al.*,2018*).*

### 4- Drop Collapse Assay

This experiment was performed by Jain *et al.*, 1991, which focused on the destabilization of a liquid drop by a cell free extract containing biosurfactant. The drop collapse method is rapid and easy to use, as it just requires a small sample volume and no special equipment. Culture supernatant dropped on an oil-coated solid surface as a result of this. The polar water molecules were repelled off the hydrophobic surface if the liquid did not contain surfactants, and the drops remained stable. When liquid drops contain surfactants, the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced, causing the drops to spread or even collapse.Drop stability is influenced by surfactant concentration, which is linked to surface and interfacial tension. All 13 isolates were used to revealed the production of biosurfactant. Eight isolates (*Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, Micrococcus luteus, Delftia acidovorans, Pseudomonas putida, Pseudomonas aeruginosa, and Pseudomonas Stutzeri*) showed good results in the drop collapse test (table 1,Figure 4). According to Erum *et al.*, 2012, positive cultures for oil drop collapse resulted in better biosurfactant production and were likely involved in lowering surface and interfacial tension between oil and water. The drop-collapse technique is a sensitive and straightforward test with a number of benefits, including the need for a small number of samples, speed and ease of execution, and the need of specialized equipment (Shoeb *et al.*,2015).

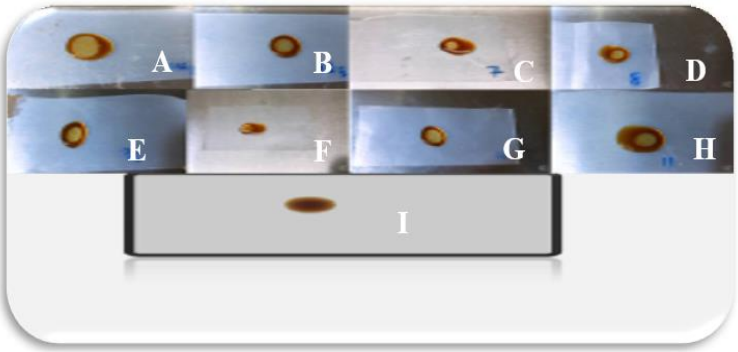


**Figure 4:** Drop collapse assay for gram positive and gram negative bacteria . Producing biosurfactant (A: Kocuria kristinae ,B: Staphylococcus warneri, C: Staphylococcus haemolyticus, D: Micrococcus luteus , E:Delftia acidovorans, F:Psedomonas putida, G:Pseudomonas aeruginosa , and H: Pseudomonas stutzeri), (I) negative control using only oil.

### 5- Emulsification Method

An indirect method for screening biosurfactant synthesis is the emulsification assay. It was assumed that if the cell free culture broth contained biosurfactant, the hydrocarbons would be emulsified. The hydrophobic substrate in this case was crude oil. Emulsification ability was regarded as a reliable test for identifying isolates that produce biosurfactant (Goswami and Deka ,2019). Table 1, Figure 5 and Figure 6 show the E24 of all eight organisms. Four gram positive isolates and four gram negative isolates showed the most emulsification. Gram positive isolates (*Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, and Micrococcus luteus*) had Emulsification index values of 104,96.3,88.5, and 81.8 percent, respectively. Gram negative bacteria (*Delftia acidovorans, Pseudomonas putida, Pseudomonas aeruginosa, and Pseudomonas Stutzeri*) have Emulsification index values of 96,92,87.4, and 76 percent, respectively. Femi-Ola et al., 2015 assessed the E24 value of isolates for strain detection and found 53,13 percent emulsification activity with kerosene. For emulsification, Ann Joice and Parthasarathi (2014) used a variety of hydrocarbon and vegetable oils. They discovered that PBSC1, a biosurfactant isolated from P. aeruginosa, had the best emulsification activity against crude oil. Velmurugan *et al.*, 2015 reported a 44 percent emulsification index of biosurfactant produced by isolate H11 against kerosene.

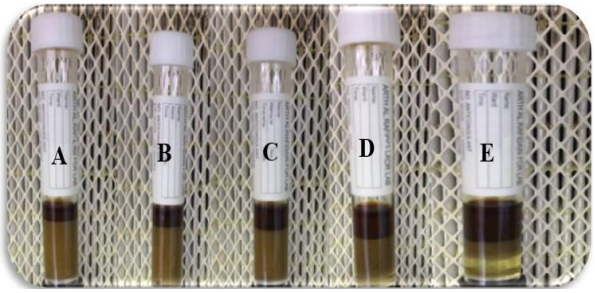


**Figure 5:** Emulsification assay for gram positive bacteria(A: Kocuria kristinae, B:Staphylococcus warneri, C:Staphylococcus haemolyticus, and D:Micrococcus luteus . Producing biosurfactant, E: negative control using only oil.

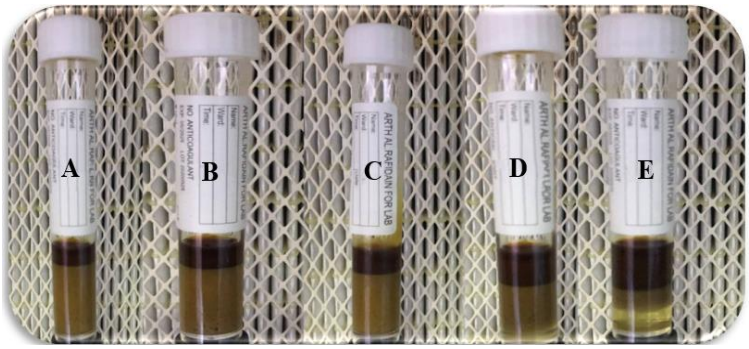


**Figure 6:** Emulsification assay for gram negative bacteria(A: Delftia acidovorans, B:Pseudomonas putida , C:Pseudomonas aeruginosa, and D:Pseudomonas Stutzeri. Producing biosurfactant, E: negative control using only oil.

### 6- Bacterial Adhesion To Hydrocarbons (BATH)

Cell adhesion with hydrophobic materials like diesel oil is considered an indirect technique to screen bacteria for biosurfactant production because cells adhere themselves to oil droplets by producing surface active molecules termed biosurfactant. The BATH test demonstrated that all eight of the isolates used in this study were positive, indicating that the bacteria preferred hydrophobic substrate. Cell adhesion to crude oil for positive isolates ( *Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, and Micrococcus luteus)* that showed 95.2, 71.5, 66.8 and 56.7% respectively, while gram negative bacteria (*Delftia acidovorans, Pseudomonas putida , Pseudomonas aeruginosa, and Pseudomonas Stutzeri*) that gave best of bacterial adhesion 97.3,83.2,77.3, and 53.2% respectively. The BATH assay demonstrated that *Kocuria kristinae* and *Delftia acidovorans* cells with crude oil had a high cell adhesion of more than 93.3 percent, which was directly connected with the biodegradation capacity observed in this study for this strain. Sauvageau *et al.*,2012 found high cell hydrophobicity and degradation for *P. aeruginosa*, which supports the findings of this investigation. Table 1 summarized the findings.

### 7- Foaming Activity

According to Abouseoud *et al.*,2008, the foaming capacity test was performed on the 8 isolates under examination. Isolates were revealed the highest foaming activity.8 isolates for gram positive and gram negative (*Kocuria kristinae, Staphylococcus warneri, Staphylococcus haemolyticus, Micrococcus luteus, Delftia acidovorans, Pseudomonas putida , Pseudomonas aeruginosa, and Pseudomonas Stutzeri*) respectively that gave the highest foaming activity. Figure 7.

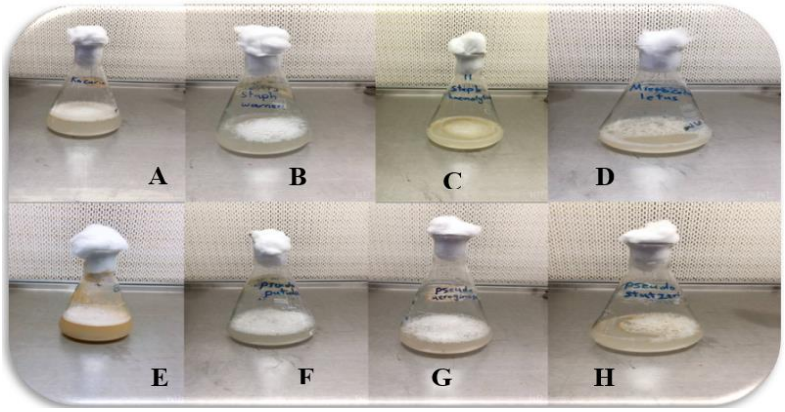


**Figure 7:** Foaming activity for gram positive and gram negative bacteria (A: Kocuria kristinae ,B: Staphylococcus warneri, C: Staphylococcus haemolyticus, D: Micrococcus luteus , E:Delftia acidovorans, F:Psedomonas putida, G:Pseudomonas aeruginosa , and H: Pseudomonas stutzeri).

## CONCLUSIONS

Eight bacterial isolates were identified as the most active biosurfactant makers utilizing the qualitative and quantitative testing, based on the approved screening methodologies. They used to screened biosurfactant four G+ve and four G-ve as: *Kocuria kristinae ,Staphylococcus warneri, Staphylococcus haemolyticus, Micrococcus luteus ,Delftia acidovorans, Pseudomonas putida, Pseudomonas aeruginosa , and Pseudomonas stutzeri* respectively, These isolates from oil having the ability to create stable biosurfactants in polluted settings Degradation of hydrocarbons .

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